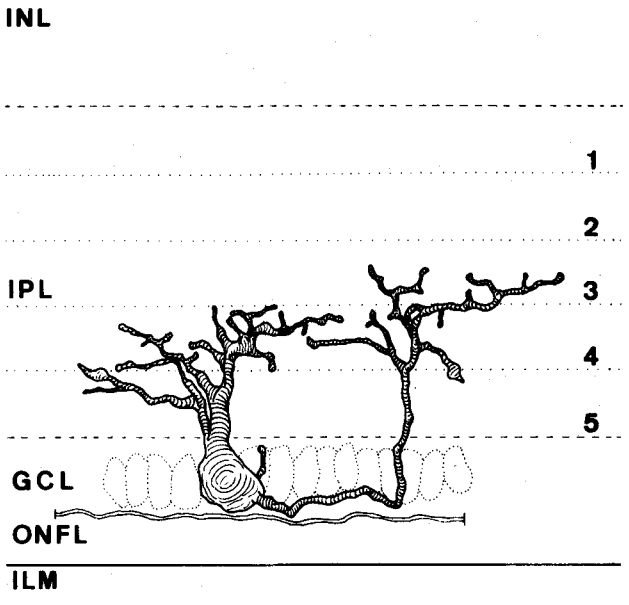

Figure 4. Camera lucida drawing of a 'short axon ganglionar cell', to point out the axonal and dendritic expansions in the different strata of the inner plexiform layer (IPL). × 600.

topies, are common occurrences in NCS development [11, 12]. On the other hand it is also known that the establishment of the retinotectal connection is an important fact in ganglion cells' survival [13]. If ganglion cells do not synapse with optic tectum cells, they degenerate and die [14, 15]. It could be thought that the axon of such a ganglion cell might have been mechanically displaced during its differentiation. But it does not degenerate because it finds a 'guide' or 'signal' [16–18] mechanism which makes possible an effective synaptic connection in the IPL. If this were certain, it would make it obvious that ganglion cells have a clear plasticity during their development, and can subsequently behave as a projection or an association neuron.

Another possibility is that these cells have the genetic information and capability necessary in order to differentiate as association neurons, with the axonal characteristics described. The fact that ganglion cell axons forming synapses with photoreceptors have been found in the retina of the primate [19] supports this hypothesis.

As the Golgi technique is a non-quantitative procedure, it is rather difficult to establish to what extent cells such as those described are a general feature of the cell population of the retina. It is possible that the cells described here represent an isolated case of neuronal ectopia. Since the existence of intraretinal axons is important, in our opinion, in retinal circuitry, we consider that further studies to provide stronger evidence are needed. Electron microscopy using the Golgi procedure is being used at present in our laboratory to study the synaptic connections of these cells.

This study was supported with grants from the Comision Interministerial de Ciencia y Tecnologia GG85-0153.

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0014-4754/89/010092-03$1.50 + 0.20/0

Effects of butyrate and insulin and their interaction on the DNA synthesis of rumen epithelial cells in culture

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Received 5 May 1988; accepted 14 September 1988

Summary. Rumen epithelial cells (REC) were incubated in the presence of various concentrations of butyrate or insulin or with both of them, to obtain information on their effect on the DNA synthesis of cultured cells. The 24-h values of ^{3}H-thymidine incorporation into cellular DNA were measured in the presence of butyrate, insulin or butyrate plus insulin. While butyrate reduced DNA synthesis, insulin produced an increase over the control. Combined butyrate plus insulin treatment influenced the incorporation of label in accordance with the relative proportion of these two substances.
Key words. Butyrate; insulin; rumen epithelial cells; DNA synthesis.

Butyrate induces characteristic biochemical changes in cultured cells [1, 2], including rumen epithelial cells (REC) [3, 4]. It increases protein synthesis and alkaline phosphatase activity and inhibits cell growth. In contrast with the last finding, observed in vitro, daily short-term intraruminal infusion of butyrate increases the mitotic index of ovine REC in vivo [5]. It has been suggested that the infused butyrate stimulated mitosis in REC not so much by a direct effect as indirectly, by elevating the insulin level [6]. This hypothetical conclusion has been supported by the experimental facts that intra-

venously administered butyrate and, to a lesser extent, propionate increased plasma insulin levels in sheep and cattle[7], and injection of butyrate into the ovine pancreatic artery stimulated insulin secretion[8]. Insulin secretion by isolated ovine pancreatic islets tended to increase in the presence of butyrate[9], and sheep intravenously treated with insulin showed an increase in the proliferation of REC[6].

It follows that both butyrate and insulin act on the proliferation of REC. To obtain more information on their action and possible interactions, we treated cultured REC with butyrate and insulin, both alone and in combination at various ratios, and measured the incorporation of ^{3}H-thymidine into cellular DNA (referred to as DNA synthesis).

Materials and methods. Isolation and culture of REC was carried out by a modified version of the method described by us earlier[10]. Papillae were taken from the caudal blind sac of the rumen of healthy merino ewes (weighing about 40 kg) after slaughter, and were digested by fractional trypsinization. The fractions containing mainly stratum spinosum and stratum basale cells were placed in plastic dishes (35 or 15 mm in diameter) for culturing in a minimal essential medium (MEM Hanks' BSS), containing 12% fetal bovine serum, 2 µmol/ml glutamine and 100 µg/ml gentamycin (buffered with 4.8 mg/ml Hepes). The insulin concentration of fetal calf serum was 7.7 µU/ml[11]. The cells were seeded onto collagen gels, Vitrogen 100 in culture dishes by the method of Yang et al.[12], (all reagents and culture dishes were from Flow Laboratories, Ayrshire, Scotland). The cultures were incubated at 37 °C.

One-week cultures of REC were treated with sodium-*n*-butyrate (BDH Chemicals Ltd Poole, England) at 0.4, 2 or 10 µmol/ml concentration, with mixed (bovine and porcine) crystalline insulin (Chemical Works of Gedeon Richter Ltd, Budapest, Hungary) at 2, 20 or 200 µU/ml concentration, or with various combinations of both, using four replicates. According to our unpublished data, the stimulatory action of mixed insulin on the DNA synthesis of REC was similar to that of sheep insulin.

12 h later, 18.5 kBq/ml ^{3}H-thymidine was added to the cultures, and after 24 h the incorporation of label into cellular DNA was determined by the modified technique of Linna[13], with a Packard Tricarb liquid scintillation counter.

Statistical comparison of means was carried out by Student's t-test. $p < 0.01$ was accepted as significant.

Results. The 24-h values of ^{3}H-thymidine incorporation in the presence of butyrate, insulin or butyrate + insulin were related to the incorporation of label into control cells maintained in plain medium (which contained 0.9 µU/ml insulin owing to the presence of fetal calf serum). The results for the extent of ^{3}H-thymidine incorporation after treatment with insulin or butyrate alone, in experiments 1 and 2, are given in table 1. While butyrate significantly reduced ^{3}H-thymidine incorporation, insulin produced a significant increase over the control. The regression equations show the dose dependency of butyrate ($y = 57.79 \times X^{-0.137}$ $r = 0.9728$) and insulin ($y = 147.21 \times X^{0.199}$ $r = 0.9966$) action in experiment 2.

In the second part of our investigation, the combined effect of insulin and butyrate was studied. Combined butyrate and insulin treatment in experiment 1 influenced the incorporation of label in accordance with the relative proportion of the two active substances; the resulting change in thymidine incorporation compared to the control cultures was not significant (table 2). In the presence of 20 and 200 µU/ml insulin, the inhibition due to butyrate decreased significantly ($p < 0.01$), compared to the values of cultures after butyrate treatment (2 or 10 µmol/ml), but without insulin addition. Moreover, 2 µmol/ml butyrate together with 200 µU/ml insulin even resulted in an increase in thymidine incorporation (vs control cultures).

Discussion. It may be concluded from these results that butyrate decelerates, whereas insulin accelerates considerably the rate of DNA synthesis. In addition, we studied the combined effect of these substances, because in the earlier in vivo and in vitro investigations the effects of butyrate and insulin were examined separately. Their combined effect caused a slight, not significant change of thymidine incorporation compared to the control value. These results suggest that insulin and butyrate can compensate each other's effect on DNA synthesis. Since butyrate stimulates the secretion of insulin, there is reason to postulate that the two active molecules may also simultaneously develop their opposing effects in vivo. Generally it seems that the predominance of the decelerating effect of butyrate or the accelerating effect of insulin depend on the relative proportion of the two active substances.

Table 1. The influence of insulin or butyrate on the ^{3}H-thymidine incorporation of rumen epithelial cells

Treatment	^{3}H-thymidine incorporation (% of control[a]) Exp. 1	Exp. 2
Insulin (µU/ml)		
2	ND	+ 15 ± 13[NS]
20	+122, + 146[b]	+ 81 ± 22*
200	+228[c] ± 12**	+185 ± 23**
Butyrate (µmol/ml)		
0.4	ND	−55 ± 4**
2	−66 ± 18*	−67 ± 2**
10	−78 ± 23*	−71 ± 7**

The cells were cultured for 36 h with insulin or butyrate as described in 'Materials and methods'. The values for ^{3}H-thymidine incorporation of cells grown in plain medium were 455 dpm/10^3 cells in exp. 1 and 150 dpm/10^3 cells in exp. 2. [a] % of ^{3}H-thymidine incorporation of cells grown in plain medium. Control was taken as 0%. Values are means ± SD of four replicates. [b] $n = 2$, [c] $n = 3$. * $p < 0.01$, ** $p < 0.001$ (vs control value). NS, not significant; ND, not done.

Table 2. Combined effect of insulin plus butyrate on the ^{3}H-thymidine incorporation of rumen epithelial cells

Treatment Insulin (µU/ml)	^{3}H-thymidine incorporation (% of control[a]) Butyrate (µmol/ml) 0	2	10
0	0	−66 ± 18[b]	−78 ± 23[b]
20	+122, + 146[b]	− 8 ± 12[c]	−29 ± 21[c]
200	+228 ± 12[b]	+28 ± 22[c,d]	−16 ± 11[c,d]

The cells were cultured for 36 h with insulin plus butyrate as described in 'Materials and methods'. The value for ^{3}H-thymidine incorporation of cells grown in plain medium was 455 dpm/10^3 cells. [a] % of ^{3}H-thymidine incorporation of cells grown in plain medium. Values are means ± SD of four replicates. [b] data are given in table 1, too. [c] $p < 0.01$ significant differences within one column (vs cultures without insulin treatment). [d] $p < 0.001$ significant differences within one line (vs cultures without butyrate treatment).

Acknowledgment. This work was supported by OTKA 1538 grant, awarded by the Ministry of Agriculture and Food, Budapest, Hungary.

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0014-4754/89/010094-03$1.50 + 0.20/0

Presence of benzodiazepine binding sites (receptors) and amplification thereof by imprinting in *Tetrahymena*

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Summary. Live *Tetrahymena* cells bound ^{3}H-diazepam specifically, as demonstrated by autoradiographic evidence of displacement of about 25% of labeled diazepam in the presence of a 1000-fold amount of cold diazepam. The ^{3}H-diazepam bound to membrane preparations isolated from untreated (control) cells was not displaced by cold diazepam, whereas cells involved in primary interaction (imprinting) with diazepam showed amplification and specificity of diazepam binding in both in vivo (cell suspension) and in vitro (pellicle) systems, as well as displacement of bound label in the presence of 1000-fold cold diazepam. It appears that diazepam induced imprinting and, consequently, also the formation of specific receptors in *Tetrahymena*.
Key words. Hormonal imprinting; receptors; benzodiazepine; *Tetrahymena*.

The unicellular organism *Tetrahymena* does contain certain vertebrate hormones [1-4], and is able to respond to these, in many cases even specifically [5-7]. For example histamine, which stimulates phagocytosis in higher organisms [8, 9], has a similar effect on *Tetrahymena* [10], and the binding sites presented by the membrane of the unicellular organism can differentiate histamine from its antagonists [11]. Phagocytosis stimulant action and selectivity have also been observed with serotonin [10, 11]. Insulin [12] and adrenalin [13] enhance the glucose uptake of the protozoan, and the effect of polypeptide hormones is measurable by stimulation of RNA synthesis [14]. *Tetrahymena* also presents receptors for opioids, detectable by displacement of bound label [15].

Primary interaction with a hormone amplifies the potential binding sites or, if such structures are lacking, induces the formation of these [5-7]. This phenomenon, termed hormonal imprinting, endows the unicellular organism with a 'memory' of the primary interaction, which persists over as many as 500 generations [16]. The increased responsiveness to the hormone shown by imprinted cells can be explained by a greater binding capacity, which has been studied mainly in connection with polypeptide hormones.

In the present study we investigated whether or not *Tetrahymena* also possessed, or could present as a result of imprinting, receptors for a non-hormone (drug) molecule acting at receptor level.

The experiments were performed partly by autoradiography, partly on membrane preparations.

1. *Autoradiography. Tetrahymena pyriformis* GL cells, maintained in 0.1% yeast extract containing 1% Bacto tryptone medium (Difco, Michigan, U.S.A.) under continuous shaking at 28 °C, were used. One-day mass cultures were treated or not treated with 1 mM diazepam solution (in ethanol) for 24 h (the ethanol content of the medium was 0.1%). After treatment the cells were washed twice by centrifugation, and were returned to plain medium for one day or one week (with a single passaging in the latter case). Finally the pretreated and not pretreated mass cultures were incubated in the presence of ^{3}H-diazepam (N-methyl-^{3}H, Amersham, England; sp. act. 3.15 TBq/mmol), added at 0.1 ng/ml, 1 ng/ml or 10 ng/ml concentration for 30 min. At the 1 ng/ml level of treatment, part of the cultures were additionally treated with a 10-, 100- or 1000-fold amount of non-labeled (cold) diazepam.

The cells were incubated for 30 min at 4 °C, fixed in 2% glutaraldehyde (in Losina solution), embedded in araldite, and cut into semi-thin sections. These were coated with Ilford G5 emulsion and exposed for 6 months, then developed in an ORWO R9 developing solution. Quantitative autoradiography was based on grain counting exclusively above those cells whose nucleus was visible in the longitudinal section. Inter-group differences were analyzed for significance with Student's two-sample t-test.

2. *Examination of cytoplasmic membrane preparations. Tetrahymena* cells treated with 10^{-6}M diazepam for 48 h were washed in three changes of sterile Losina solution, and were returned to fresh plain medium for culturing for a further period of 48 h at 28 °C, under mild shaking. A control series of untreated cells was set up in parallel in diazepam-free medium. The cells were washed and transferred to fresh medium after 48 h, exactly like the treated series. The cell density of the 48-h cultures was 10^5/ml.

For binding studies the cells were separated from the nutrient medium by centrifugation at 400 × g for 10 min and pellicle preparations were made as described by Nozawa and Thompson [17]. The homogenate was centrifuged at 100 × g for 10 min at 4 °C, the supernatant and the layer above the whole-cell pellet was withdrawn, the sedimented cells were washed in two changes of 10 mM TRIS-HCl buffer (at 10,000 × g for 10 min at 4 °C) and were resuspended in the same buffer solution. The protein content of the suspension, determined according to Lowry et al. [18], was 1.84 mg/ml.

For determination of ^{3}H-diazepam binding we incubated 160 µl pellicle preparation in the presence of 20 µl 20 nM ^{3}H-diazepam and 20 µl 20 µM non-labeled diazepam or 20 µl buffer for 2 h at 4 °C. After incubation the suspension