Effect of sodium butyrate on the stimulation of casein gene expression by prolactin

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Sodium butyrate, but not isobutyrate, inhibits prolactin action on the induction of casein synthesis and casein mRNA accumulation in rabbit mammary explants. Sodium butyrate specifically prevents the generation of the prolactin relay which can be released from isolated membranes incubated with prolactin and which stimulates directly casein gene transcription when added to isolated mammary nuclei. This indicates that sodium butyrate exerts its inhibitory action essentially at the membrane level.

Casein synthesis   *Casein mRNA*   *Casein gene transcription*   *Sodium butyrate*
Prolactin intracellular relay

1. INTRODUCTION

Sodium butyrate and several other short-chain fatty acids are known to modify the activity of many cell types. Sodium butyrate inhibits cell replication, it acts as inducer or inhibitor for cell differentiation, and it modifies cell shape possibly through its effect on cytoskeleton (review [1–3]). A well-established action of sodium butyrate is the inhibition of histone deacetylase which results in hyperacetylation of these proteins and in an inhibition of DNA replication. Sodium butyrate provokes hyperphosphorylation of nuclear proteins HMG 14 and HMG 17 [4,5] which are associated with active chromatin. Thus it is believed that the nucleus is the essential cell compartment in which sodium butyrate acts.

The expression of casein gene is under hormonal control in which prolactin, a protein hormone, plays the major role of inducer. Induction of casein synthesis by prolactin is accompanied by an accumulation of the corresponding mRNA. This accumulation results from two independent phenomena: an enhancement of casein gene

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transcription and a stabilization of gene product [6,7]. Membranes containing prolactin receptors and incubated with the hormone release a factor which accelerates specifically the transcription of β-casein gene when added to isolated mammary nuclei [8,9]. Given the specificity of this factor, it has been considered as the prolactin intracellular relay or second messenger.

This work has been done to determine whether sodium butyrate affects prolactin action on casein gene expression. It has been observed that sodium butyrate, when added to the culture medium of mammary explants, counteracts prolactin for the induction of casein synthesis. Evidence is presented that this effect is elicited essentially at the membrane level since mammary membranes do not release the prolactin intracellular relay when they are incubated in the presence of sodium butyrate.

2. MATERIALS AND METHODS

Mammary fragments explanted from pseudopregnant rabbits were cultured for one day in medium 199 in the presence of various concentrations of sodium butyrate or isobutyrate (Pro-

labo) and in all cases with beef insulin (5 μg/ml) with or without ovine prolactin (NIH-PS9) 100 ng/ml. At the end of the cultures, the tissue was incubated for 3 h with ^{14}C-labelled amino acids and casein synthesis was then evaluated using an anti-casein antibody [10].

The concentration of α_{s1}-casein mRNA in total mammary nucleic acids of explants was evaluated with a labelled nick-translated plasmid carrying the DNA sequence complementary to α_{s1}-casein mRNA and kindly provided by Dr J.P. Kraehen-buhl [11]. Conditions of hybridization were those defined in [12].

The number of prolactin receptors in membranes was determined essentially as in [13]: ^{125}IhGH taken as the lactogenic hormone (~50 μCi/μg) was incubated with crude microsomes overnight at 25°C (100000 cpm labelled hormone, with 300 μg membrane protein). The aspecific binding was evaluated by incubating membranes with a large excess of unlabelled prolactin in similar conditions. The resulting blank was subtracted and results are expressed as the percentage of the cpm of the labelled hormone which is specifically bound to membranes.

The capacity of mammary membranes to release the prolactin intracellular relay was tested by incubating crude microsomes with prolactin in the presence or in the absence of sodium butyrate for 1 h at 25°C. The membrane supernatant was then added to mammary nuclei isolated from lactating rabbit treated for 4 days with CB 154 to suppress prolactin secretion and thus to de-induce casein gene transcription. Nuclei were incubated in the presence of Hg-UTP. The resulting neosynthesized mercurated RNA was selectively retained on a SH-Sepharose column and it was eluted in the presence of β-mercaptoethanol. The concentration of β-casein mRNA sequences in the eluate, which reflects the transcription rate of the gene in the isolated nuclei, was evaluated using a ^{125}I-labelled cDNA probe [8,9]. Alternatively, nuclei were incubated in the presence of Hg-UTP and [α-^{32}P]CTP (Amersham, 3000 Ci/mmol) and the labelled RNA retained and eluted from the SH–Sepharose column was incubated in the presence of a nitrocellulose filter to which was bound a plasmid containing the DNA sequence complementary to β-casein (the generous gift of Dr J.P. Kraehenbuhl).

3. RESULTS

3.1. Effect of sodium butyrate on the induction of casein synthesis

Addition of prolactin to the culture medium of mammary explants results in the induction of casein synthesis. This effect of prolactin was progressively inhibited with increasing concentration of sodium butyrate in the medium and the inhibition was complete at 25 mM (fig.1). As a matter of comparison, sodium isobutyrate was essentially ineffective in this concentration range. The effect of sodium butyrate was not due to a general cytotoxic effect since total basal protein synthesis was not affected (table 1), as it has been observed in other cell types. However, the slight stimulation of total protein synthesis which takes place in the presence of prolactin was abolished by butyrate but not by isobutyrate (table 1).

At 10 mM, butyrate was only partly inhibitory. This value is rather elevated in comparison to that observed with other biological systems where 5 mM proved to be sufficient to elicit the full inhibition [14–17]. This discrepancy may be attributed to the fact that mammary explants from pseudopregnant rabbits contain a substantial proportion of lipids which may trap butyrate and isobutyrate aspecifically. This interpretation is
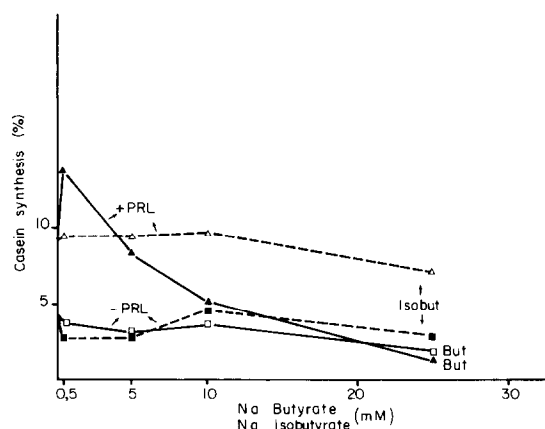

Fig.1. Effect of various concentrations of sodium butyrate and isobutyrate on the induction of casein synthesis by prolactin. Results are expressed as the percentage of total labelled proteins which are precipitated by the anti-casein antibody. In all cases, insulin (5 μg/ml) and cortisol (500 ng/ml) were present.
Prolactin (PRL) was present at 100 ng/ml.

Table 1

Effect of isobutyrate and butyrate on total protein synthesis

	Control	Isobutyrate	Butyrate
I	9320	6450	6300
I + PRL	13700	14500	6100

Isobutyrate and butyrate were added with insulin (I, 5 μg/ml) and prolactin (PRL, 100 ng/ml) at 25 mM. At the end of the culture (24 h), the mammary explants were incubated for 3 h in Krebs medium containing ^{14}C-labelled amino acids (10 μCi/ml). Total protein synthesis was estimated after a precipitation with trichloroacetic acid. Results which are the mean of two independent cultures are expressed as cpm/mg tissue

Fig.2. Effect of sodium isobutyrate and butyrate on the induction of α_{s1}-casein mRNA accumulation by prolactin. Explants were cultured for 24 h in the presence of insulin (I, 5 μg/ml), prolactin (PRL, 100 ng/ml) and the agents at 25 mM. At the end of the culture, nucleic acids were extracted by phenol method and increasing amounts were bound to nitrocellulose filters. Filters were incubated for 24 h at 68°C in the presence of ^{32}P nick-translated plasmid (10^8 cpm/g). The hybridization medium contained 10 cpm/ml, 0.6 M NaCl, 20 mM Tris (pH 7.5), 0.13 sodium dodecylsulfate, 5 A_{260}/ml yeast RNA and 10% dextrane sulfate. After extensive washing in the absence of the probe the cpm bound to filters were evaluated by scintillation counting.

validated by the fact that butyrate which is known to be a potent inhibitor of DNA synthesis [16] did not reduce DNA synthesis at < 10 mM (not shown).

Sodium butyrate but not isobutyrate totally prevented the accumulation of α_{s1}-casein mRNA provoked by prolactin (fig.2). This demonstrates that sodium butyrate affects one of the essential prolactin actions in the mammary cell.

3.2. *Effect of sodium butyrate on prolactin binding to receptors*

To determine whether the effect of sodium butyrate was not simply due to the inhibitor of prolactin binding to its receptors, mammary membranes were incubated with the labelled hormone and in the presence or in the absence of butyrate. At ⩽ 50 mM, sodium butyrate or isobutyrate did not interfere with the binding of prolactin to its receptors (table 2). Hence, sodium butyrate does not alter the binding of prolactin to its receptors.

Prolactin is known to provoke a down-regulation of its own receptors [13]. To determine whether sodium butyrate affects this process, prolactin was added to the culture medium of explants with or without the agent. Butyrate added to the culture medium of explants significantly reduced the basal level of prolactin receptors but it did not prevent the down-regulation to take place (table 3). The slight effect of butyrate may result from a direct action with the membranes or from a partial inhibition of receptor biosynthesis. This lowering of prolactin receptor number in the presence of
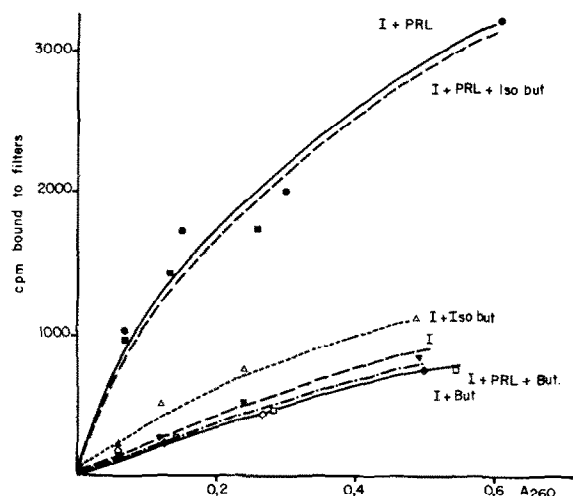
Table 2

Effect of sodium isobutyrate and butyrate on the binding of prolactin to its receptors

	Control	Na Iso-butyrate	Na butyrate
Prolactin receptors % specific binding	32.9%	31.7%	36.2%

Crude microsomes prepared from the mammary gland of a lactating rabbit were incubated with ^{125}I-hGH (100000 cpm) in the presence or in the absence of the two agents at 50 mM. At the end of the incubation, membranes were pelleted and the labelled hormone specifically bound to membranes was evaluated using a gamma counter (section 2). Results which are the mean of duplicate determinations are expressed as the percentage of the total cpm of the labelled hormone which are specifically bound to membranes

Table 3

Effect of sodium isobutyrate and butyrate on the number of prolactin receptors

Culture medium	I	I + Iso	I + But	IP	IP + Iso	IP + But
Prolactin receptors % specific binding	11.7%	13.8%	5.9%	6.4%	8.4%	2.9%

Mammary explants from pseudopregnant rabbit were cultured for 24 h in the presence or in the absence of the agents at 25 mM. Insulin (I) and prolactin (PRL) were present at 5 μg/ml and 100 ng/ml, respectively. At the end of the culture, microsomes were prepared and their content in prolactin receptors was determined using ^{125}I-hGH as a marker (table 2 legend; section 2). Results which are the means of two independent cultures are expressed as the percentage of the total cpm of the labelled hormone which is specifically bound to membranes
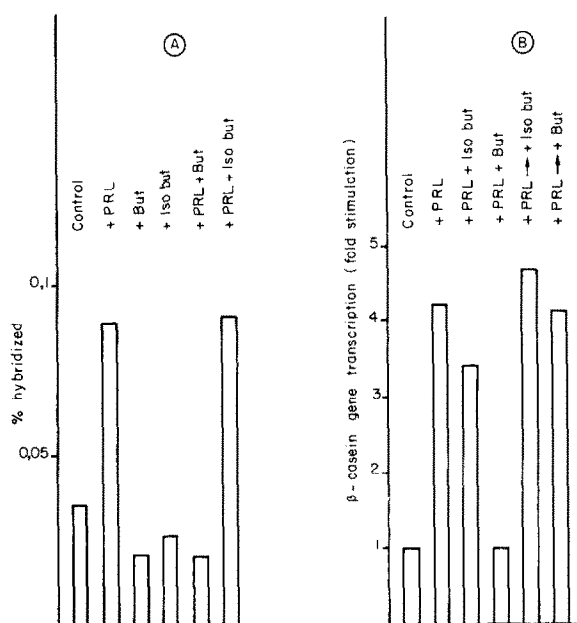

Fig.3. Effect of sodium isobutyrate and butyrate on the release of prolactin intracellular relay. Mammary membranes were incubated with or without prolactin (1 μg/ml) in the presence or in the absence of 25 mM agent. The membrane supernatants were added to the incubation medium of nuclei synthesizing RNA. (A) Nuclei were incubated in the presence of [α-^{32}P]CTP and Hg-UTP. The labelled RNAs were hybridized to plasmid bound to nitrocellulose filters. Results are expressed as the percentage of the RNA which is specifically hybridized to the filters. (B) Nuclei were incubated in the presence of Hg-UTP and the mercurated RNAs were incubated with a ^{125}I-casein cDNA probe. The last two bars were obtained when isobutyrate and butyrate (10 mM) were added to the membrane supernatants after the incubation with prolactin.

butyrate might contribute to its inhibitory action on casein gene expression.

3.3. Effect of sodium butyrate on the release of prolactin intracellular relay

Incubation of mammary membranes with prolactin provokes the generation of factor which stimulates β-casein gene transcription when added to isolated nuclei [8,9] (fig.3A,B). When incubation of membranes with prolactin was carried out in the presence of 25 mM sodium butyrate, the membrane supernatant was totally devoid of activity. In contrast, sodium isobutyrate was without any effect. This inhibitory effect of butyrate was not exerted at the nuclear level in the cell-free system. Indeed, addition of butyrate at 10 mM (10-times the actual concentration in the above experiment) to nuclei with a membrane supernatant prepared independently with prolactin, but in the absence of butyrate, did not prevent the prolactin relay to act (fig.3B). Sodium butyrate thus appears to act directly on the membranes by preventing the generation of the prolactin relay.

4. CONCLUSION

These data are an example of the inhibitory action of butyrate on cell differentiation [14–17]. The essential information, that the chemical agent acts directly on membranes, is not surprising, given the lipid nature of butyrate. This observation does not exclude a simultaneous effect on nuclei. However, an experiment (not detailed here) indicated that the action of the prolactin relay when it is added to the culture medium of isolated mam-

mary cell [18] is only partly inhibited by butyrate present in the culture medium. The effect of the prolactin relay on nuclei is assumed to be exerted via a dephosphorylation of nuclear proteins [19] and sodium butyrate is known to provoke a hyper-phosphorylation of nuclear proteins HMG 14 and 17 [4,5]. If this hyperphosphorylation takes place in the mammary cell, it is most likely not the essential action of butyrate. It is rather at the membrane level that butyrate exerts most of its effect. This might be also the case for egg white protein genes which are stimulated by oestrogens and protein factors having receptors on the cell membrane [20] and which are strongly inhibited by sodium butyrate [14]. When sodium butyrate acts as an inducer [21–30], it might act at the membrane level by mimicking the effect of natural inducers. Most likely, the action of butyrate reported here does not reflect a biological event, although it is tempting to imagine that the accumulation of milk which contains butyrate exerts part of its feedback mechanism on milk synthesis via this short-chain fatty acid. Whatever interpretation can be given for these experiments, as colchicine [9] and phorbol esters (in preparation) which both inhibit the generation of the prolactin relay, butyrate can be considered as a tool for the study of the mechanism of prolactin action.

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