BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Cholera toxin stimulates type II pneumocyte proliferation by a cyclic AMP-independent mechanism

Bruce D. Uhal [a],*, Michael Papp [a], Kevin Flynn [a], Mary E. Steck [b]

[a] *Lung Cell Kinetics Laboratory, Cardiovascular Institute, Michael Reese Hospital, 2929 S. Ellis Ave., Rm. 405KND, Chicago, IL 60616, USA*
[b] *Department of Cellular and Molecular Physiology, M.S. Hershey Medical Center, Hershey, PA 17033, USA*

Abstract

Cholera toxin (CT) stimulated DNA synthesis by low-density primary cultures of adult rat type II pneumocytes (T2P) in a dose-dependent manner, either in the presence or the absence of serum. In the presence of 1% rat serum, 1 µg/ml CT also stimulated a 50% increase in cell number over 8 days of incubation ($P < 0.01$); this was in addition to a 2-fold increase in cell number induced by the serum alone ($P < 0.05$). The same dose of CT also elevated intracellular cAMP and the total activity of protein kinase A (both $P < 0.01$), suggesting toxin stimulation of T2P proliferation by a cAMP-dependent mechanism. However, the effect of CT on DNA synthesis could not be mimicked by 8-bromoadenosine 3′:5′-cyclic monophosphate (8-bromo-cAMP), nor by N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl-cAMP), each tested over a wide range of concentrations. L-Isoproterenol stimulated surfactant secretion by over 5-fold ($P < 0.01$), but neither the β-agonist, forskolin nor 3-isobutyl-1-methylxanthine had any significant effect on DNA synthesis. The purified B-subunit of CT stimulated DNA synthesis to the same degree as did the holotoxin, either in the presence or the absence of rat serum. In contrast, the purified A-subunit had no significant effect. These data suggest that cholera toxin stimulates type II pneumocyte proliferation through a mechanism that is independent of cAMP, protein kinase A and toxin-catalyzed ADP-ribosylation. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Alveolar epithelium; Signal transduction; ADP-ribosylation

1. Introduction

Proliferation and differentiation of type II pulmonary alveolar epithelial cells is believed to be responsible for normal turnover of the alveolar epithelium and alveolar repair after lung injury [1]. Various forms of lung injury result in the proliferation of type II cells, which are believed to replenish both the type I and type II alveolar epithelial cell populations [2,3]. The rate of alveolar repair by this mechanism is an important determinant in the development of pulmonary fibrosis [4], and uncontrolled proliferation of type II pneumocytes may lead to lung carcinoma [5]. Thus, an understanding of the regulation of type II cell proliferation is crucial to the definition of normal and abnormal lung injury/repair mechanisms. Further, elucidation of signaling pathways involved in both positive and negative control of this process will provide a foundation from

---

* Corresponding author. Fax: (312) 7912788;
E-mail: bdu1@earthlink.net

which to design therapeutic strategies for the manipulation of alveolar repair.

A high percentage of chemically induced mouse lung tumors are now known to derive directly from type II pneumocytes [5,6]. Studies of mouse lung tumorigenesis have thus provided important insights into the control of type II cell proliferation within these tumors. Glucocorticoids are known to inhibit the genesis of urethane-induced tumors, as well as the proliferation of tumor-derived type II cell lines; the inhibitory action is believed to be mediated by protein kinase C [7]. In studies of tumorigenic type II cell lines [8], both isoforms of the regulatory subunit of protein kinase A were found to be less susceptible to activation by cAMP, as a result of either decreased expression (RI subunit) or altered state of phosphorylation (RII). These and other observations have led to the hypothesis that the cAMP/protein kinase A-mediated pathway is inhibitory to the growth of type II cell-derived mouse lung tumors [8,9].

In related work, a number of laboratories have adopted the model of type II cells in primary culture to investigate the influence of extracellular matrix components [10,11], purified polypeptide growth factors [12,13] and other growth-regulatory substances on the proliferation of these cells. The most potent of these appear to be factors which act through tyrosine kinase type receptors, such as those for epidermal growth factor [14], insulin-like growth factor I [15] and the fibroblast growth factors [12]. DNA synthesis by primary adult rat type II cells is also stimulated by cholera toxin [13], which was shown earlier [16] to stimulate surfactant secretion in primary cultures by a cAMP-dependent mechanism. Together, these observations suggested a stimulatory role for cAMP/protein kinase A in the proliferation of primary type II cells, in contrast to the type II cells within chemically induced mouse lung tumors. As a test of that hypothesis, we studied the influence of membrane-permeable cAMP analogs and agents capable of elevating intracellular cAMP on type II cell proliferation in primary culture. In this report we demonstrate that cAMP has no apparent influence on the proliferation of primary type II cells, and conclude that the stimulatory action of cholera toxin is mediated through a cAMP-independent mechanism.

2. Materials and methods

2.1. Animals and reagents

Male Sprague-Dawley rats (200 g) were obtained from Charles River Laboratories and were housed in the Department of Comparative Medicine, the Milton S. Hershey Medical Center as described previously [17]. Materials for cell isolation and culture were purchased from sources described elsewhere [17]. Cholera holotoxin (type Inaba B) was obtained from Calbiochem (La Jolla, CA). Purified A-subunit and B-subunit of cholera toxin, forskolin, L-isoproterenol and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO), as were 8-bromoadenosine $3':5'$-cyclic monophosphate (8-bromo-cAMP), $N^6,2'$-O-dibutyryladenosine $3':5'$-cyclic monophosphate (dibutyryl-cAMP), and deoxyribonuclease I (DNase I). Cell dissociation solution (PET) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-vimentin monoclonal antibody (clone V9), monoclonal antibody AE3, FITC- and rhodamine-conjugated anti-mouse IgG and 5-bromo-2-deoxyuridine were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were of reagent grade, or were purchased from sources described earlier.

2.2. Pneumocyte isolation and culture

Type II pneumocytes were isolated by a protocol described previously [17]. Briefly, rat lungs were perfused in situ to remove blood and were then excised. The isolated lungs were instilled three consecutive times with 10 ml of a mixture of elastase (30 U/ml, ICN Nutritional Biochemicals, Cleveland, OH) and 0.05% $BaSO_4$ for a total of 20 min incubation at 37°C. The lungs were then reinstilled with 10 ml of a mixture of 50% fetal bovine serum (FBS) and DNase I (240 µg/ml) to inhibit the elastase reaction. After 5 min at 20°C, the lobes were freed of large airways and other tissues and minced to produce 1 mm^2 fragments. The minces were agitated for 10 min at 20°C and filtered through 160 µm nylon mesh. The resulting suspension was centrifuged for 6 min at $315 \times g$, and the cell pellet resuspended in Dulbecco's modified Eagle's medium (DMEM) and layered over a 2-step gradient (density = 1.04 over

1.08) of Percoll in DMEM. The gradient was centrifuged at $400 \times g$ for 20 min at 4°C, and a fraction of cells enriched in type II pneumocytes was recovered from the 1.04–1.08 interface. The cells were washed once, resuspended in DMEM plus 10% FCS and plated at $3–4 \times 10^6$/ml for 30 min to remove macrophages [18]. Depending on experimental requirement, non-adherent cells were either recovered directly or plated on tissue culture plastic at the indicated densities in DMEM plus 10% FBS. After 24 h at 37°C (referred to as culture day 1), the adherent cells were washed and the medium was replaced with RPMI 1640 supplemented with the indicated sera. The medium was changed every 48 h in experiments of longer than 72 h duration. Rat sera were collected from the same animals as those used for cell culture by cardiac puncture immediately before in situ perfusion of the lungs. On culture day 1, adherent type II cells were identified as more than 95% pure by positive acridine orange staining [18] and immunoreactivity to monoclonal AE3, as well as negative anti-vimentin immunoreactivity [19]. Prior to immunocytochemistry, adherent cells were fixed with 70% ethanol at −20°C. The cells were washed and then incubated with a solution of 1% bovine serum albumin (BSA) for 1 h at 37°C. Monoclonal antibodies were then applied for 1 h at 37°C at 1/100 dilution in 1%BSA/PBS. Secondary antibodies consisting of rhodamine-conjugated anti-mouse IgG were applied for 30 min at 37°C.

2.3. Toxin and agonist incubations

Cell incubations with cholera toxin were conducted essentially as described previously [16]. Before application to cultured cells, cholera holotoxin or purified subunits were activated at 37°C for 10 min in the presence of 5 mM dithiothreitol (DTT). Culture media for all toxin and control incubations therefore contained 100 μM DTT, which had no effect on DNA synthesis (not shown). Dimethylsulfoxide (DMSO) was used as the diluent for forskolin, dibutyryl-cAMP and IBMX; similarly, DMSO had no effect when included in control incubations in the absence of agonist. All incubations with toxin or agonists were conducted by the same general design: type II cells were cultured for a total of 72 h; toxin, agonist (or vehicle) were added to the medium after

24 h, and labeled thymidine was added after 48 h. At 72 h, the cells were washed and harvested with trichloroacetic acid and sodium hydroxide for radioactivity and/or protein determinations as described earlier [16]. Alternatively, cells were harvested with ethanol for isolation of disaturated phosphatidylcholine [20] or with 1 N HCl for determination of intracellular cAMP [16]. For determination of total protein kinase A activity, cells were harvested after 1 h of incubation by scraping into cold PBS containing 1 μM phenylmethylsulfonyl fluoride (PMSF). The resulting cell suspension was then sonicated at 0°C for 60 s with a Branson sonicator and the sonicates were stored at −20°C.

2.4. Assays and general methods

Cell number was measured by flow cytometric counting of cell nuclei prepared from adherent cells as described earlier [19]; the effect of cholera toxin on cell number was determined after 8 days of incubation. Rates of cell cycle progression were obtained by flow cytometric analyses of the relative movement (RM) of 5-bromo-2-deoxyuridine (BrdUrd)-positive cells over 5 h of in vitro pulse-labeling as described earlier [22]. Briefly, the cells are pulse-labeled with 10 μM BrdUrd in the indicated culture media for 1 h. At 2.5, 5 and 7.5 h after the end of the pulse period, the cells are quickly trypsinized from the culture surface, fixed in 70% ethanol and prepared for bivariate FACS analysis of DNA content versus BrdUrd content with propidium iodide and anti-BrdUrd-fluorescein as described earlier (DNAdist). The rates of S-phase and G_2/M-phase transit are calculated from the relative movement (RM) of the BrdUrd-positive cell population with respect to the BrdUrd-negative diploid (G_0/G_1) and tetraploid (G_2/M) subsets over the 7.5 h post-pulse interval. Time-dependent movement of the BrdUrd-positive population toward the G_2/M position indicates replicative DNA synthesis, whereas BrdUrd incorporation without movement indicates DNA repair. Similarly, a time-dependent disappearance of BrdUrd-positive tetraploid cells together with a reciprocal appearance of BrdUrd-positive diploid cells indicates mitoses of the BrdUrd-positive cohort. The slopes and x-intercepts of lines plotted through the 2.5, 5 and 7.5 h time points yield rate information (for details, see

[22]). Cell cycle transit data were obtained on day 4 of primary culture.

Cellular protein and incorporated radioactivity were assessed by standard methods as described previously [17]. Lactate dehydrogenase was measured by a spectrophotometric method [16] applied to aliquots of cell-free medium collected over the last 48 h of culture. Total protein kinase A activity was measured in cell sonicates with a peptide pseudosubstrate assay (Upstate Biotechnology, Lake Placid, NY) performed in the presence of excess exogenous cAMP. Intracellular cAMP was assessed with a colorimetric immunoassay kit (Advanced Magnetics, Cambridge, MA) applied to neutralized 1 N hydrochloric acid extracts of adherent cells [16]. Surfactant secretion was measured as described earlier [16] as the percentage of total cellular disaturated phosphatidylcholine released from prelabeled cells over a 90 min incubation in the presence of agonist or vehicle.

3. Results

3.1. Type II cell proliferation in low-density primary culture

Previous efforts to induce type II pneumocyte proliferation in vitro have been unsuccessful, resulting in stimulation of DNA synthesis followed by cell cycle block in S-phase [21] or G_2/M-phases [12,22]. In the present study, we therefore employed a low-density primary culture system which supports cell division. As shown in Fig. 1 (bottom panel), adult rat type II cells increased in number by 3-fold over 8 days in culture in the presence of 1.0% rat serum, provided that the cells were cultured at a density no greater than 0.2×10^5/cm^2. The observed increases in cell number were not significantly influenced by changes in the number of binucleated cells under these conditions (see Section 3.2). Medium supplemented with 1.0% fetal bovine serum (FBS) had no effect on cell number, despite the fact that this medium could stimulate DNA synthesis (not shown).

Cells cultured at a density just 3-fold higher (Fig. 1, upper panel) did not increase in number, even in the presence of rat serum, and despite the fact that they were only 50–60% confluent after 8 days in culture (not shown). Type II cells cultured at the lower
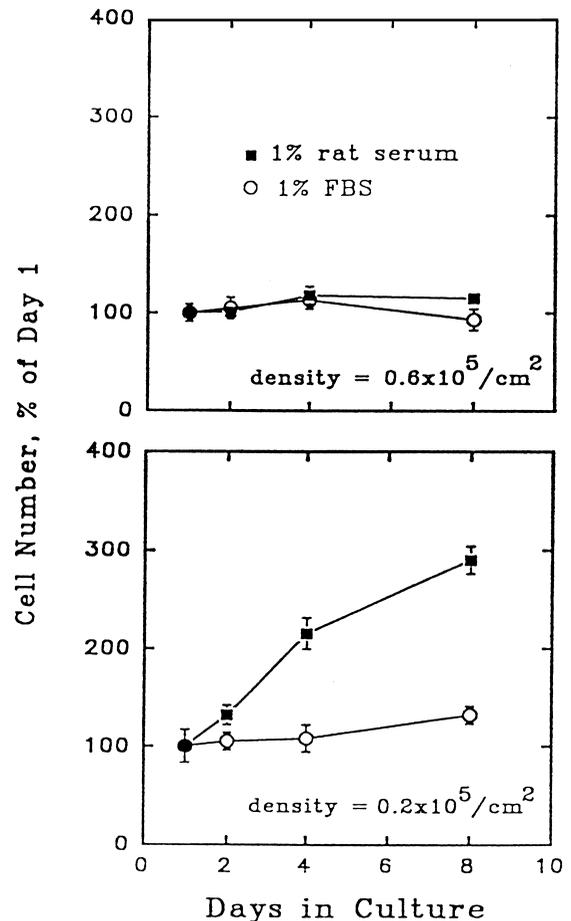

Fig. 1. Primary adult rat type II pneumocytes cultured at low density proliferate in response to normal rat serum. (Top panel) Type II cells plated at 0.6×10^5/cm^2 fail to increase in number despite exposure to rat serum or fetal bovine serum (FBS). (Bottom panel) Cells cultured at lower density $(0.2 \times 10^5$/cm$^2)$ increase 3-fold in number over 8 days in culture in the presence of normal rat serum, but not in the presence of FBS. Cells were isolated and plated on tissue culture plastic as described in Section 2; all data points represent the mean ± S.E.M. of at least three determinations in two separate experiments.

density began to form colonies at 2–3 days after the addition of rat serum and became confluent after 9–10 days. At day 2 after isolation, more than 95% of the cells contained inclusion bodies which stained positively classical lamellar body stains (see Section 2). At confluence, more than 90% of the cells reacted positively with monoclonal antibodies to cytokeratins 7 and 8, but negatively with monoclonal antibodies to vimentin (not shown), confirming the epithelial origin of the cells which proliferated in response to the rat serum. All subsequent experi-
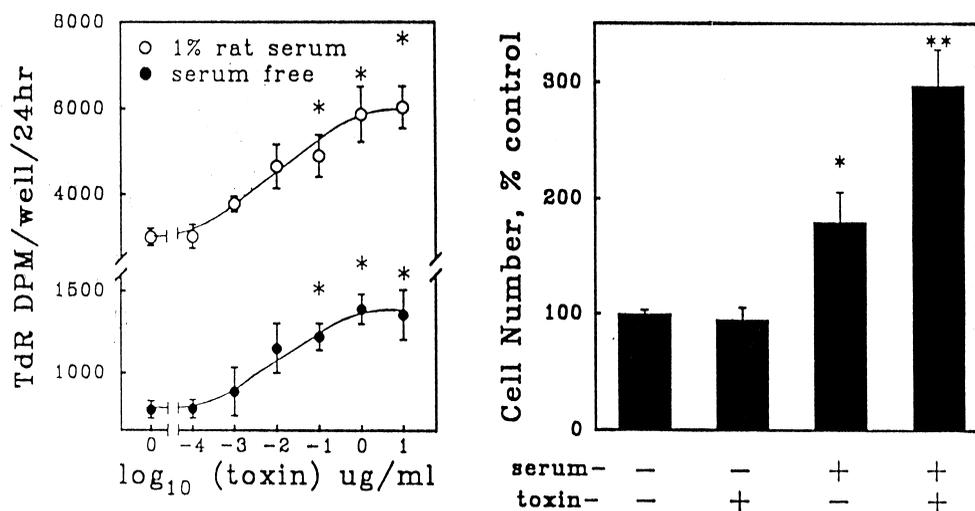
Fig. 2. Cholera toxin stimulates type II pneumocyte DNA synthesis in a dose-dependent manner. (Left panel) In the presence or absence of rat serum, cholera holotoxin stimulates thymidine incorporation into acid-precipitable material. Type II cells were cultured at low density for 72 h; toxin (or vehicle) was added to the medium after 24 h, and labeled thymidine was added after 48 h. At 72 h, the cells were washed and harvested for radioactivity and protein determinations as described in Section 2. Lactate dehydrogenase release was unaffected except at the highest dose of toxin. Values represent the mean $\pm$ S.E.M. of at least three determinations in two separate experiments; *$P < 0.05$ compared to no toxin by Analysis of Variance and Dunnett's test. (Right panel) Normal rat serum stimulated a 2-fold increase in type II cell number when applied over 8 days in low-density primary culture (*$P < 0.05$). The subtoxic dose of 1 μg/ml cholera toxin induced a further increase in cell number in the presence of serum (**$P < 0.01$ vs. serum alone), but had no effect on cell number in the absence of rat serum. Preparation of toxin and counting of cells are described in Section 2.

ments were performed with cells cultured at the lower density of $0.2 \times 10^5/cm^2$.

3.2. Stimulation of type II cell DNA synthesis and cell division by cholera toxin

Cholera holotoxin stimulated type II cell DNA synthesis in a dose dependent manner, either in the presence or absence of rat serum (Fig. 2, left panel). Only the highest toxin dose (10 μg/ml) induced mild cytotoxicity after 24 h of cell exposure (see Section 2); doses one and two orders of magnitude lower were capable of stimulating DNA synthesis by about 2-fold. At the subtoxic dose of 1.0 μg/ml, cholera toxin also induced an increase in type II cell number, provided that it was applied in the presence of rat serum (Fig. 2, right panel). In the absence of rat serum, the toxin had no effect on cell number despite the ability to stimulate thymidine incorporation at the same concentration (left panel). As shown in Table 1, flow cytokinetic analyses of bromodeoxyuridine incorporation (BrdUrd, see Section 2) indicated that cholera toxin by itself (+cholera, serum-free) increased by 10-fold the percentage of cells which

incorporated the thymidine analog. However, the BrdUrd-positive cells without serum exhibited no detectable progression through S- or G_2/M-phases over time (Table 1, denoted NP), suggesting that toxin-stimulated BrdUrd incorporation may have resulted from DNA repair or from aborted initiation of replication. In contrast, the same dose of toxin applied in the presence of serum induced a 5-fold decrease in

Table 1
Effects of cholera toxin on cell cycle progression by type II cells in low-density primary culture

	S-phase[a] (%)	T_S^{b} (h)	$T_{G2/M}$ (h)
Serum-free			
No toxin	0.5 ± 0.4	NP[c]	NP
+cholera (1 μg/ml)	4.2 ± 0.5	NP	NP
1% rat serum			
No toxin	4.3 ± 0.7	12.0	10.3
+cholera (1 μg/ml)	7.7 ± 1.5	7.9	2.1

[a]Determined as % BrdUrd-positive by flow cytometry; values are means $\pm$ S.E.M. of three determinations.
[b]Cell cycle transit times were determined by flow cytokinetic assay as described earlier [22]; T_S, duration of S-phase; $T_{G2/M}$, duration of G2/M-phases.
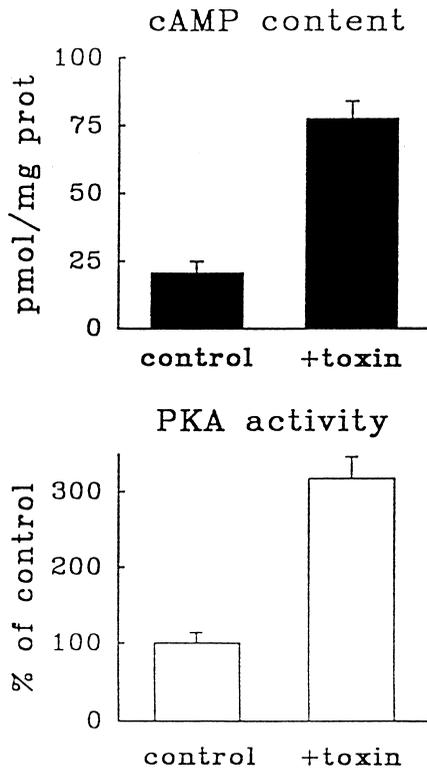[c]NP, no cell cycle progression detectable.

Fig. 3. Cholera toxin elevates intracellular cAMP and total protein kinase A activity in primary type II cells. (Top panel) Type II cells cultured at low density for 24 h (+rat serum) were exposed to a subtoxic dose of cholera holotoxin (1 μg/ml). Five minutes later, cells were harvested with 1 N HCl for determination of intracellular cAMP as described in Section 2. (Bottom panel) Type II cells were cultured and exposed to toxin as described in the panel above. One hour after exposure to toxin, the cells were harvested by scraping and sonication for determination of total protein kinase A activity as described in Section 2. All values represent the mean ± S.E.M. of at least four observations; by Student's t-test, values from toxin-treated cells are significantly different from the control at $P < 0.01$.

type II cell membrane fractions. On the basis of sensitivity toward GTP analogs and their apparent molecular weights, two of these substrates were presumed to be the two isoforms of the stimulatory G-protein Gsα. Under the culture conditions employed in this study (Fig. 3), exposure of type II cells
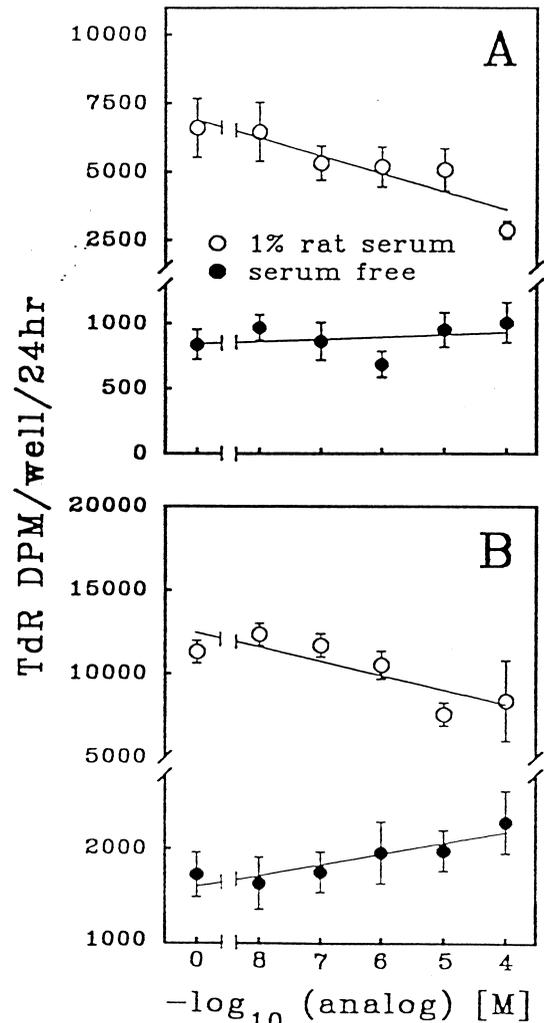

Fig. 4. Cyclic AMP analogs have no effect on type II cell DNA synthesis in low-density primary culture. (A) Type II cells were incubated with the indicated concentrations of 8-bromo-cAMP in the presence or absence of 1.0% normal rat serum. Cells were cultured at low density for 72 h; analog (or vehicle) was added to the medium after 24 h, and labeled thymidine was added after 48 h. At 72 h, the cells were washed and harvested as in the legend to Fig. 2. (B) Type II cells were incubated with dibutyryl-cAMP in the same manner as that described in A. Neither analog significantly influenced DNA synthesis at any concentration; all values represent the mean ± S.E.M. of at least four observations.

the G_2/M-phase transit time ($T_{G2/M}$, +cholera) compared to transit induced by serum alone. The faster rate of cell cycle transit was reflected in the lower percentage of binucleated cells (Table 2) observed in the presence of toxin and serum (although these were not quite significant statistically).

3.3. Stimulation of type II cell adenylate cyclase by cholera toxin

In earlier work with type II cells exposed to cholera toxin in high density culture [16], we identified substrates for toxin-dependent ADP-ribosylation in
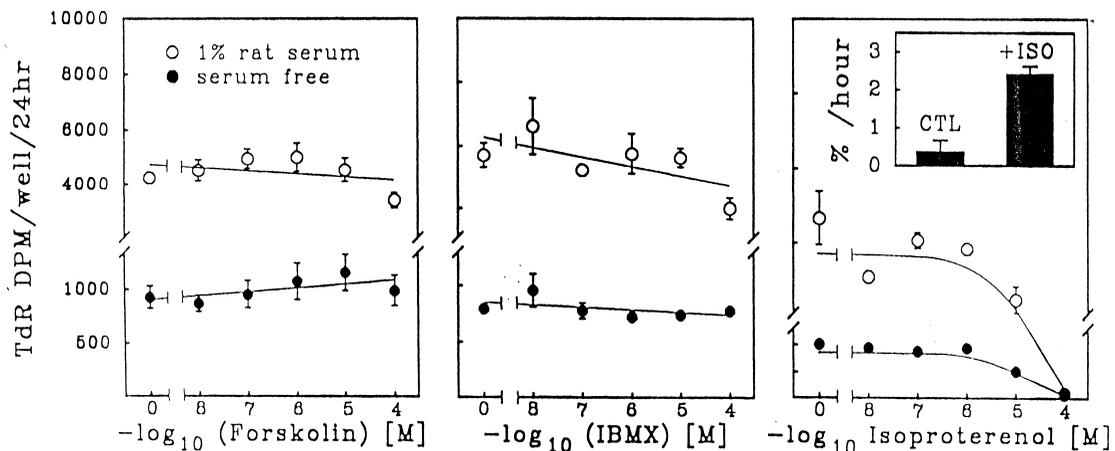
Fig. 5. Agents capable of elevating intracellular cAMP have no effect on DNA synthesis by type II cells in low-density primary culture. Type II cells were incubated with the indicated concentrations of forskolin (left panel), isobutylmethylxanthine (IBMX, middle panel) or isoproterenol (right panel) in the presence or absence of rat serum. Cells were preincubated with each agent for 24 h before measurement of DNA synthesis over the subsequent 24 h as in Figs. 3 and 4. The highest concentration of isoproterenol (10^{-4} M) induced cytotoxicity as measured by lactate dehydrogenase release (see Section 2); all other values were not significantly different from the zero dosage. (Inset) Incubation of type II cells with 10^{-5} M isoproterenol (+ISO) for 1.5 h stimulated secretion of pulmonary surfactant by 5-fold; by Student's t-test, $P < 0.01$ compared to control (CTL). Secretion was measured as the percentage of total cellular disaturated phosphatidylcholine released per hour; all values represent the mean ± S.E.M. of at least four observations.

to the subtoxic dose of cholera toxin (+rat serum) elevated intracellular cAMP (top panel), presumably as a result of activation of adenylate cyclase through the ADP-ribosylation of Gsα isoforms [23]. One hour after exposure to cholera toxin, the same cells exhibited a 3-fold increase in the total activity of protein kinase A (bottom panel), consistent with the premise that the subtoxic dose of toxin was capable of eliciting a protein kinase A-dependent signaling cascade in these cells (see Fig. 5).

3.4. Cyclic AMP independence of the cholera toxin effect

The ability of cholera toxin to elevate intracellular cAMP and total protein kinase A activity suggested that the toxin-dependent stimulation of type II cell proliferation was a cAMP-mediated mechanism. However, neither 8-bromo-cAMP (Fig. 4A) nor N^6-O^2-dibutyryl-cAMP (Fig. 4B) were capable of stimulating DNA synthesis by type II cells in low-density primary culture, either in the presence or absence of rat serum. Not only did cAMP analogs fail to stimulate type II cell DNA synthesis, but so did forskolin (Fig. 5, left panel), isobutylmethylxanthine (IBMX, middle panel), and isoproterenol (right panel), re-

gardless of whether these agents were applied in the presence or absence of rat serum. As shown in the inset (right panel), 10^{-5} M isoproterenol was capable of stimulating surfactant secretion by over 5-fold in replicate wells of the low-density cell preparations, but the same concentration of the β-agonist had no effect on DNA synthesis. Although the 10^{-4} M isoproterenol was significantly inhibitory for DNA synthesis, this dose was also mildly cytotoxic over a 24 h incubation (not shown). Forskolin at 10^{-4} M induced a potent and sustained elevation of intracellular cAMP for up to 18 h of primary culture (Fig. 6), but had no effect on DNA synthesis (Fig. 5).

Table 2
Binucleated type II pneumocytes in low-density culture

Treatment	% of total
Serum-free (SF)	4.88+0.58
+rat serum (RS)	4.00+0.53
+toxin (SF)	3.88+0.30
+toxin (+RS)	3.00+0.42

Data are the mean ± S.E.M. of eight observations from two separate experiments. Values were obtained at 8 days of primary culture under the same low-density conditions as in Fig. 2.
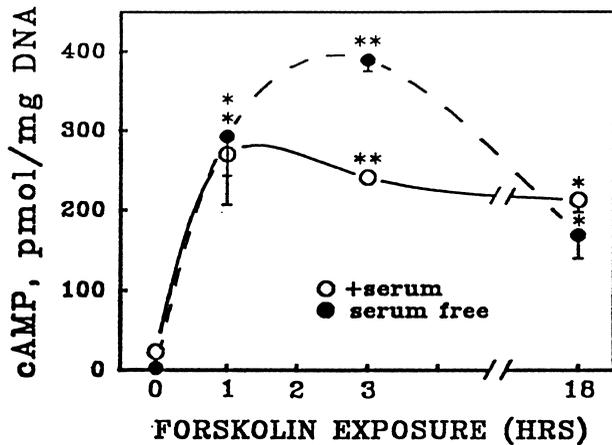
Fig. 6. Forskolin induces sustained elevation of cAMP in primary type II pneumocytes. Type II cells at 2 days of primary culture were incubated with 10^{-4} M forskolin for the indicated number of hours and harvested as described in Section 2. Intracellular cAMP was measured by EIA. *$P < 0.05$; **$P < 0.01$ compared to time zero by Dunnett's test. Values are the mean ± S.E.M.; where not visible, error bars fall within symbols.

3.5. Independence of cholera toxin action on ADP-ribosylation

In a functional sense, the cholera holotoxin consists of two subunits; the A-subunit, which contains the ADP-ribosyltransferase activity, and the B-subunit, which has no known enzymatic activity, but facilitates binding of the holotoxin to the cell surface [23]. As shown in Fig. 7, the purified cholera toxin B-subunit alone was capable of stimulating DNA synthesis by type II cells in low-density culture, either in the presence or absence of rat serum (left panel). In the presence of serum, the B-subunit stimulated DNA synthesis to the same degree as did the holotoxin; in serum-free media, the B-subunit was also stimulatory, but it was roughly 10-fold less potent. In contrast, the purified A-subunit had no significant effect on type II cell DNA synthesis, either in the presence or the absence of serum (right panel).

4. Discussion

The role of intracellular cAMP and protein kinase A in the regulation of cell proliferation remains controversial, largely because of the fact that cAMP is inhibitory for the proliferation of some cells and stimulatory for others. Agents capable of elevating or mimicking intracellular cAMP can inhibit the proliferation of fibroblast cell lines, T-cells and neuroblastoma cells, for example [24], but can potently
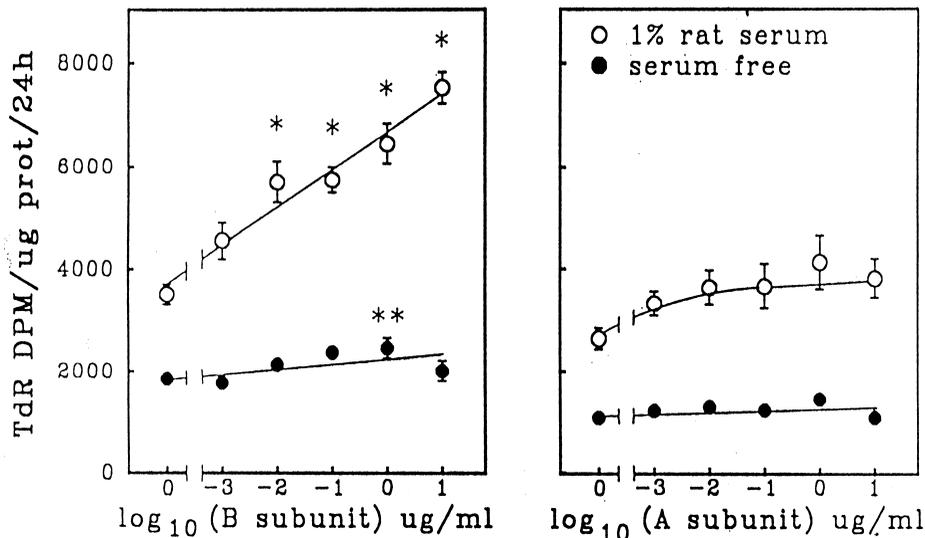

Fig. 7. Purified cholera toxin B-subunit stimulates DNA synthesis by type II cells in the presence or absence of rat serum. Low-density cultures of type II cells were incubated with the indicated concentrations of purified cholera toxin B-subunit (left panel) or purified A-subunit (right panel) in the presence or absence of rat serum. Cells were preincubated with each subunit for 24 h before measurement of DNA synthesis over the subsequent 24 h as in Figs. 2–4. All values represent the mean ± S.E.M. of at least four observations; *$P < 0.01$ and **$P < 0.05$ compared to the zero dose by Analysis of Variance and Dunnett's test. The purified A-subunit had no significant effect either in the presence or absence of rat serum.

stimulate the proliferation of well-differentiated epithelial cells such as the parotid cell [25] or thyrocyte [26]. In these cell types, the stimulatory effect of cAMP is elicited without phosphorylation of the 42–44 kDa mitogen-activated protein kinases [27], which are induced by growth factors which act through receptors coupled to protein tyrosine kinases (PTK) and/or protein kinase C (PKC). Further, those growth factors invoke a pattern of protein phosphorylation that is distinct from that induced by cAMP [27]. Thus, the actions of cAMP on cell proliferation are likely exerted at points downstream from the signal transduction pathways activated by PTK- and PKC-mediated growth factors. Consistent with these observations, recent evidence from rat fibroblast cell lines suggests that cAMP alters the interaction between the protein kinase raf-1 and the activated form of ras, presumably through alterations in the phosphorylation state of raf-1 [28,29]. The relevance of these findings with respect to the variability in cAMP action on cell proliferation remains to be determined.

An inhibitory role for cAMP in the proliferation of type II pneumocytes was suggested by the work of Lange-Carter et al. [8], who have identified alterations in both isoforms of the regulatory subunit of protein kinase A within tumorigenic cell lines of type II cell origin. In some cell lines, the amount of the RI isoform is reduced and the extent of phosphorylation of the RII subunit is increased; these alterations are consistent with reduced susceptibility of the protein kinase A holoenzyme to activation by the cyclic nucleotide [8]. These and other observations have led to the hypothesis that the growth of type II cell-derived tumors is less resistant to the inhibitory action of cAMP on pneumocyte proliferation. To our knowledge, the direct effect of cAMP analogs or cAMP-dependent agonists on the proliferation of type II cells in vivo or in vitro has not been investigated.

Indirect evidence consistent with a stimulatory role for cAMP in the proliferation of primary type II pneumocytes was first provided by Leslie et al. [13], who found that cholera toxin stimulated DNA synthesis by adult rat type II cells cultured at high density. Under the conditions of that study, cholera toxin stimulated DNA synthesis but did not induce increases in cell number. Later work by the same group [12] found that type II cells stimulated with fibroblast growth factors (FGFs) undergo increases in DNA synthesis accompanied by a large increase in the number of binucleated cells. In the present study, the toxin by itself stimulated DNA synthesis by cells with S-phase DNA content, but without cell progression through S- or G_2/M-phases, suggesting induction of DNA repair or initiation of DNA replication that was aborted in the absence of additional growth factors. To our knowledge, such an effect of cholera toxin has not been reported earlier, and may be unique to type II cells. When contrasted with the studies of high density cultures by Leslie et al. [13], the fact that the addition of serum at lower cell densities permitted cell division without the accumulation of binucleated cells suggests that traverse of G_2/M-phases in type II cells is particularly sensitive to cell density. These findings are consistent with our earlier investigations of the type II cell cycle in vivo and in short-term primary culture [22].

Our presumption that the stimulatory effect of cholera toxin was mediated by cAMP was consistent with earlier observations from this laboratory [16], which demonstrated that type II cells possess membrane-associated cholera toxin substrates which were functional in β-agonist-stimulated surfactant secretion. Those studies were conducted with high-density primary cultures; in the present work it was found that the ability of cholera toxin or isoproterenol to elevate intracellular cAMP or surfactant secretion, respectively, were maintained in low-density primary cultures as well. Under these conditions, the failure of cAMP analogs and cAMP-elevating compounds to induce DNA synthesis provide strong evidence of the cAMP independence of the cholera toxin effect on type II cell proliferation.

Cholera toxin action independent of the cAMP-mediated pathway has been described; the toxin inhibits chemotaxis of the RAW264 mouse macrophage cell line [30], enhances the expression of MHC class II molecules by mouse B cells [31] and induces the expression of the immediate-early response gene JE [32], all by cAMP-independent mechanisms. In addition, cAMP-independent toxin effects on cell proliferation have been implicated in several experimental systems. The cholera holotoxin inhibits the growth of human small-cell lung carcinoma cell lines [33], an effect that could not be reproduced with forskolin or cAMP analogs. Further, the purified

B-subunit of cholera toxin inhibited mitogen-stimu-lated B-cell proliferation [31], but enhanced EGF-in-duced DNA synthesis in primary cultures of rat hep-atocytes [34]. The B-subunit of cholera toxin facilitates binding of the cholera holotoxin to the cell surface via the cholera toxin receptor, the G_{M1} ganglioside [23], and the A_1-subunit contains the ADP-ribosyltransferase activity. Thus, cholera toxin effects that are reproducible with purified B-subunit, but not with purified A-subunit, are independent of the activation of adenylate cyclase through ADP-ri-bosylation of $Gs\alpha$.

At present, the mechanism by which the purified B-subunit of cholera toxin stimulates cell prolifera-tion is unclear. The function of the B-subunit within the holotoxin appears to be the aggregation of mem-brane-associated G_{M1} gangliosides to form a pore, through which the A-subunit can gain access into the cell [23]. Evidence from neuronal cells [35], rat lymphocytes [36] and 3T3 fibroblasts [37] suggests that the B-subunit-G_{M1} ganglioside complex modu-lates a rapid rise in intracellular free calcium, which may be involved in the mitogenic response of some cells to the isolated B-subunit. However, other evi-dence suggests that the rise in calcium may not be sufficient to invoke mitogenesis [38]. These observa-tions are consistent with the finding that calcium ionophores can induce expression of c-myc and c-fos, but cannot induce DNA synthesis in the ab-sence of other growth factors [39]. Interestingly, the isolated B-subunit of cholera toxin inhibits the pro-liferation of ras-transformed NIH3T3 cells, but stim-ulates that of quiescent NIH3T3 cells [40]. These findings are paralleled by the observation that chol-era toxin inhibits the proliferation of human lung carcinoma cell lines [33], but as shown in this report, stimulates the proliferation of primary type II cells. The significance of these findings will be interesting topics for future inquiry.

Acknowledgements

References

[1] R.J. Mason, M.C. Williams, Type II alveolar epithelial cells, in: R.G. Crystal, J.B. West (Eds.), The Lung: Scientific Foundations, Raven Press, New York, 1991.

[2] M.J. Evans, L.J. Cabral, R.J. Stephens, G. Freeman, Trans-formation of alveolar type II cells to type I cells following exposure to NO_2, Exp. Mol. Pathol. 22 (1975) 142–150.

[3] I. Adamson, D. Bowden, The type II cell as progenitor of alveolar epithelial regeneration, Lab. Invest. 30 (1974) 35–42.

[4] H. Witschi, Responses of the lung to toxic injury, Environ. Health Perspect. 85 (1990) 5–13.

[5] L.G. Thaete, A.M. Malkinson, Cells of origin of primary pulmonary neoplasms in mice: morphologic and histochem-ical studies, Exp. Lung Res. 17 (1991) 219–228.

[6] S. Rehm, D.E. Devor, J.R. Henneman, J.M. Ward, Origin of spontaneous and transplacentally induced mouse lung tu-mors from alveolar type II cells, Exp. Lung Res. 17 (1991) 181–195.

[7] K.A. Droms, A.M. Malkinson, Mechanisms of glucocorti-coid involvement in mouse lung tumorigenesis, Exp. Lung Res. 17 (1991) 359–370.

[8] C.A. Lange-Carter, A.M. Malkinson, Alterations in the cAMP signal transduction pathway in mouse lung tumori-genesis, Exp. Lung Res. 17 (1991) 341–357.

[9] A.M. Malkinson, M.S. Butley, Alterations in cyclic adeno-sine $3':5'$-monophosphate-dependent protein kinases during normal and neoplastic lung development, Cancer Res. 41 (1981) 1334–1341.

[10] S.R. Rannels, C.S. Fisher, L.J. Heuser, D.E. Rannels, Cul-ture of type II pneumocytes on a type II cell-derived fibro-nectin-rich matrix, Am. J. Physiol. 253 (1987) C759–C765.

[11] S.R. Rannels, J.A. Yarnell, C.S. Fisher, J.P. Fabisiak, D.E. Rannels, Role of laminin in maintenance of type II pneumo-cyte morphology and function, Am. J. Physiol. 253 (1987) C835–C845.

[12] C.C. Leslie, K. McCormick-Shannon, R.J. Mason, Heparin-binding growth factors stimulate DNA synthesis in rat al-veolar type II cells, Am. J. Respir. Cell. Mol. Biol. 2 (1990) 99–106.

[13] C.C. Leslie, K. McCormick-Shannon, P.C. Robinson, R.J. Mason, Stimulation of DNA synthesis in cultured rat alveo-lar type II cells, Exp. Lung Res. 8 (1985) 53–66.

[14] T.O. Messmer, R. Armour, R.W. Holley, Factors influencing the growth of alveolar type II cells isolated from rat lungs, Exp. Cell Res. 142 (1982) 417–426.

[15] A.K. Tanswell, P.J. Byrne, R.N. Han, J.D. Edelson, V.K.M. Han, Limited division of low-density adult rat type II pneu-mocytes in serum-free culture, Am. J. Physiol. Lung Cell. Mol. Physiol. 4 (1991) L395–L402.

[16] V.O. Rybin, B.D. Uhal, L.A. Russo, D.E. Rannels, ADP-ribosylation of type II pulmonary epithelial cell G-proteins, Am. J. Physiol. 260 (1991) L539–L547.

[17] B.D. Uhal, G.D. Hess, D.E. Rannels, Density-independent isolation of type II pneumocytes after partial pneumonectomy, Am J. Physiol. (1989) C515–C521.

[18] B.D. Uhal, S.R. Rannels, D.E. Rannels, Flow cytometric identification and isolation of hypertrophic type II pneumocytes after partial pneumonectomy, Am. J. Physiol. (1989) C528–C536.

[19] B.D. Uhal, D.E. Rannels, DNA distribution analysis of type II pneumocytes by laser flow cytometry: technical considerations, Am. J. Physiol. (1991) L296–L306.

[20] B.D. Uhal, M.D. Etter, Type II pneumocyte hypertrophy without activation of surfactant biosynthesis after partial pneumonectomy, Am. J. Physiol. 264 (1993) L153–L159.

[21] A. Clement, J. Campisi, S.R. Farmer, J.S. Brody, Constitutive expression of growth-related mRNAs in proliferating and nonproliferating lung epithelial cells in primary culture; evidence for growth-dependent translational control, Proc. Natl. Acad. Sci. USA 87 (1990) 318–322.

[22] B.D. Uhal, A flow cytometric study of the type II pneumocyte cell cycle in vivo and in vitro, Cytometry 15 (1994) 46–52.

[23] J. Moss, M. Vaughn, ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial toxins, Adv. Enzymol. 61 (1990) 303–378.

[24] I.H. Pastan, G.S. Johnson, W.B. Anderson, Role of cyclic nucleotides in growth control, Annu. Rev. Biochem. 44 (1975) 491–522.

[25] B.K. Tsang, R.H. Rixon, J.F. Whitfield, A possible role for cAMP in the initiation of DNA synthesis by isoproterenol-stimulated parotid gland cells, J. Cell. Physiol. 10 (1980) 19–26.

[26] F. Lamy, P.P. Roger, R. Lecocq, J.E. Dumont, Differential protein synthesis in the induction of thyroid cell proliferation, Eur. J. Biochem. 155 (1986) 265–272.

[27] J.E. Dumont, J.C. Jauniaux, P.P. Roger, The cyclic AMP-mediated stimulation of cell proliferation, Trends Biochem. Sci. 14 (1989) 67–71.

[28] J. Wu, P. Dent, T. Jelinek, A. Wolfman, M.J. Weber, T.W. Sturgill, Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′-5′-monophosphate, Science 262 (1993) 1065–1069.

[29] S.J. Cook, F. McCormick, Inhibition by cAMP of ras-dependent activation of raf, Science 262 (1993) 1069–1072.

[30] R.R. Aksamit, P.S. Backlund, G.L. Cantoni, Cholera toxin inhibits chemotaxis by a cAMP-independent mechanism, Proc. Natl. Acad. Sci. USA 82 (1985) 7475–7479.

[31] M.L. Francis, J. Ryan, M.G. Jobling, R.K. Holmes, J. Moss, J.J. Mond, Cyclic AMP-independent effects of cholera toxin on B-cell activation, J. Immunol. 148 (1992) 1999–2005.

[32] S.A. Qureshi, K. Alesandropoulos, C.K. Joseph, R. Spangler, D.A. Foster, Cholera toxin induces expression of the immediate-early response gene JE via a cyclic AMP-independent signaling pathway, Mol. Cell. Biol. 11, (1) (1991) 102–107.

[33] G. Kaur, J. Viallet, J. Laborda, O. Blair, A.F. Gazdar, J.D. Minna, E.A. Sausville, Growth inhibition by cholera toxin of human lung carcinoma cell lines: correlation with GM1 ganglioside expression, Cancer Res. 52 (1992) 3340–3346.

[34] H. Mitsui, M. Iwamori, N. Hashimoto, H. Yamada, Y. Ikeda, G. Toda, K. Kurokawa, Y. Nagai, The B-subunit of cholera toxin enhances DNA synthesis in rat hepatocytes induced by insulin and epidermal growth factor, Biochem. Biophys. Res. Commun. 174 (1991) 372–378.

[35] D. Milani, M.C. Minozzi, L. Petrelli, D. Guidolin, S.D. Skaper, P.E. Spoerri, Interaction of ganglioside GM1 with the B subunit of cholera toxin modulates intracellular free calcium in sensory neurons, J. Neurosci. Res. 33 (1992) 466–475.

[36] S.J. Dixon, D. Stewart, S. Grinstein, S. Spiegel, Transmembrane signaling of the B-subunit of cholera toxin: increased cytoplasmic free calcium in rat lymphocytes, J. Cell Biol. 105 (1987) 1153–1161.

[37] S. Spiegel, C. Panagiotopoulos, Mitogenesis of 3T3 fibroblasts induced by endogenous gangliosides is not mediated by cAMP, protein kinase C or phosphoinositide turnover, Exp. Cell Res. 177 (1988) 417–427.

[38] S. Spiegel, Inhibition of protein kinase C-dependent cell proliferation by interaction of endogenous ganglioside GM1 with the B-subunit of cholera toxin, J. Biol. Chem. 264 (1989) 16512–16517.

[39] J.P. Moore, J. Todd, T.R. Hesketh, J.C. Metcalfe, c-fos and c-myc gene activation, ionic signals and DNA synthesis in thymocytes, J. Biol. Chem. 261 (1986) 8158.

[40] S. Spiegel, P.H. Fishman, Gangliosides as bimodal regulators of cell growth, Proc. Natl. Acad. Sci. USA 84 (1987) 141–147.