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ORIGINAL ARTICLE

Influence of melatonin on chicken lymphocytes *in vitro*: involvement of membrane receptors

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Abstract

OBJECTIVES: Time-dependent melatonin effects on chicken lymphocyte proliferation *in vitro* and the involvement of cAMP in melatonin signal transduction were examined.

MATERIALS AND METHODS: Splenocytes and peripheral blood mononuclear cells (PBMC) were cultured *in vitro* in the presence of melatonin, phytohemagglutinin, luzindole, dibutyrylcAMP (dbcAMP), forskolin and vasoactive intestine peptide (VIP). Proliferation was measured by [^{3}H]-thymidine incorporation in cultures carried out for 24, 36, 48 and 72 h. Cyclic AMP formation was assessed by radioimmunoassay in cells incubated for 30 min. or 24 h.

RESULTS: Melatonin stimulated the spontaneous proliferation in short-term (36 and 48 h) splenocyte cultures and had no effect in 72 h cultures. It inhibited mitogen-stimulated proliferation already in 24 h cultures and this effect was observed regardless of the time of the culture. Both melatonin effects were antagonized by luzindole – membrane-bound melatonin receptor antagonist. Forskolin and dbcAMP caused a significant inhibition of proliferation of splenocytes and PBMC cultured for 24 or 72 h, respectively. Melatonin inhibited the cAMP formation (30 min. of incubation) stimulated by adenosine cyclase activators – forskolin and VIP, but added alone failed to affect the cAMP concentration. In mitogen-stimulated splenocytes cultured for 24 h Mel caused an increase in cAMP correlated with the inhibition of cell proliferation.

CONCLUSIONS: Melatonin effects on chicken splenocytes appears time- and activation-dependent: in short-term cultures it stimulates spontaneous and inhibits mitogen-activated proliferation, probably via membrane-bound, luzindole-sensitive melatonin receptors. Incubation with melatonin for 30 min. inhibits cAMP formation, but in 24 h cultures it increases cAMP concentration leading to inhibition of proliferation.

Abbreviations

AC	adenylate cyclase
cAMP	cyclic adenosine monophosphate
dbcAMP	dibutyryladenosine cyclic monophosphate
Mel	melatonin, N-acetyl-5-methoxytryptamine
2-ME	2- mercaptoethanol
MEM	Minimal Essential Eagle medium
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PHA	phytohemagglutinin
PKC	protein kinase C
PTX	pertussis toxin
RZR/ROR	retinoid Z receptor/related orphan receptor
SI	stimulation index
VIP	vasoactive intestine peptide

Introduction

Melatonin (Mel) is the chief hormone produced and released by the pineal gland with the circadian pattern reaching a peak in the dark phase [1]. It plays a crucial role in the regulation of circadian rhythms in all vertebrate groups examined, by synchronizing the organisms with the environmental light conditions [2]. The findings of the last 30 years revealed more complex functions of Mel, including its role in immunoregulation.

It has been shown in the *in vitro* studies that Mel inhibits proliferation of fully activated mammalian and avian lymphocytes [3, 4] as well as the numerous cell lines [5, 6], but stimulatory or no effect were also described [7, 8, 9]. The mechanism(s) whereby Mel exerts its intracellular effect(s) are still unknown and several hypotheses have been proposed. It was found that Mel modulates the cytokine secretion [9, 10], causes an arrest of the cell cycle [11], induces apoptosis [12], and influences the cytoskeleton rearrangement [13]. On the other hand, it acts as a free radical scavenger [14] and an anti-apoptotic agent [15].

The versatility of cellular effects caused by Mel seems to result from the diversity of Mel binding sites and, therefore, from the intracellular signalling pathway involved. Specific Mel membrane binding sites belonging to the superfamily of G-protein-coupled receptors have been identified and cloned [16]. Numerous reports suggest that Mel can inhibit adenylyl cyclase (AC) via pertussis toxin (PTX) sensitive G_i proteins [see 17, 18] albeit Mel-stimulated cAMP accumulation has been found in human 293 cell line transfected with *Xenopus* Mel receptor [19] and in benign prostate epithelial cells [20]. Furthermore, it is known that Mel intracellular signalling is linked to the phosphatidylinositol turnover pathway [21, 22]. On the other hand, it has been demonstrated [23] that the modification of interleukin secretion by Mel was mediated via RZR/ROR nuclear receptors, and the oncostatic effect of Mel (antiproliferative and proapoptotic) on murine transplantable colon cancer was mediated by nuclear (RZR/ROR) receptors as well [12].

In the mechanisms involved in the control of cell proliferation the changes in the intracellular level of cAMP are included. In mammalian lymphoid cells *in vitro* cAMP influences several processes leading to the attenuation of the proliferative response; e.g. cytokine synthesis pattern [24], apoptosis processes [25] and expression of cytosolic factors regulating the cell cycle [26].

Until now, the mechanism of antiproliferative effect of Mel on avian lymphoid cells was not studied. Therefore, the aim of presented here experiments performed on chicken lymphoid cells *in vitro* was to examine: (1) - time-dependent effects of Mel on splenocyte proliferation; (2) - involvement of membrane-bound Mel receptors in its effects on proliferation; (3) - involvement of cAMP in the intracellular Mel signal transduction; (4) - interrelationship between cAMP content and cell proliferation.

Materials and Methods

Animals. The experiments were performed on 2-4-week-old Hi-line male chickens kept from hatching in controlled light (L:D = 12:12) and temperature (28 $\pm$ 2°C) conditions. Standard food and water were available *ad libitum*. Chickens were submitted to the treatment according to the Polish regulations concerning experiments on animals.

Reagents. Melatonin, phytohemagglutinin (PHA), vasoactive intestine peptide (VIP), 2-mercaptoethanol (2-ME), dibutyryladenosine cyclic monophosphate (dbcAMP), forskolin, luzindole and ethanol were purchased from Sigma (St. Louis, MO, USA). Antibiotic-Antimycotic and Lymphocyte Separation Medium were purchased from Gibco BRL (Paisley, UK). Minimal Essential Eagle medium (MEM) and PBS were obtained from Polfa (Lublin, Poland), and ^{3}H-thymidine ([6-^{3}H]dTdR) from UVVVR, Prague, Czech Republic.

Cell preparation. Splenocytes and peripheral blood mononuclear cells (PBMC) were used in the present study. Blood samples collected from jugular vein into sterile tubes containing heparine were layed on Lymphocyte Separation Medium (2:3) and allowed to separate. After centrifugation for 30 min. (200 g) a layer containing PBMC was collected, washed twice with MEM and cells were re-suspended in MEM supplemented with 1% of Antibiotic-Antimycotic and $5x10^{-5}$ M 2-ME.

After chicken decapitation spleens were isolated aseptically, pooled and homogenized with MEM in a glass homogenizer. Single cell suspensions were filtered through nylon meshes to remove the tissue debris and washed 3 times with supplemented MEM medium.

Splenocytes and PBMC were counted in hemocytometer using Natt-Herrick diluent [27] and cell viability estimated by the trypan blue exclusion test. Final suspensions were prepared using cells showing viability ≥ 98%, adjusted with the supplemented MEM medium to the final concentration of 10^7cells/ml.

Cell culture. The cell cultures were prepared and maintained according to the routine established in our laboratory for chicken lymphocyte proliferation [4, 28]. Briefly, lymphocytes (10^6 cells/well) were cultured in triplicate in 96-well microtiter plates (Falcon) in the presence of PHA (16 μg/ml and 31.25 μg/ml), dbcAMP (10^{-4} M), forskolin (10^{-4} M), luzindole (10^{-9} M, 10^{-7} M and 10^{-5} M) and/or Mel (10^{-9} M and 10^{-7} M). Control cultures consisted in the cells incubated with the culture medium alone. Cultures were carried out for 24, 36, 48 or 72 h in 41 °C, fully humidified, 5% CO_2 atmosphere. Prior to the harvesting with semiautomatic cell Harvester (Skatron) cells were pulsed for 18 hours with 1μCi/well of ^{3}H-thymi-

dine (40 MBq/ml). Incorporation of tritiated thymidine was measured by liquid scintillation spectrometry (Beckmann) and expressed as counts per minute (cpm).

Cyclic AMP assay. Cyclic AMP concentration was measured in splenocytes (10^7 cells/ml) incubated for 30 min. at 15°C in a total volume of 1 ml of PBS or cultured for 24 h with the activators of adenyl cyclase (AC): forskolin (10^{-4}M) and VIP (10^{-10} M, 10^{-8} M), PHA (16 µg/ml and 31.25 µg/ml) and Mel (10^{-9} M, 10^{-7} M), alone or in combination of the above mentioned compounds. Control cells were incubated with MEM only.

The reaction was stopped by centrifugation and cAMP was extracted from precipitate with 200 µl of 65% ethanol. Extraction was repeated twice, samples were pooled and ethanol evaporated. After being dissolved in Tris/EDTA the level of cAMP was assessed by radioimmunoassay (Amersham, UK) according to the producer assay protocol.

Statistical analysis. Radioactivity of tritiated thymidine incorporated (cpm) was expressed as mean ± SEM of triplicate cultures as well as a stimulation index (SI) calculated as the ratio of mean incorporation in the presence of mitogen to the mean cpm in the absence of mitogen. Cyclic AMP content (pmol/10^6 cells) was expressed as a mean of 4-6 replications. Analysis of variance (ANOVA) followed by the least-significant difference Student-Newman-Keuls test were used to estimate statistical differences between groups. Correlation coefficient between cAMP content and thymidine incorporation was calculated.

Results

The effect of Mel on spontaneous and PHA-activated splenocyte proliferation was examined in short-term (24, 36, 48 h) and standard (72 h) cultures. Mel alone increased the [^{3}H] – thymidine incorporation but this effect was statistically significant only in short-term cultures (Fig. 1a). Maximal splenocyte response to PHA was observed in 24 h culture and it decreased with time. On the other hand, PHA-activated proliferation was inhibited in the presence of Mel and this effect was the best seen already after 24 h of incubation (Fig. 1b).

In order to examine the involvement of membrane-bound Mel receptors in its effect on splenocyte proliferation, the antagonist of specific MT_2 receptors – luzindole – was added to the splenocyte cultures. In 24 h cultures luzindole antagonized both effects of Mel, i.e. stimulation of spontaneous (Fig. 2a), and inhibition of mitogen-activated proliferation (Fig. 2b).

Subsequently, the effect of Mel on cAMP formation in splenocytes incubated for 30 min. with the common AC activators was assessed. Mel addition caused a decrease in cAMP concentration, stimulated by both forskolin and VIP (10^{-10} M, Fig. 3). Higher dose of VIP (10^{-8} M) did not affect the cAMP content which was significantly elevated in the presence of Mel. Mel alone failed to alter the cAMP formation (insert to Fig. 3).

In the next step lymphoid cell (PBMC and splenocytes) proliferation *in vitro* was examined in the presence of fac-

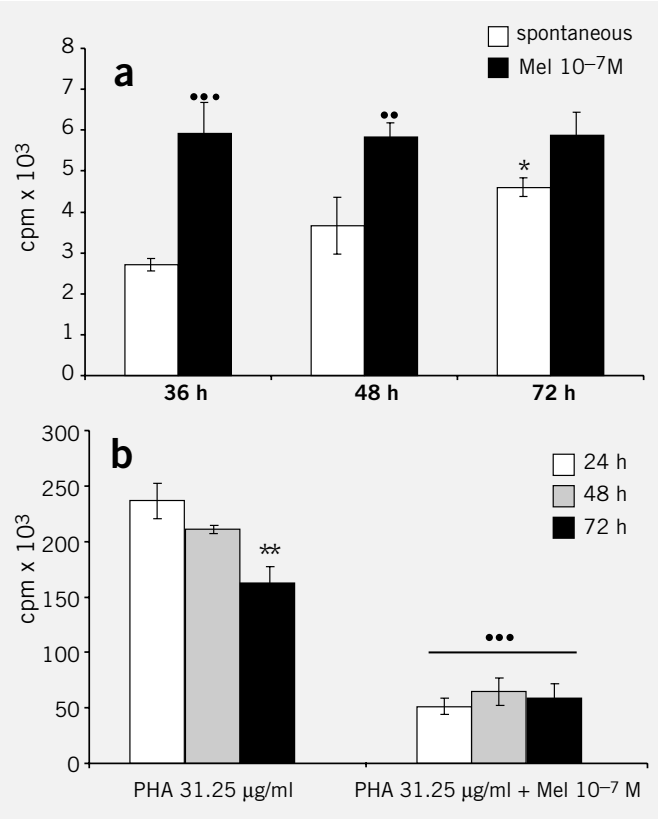

Fig. 1. Effect of Mel on spontaneous (a) and PHA-activated (b) chicken splenocyte proliferation in cultures carried out for 24, 36, 48 and 72 h. •• p< 0.01, ••• p< 0.001 vs. spontaneous (a) or PHA (b) value, * p< 0.05, ** p< 0.01 vs. 36 h (a) or 24 (h) value .

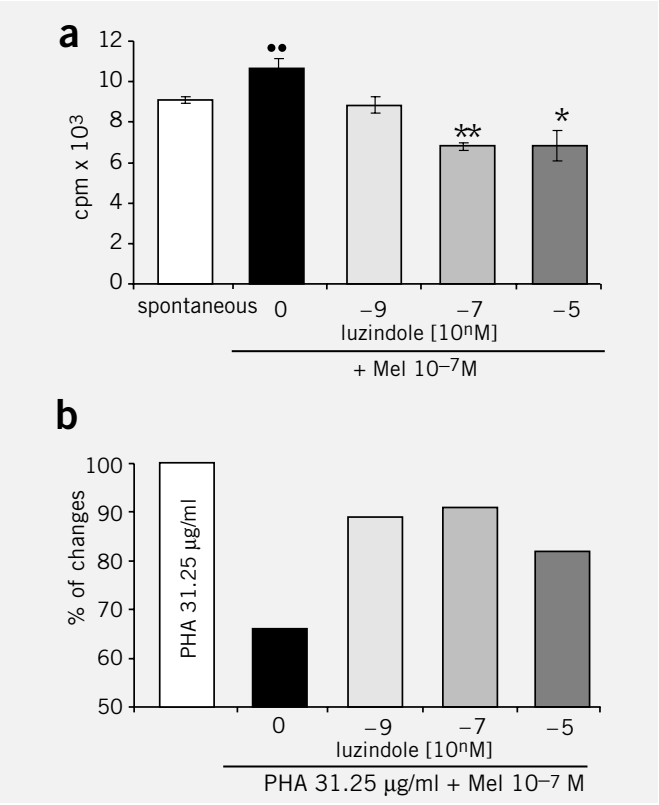

Fig. 2. Effect of luzindole on the proliferation of chicken splenocytes cultured for 24 h in the presence of Mel alone (a) or Mel and PHA (b). •• p< 0.01 vs. spontaneous value, * p< 0.05, ** p< 0.01 vs. Mel value.

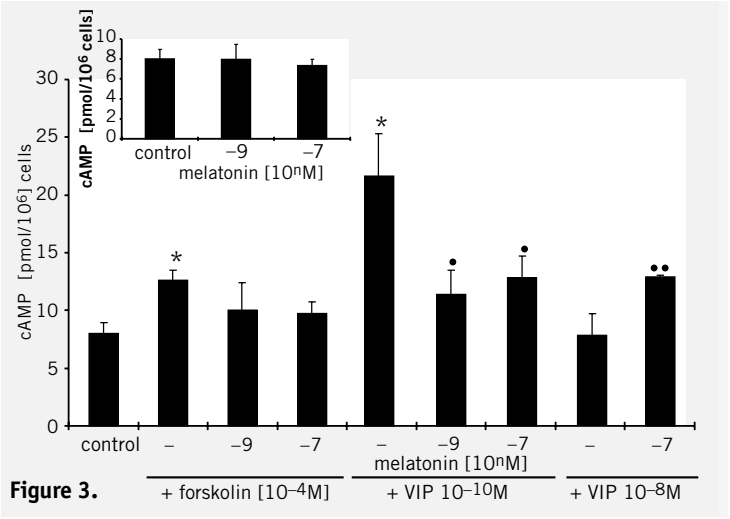

Figure 3.

Fig. 3. The effect of Mel on cAMP concentration in forskolin and VIP stimulated chicken splenocytes. The insert shows the effect of Mel on cAMP concentration in nonstimulated splenocytes. Lymphoid cells were incubate with Mel and AC activators for 30 min. at 15°C. *p< 0.05 vs. control value, • p< 0.01, •• p< 0.01 vs. respective concentration of VIP.

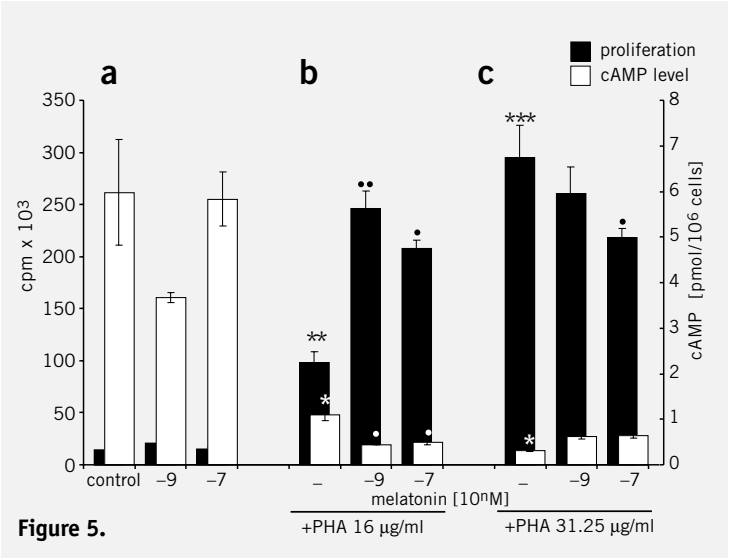

Figure 5.

Fig. 5. Effect of Mel on cAMP level (white bars, right axis) and splenocyte proliferation (black bars, left axis) of control and mitogen-stimulated cells cultured for 24 h with PHA and/or Mel.
* p< 0.05, ** p< 0.01, *** p< 0.001, vs. control value,
• p< 0.01, •• p< 0.01 vs. respective concentration of PHA

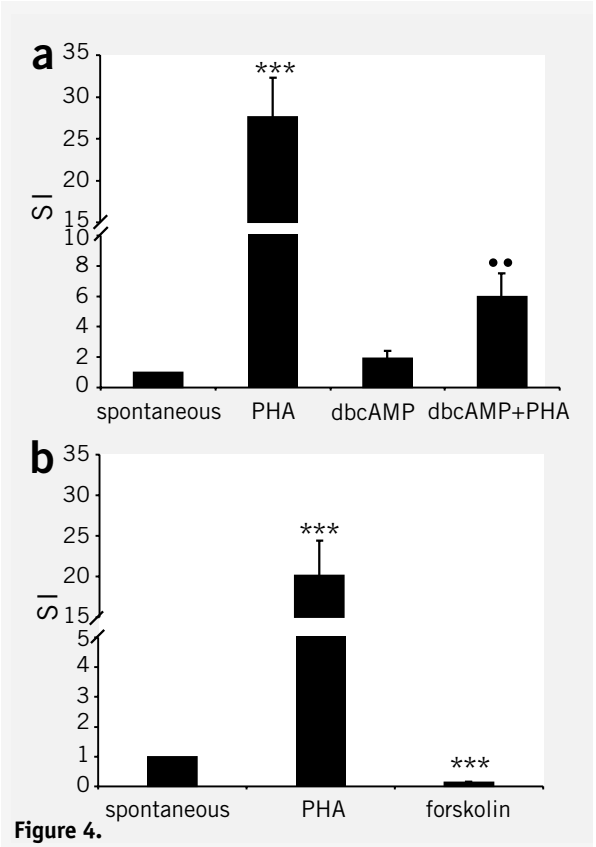

Figure 4.

Fig. 4. Inhibition of avian lymphocyte proliferation by the factors influencing intracellular cAMP concentration: a) PBMCs cultured for 72 h with dbcAMP (10^{-4} M), b) splenocytes cultured for 24 h with forskolin (10^{-4} M).
*** p< 0.001 vs. spontaneous value,
•• p< 0.01 vs. PHA (31.25 µg/ml).

tors influencing the intracellular cAMP concentration. PHA-stimulated PBMC proliferation was inhibited by dbcAMP addition, which, itself, has no effect on spontaneous proliferation (Fig. 4a). Moreover, splenocytes cultured for 24 h in the presence of forskolin exhibited a decrease in proliferation non-stimulated otherwise (Fig. 4b).

In order to examine the correlation between the effect of Mel on cAMP concentration and cell proliferation, 24 h cultures of splenocytes were carried out. In this particular case Mel alone neither influenced splenocyte proliferation nor modified intracellular cAMP content (Fig. 5a – a decrease in cAMP level observed in cell cultured with Mel concentration 10^{-9} M was not statistically significant). Both concentrations of the mitogen used caused an increase in splenocyte proliferation accompanied by a significant fall in cAMP level (Fig. 5b, c) compared to the control (Fig. 5a). In cultures stimulated with lower PHA dose (16 µg/ml), where Mel addition caused an increase in cell proliferation, a decrease in cAMP content was observed (Fig. 5b). On the contrary, in the cells stimulated with higher PHA dose (31.25 µg/ml), the effect of Mel was inhibitory (Fig. 5c). In both cases a negative correlation (R= -0.9) between cell proliferation and cAMP content was found.

Discussion

In previous study we have demonstrated that Mel alone did not alter spontaneous but inhibited PHA-activated chicken splenocyte proliferation in 72 h cultures [4]. In order to examine which step of proliferation is influenced by Mel, chicken splenocyte cultures were carried out for 24, 36, 48 and 72 h. Interestingly, Mel effects consisted of inhibition of PHA-activated and stimulation of spontaneous splenocyte proliferation were observed as early as after 24 h, suggesting Mel involvement in the

regulation of the early steps of cell cycle. As the spontaneous proliferation increased with time of culture, in present experiments (Fig. 1a) and previously [4] we were unable to demonstrate any significant effect of Mel alone in 72 h cultures. Inhibition of PHA-stimulated splenocyte proliferation by Mel, presented herein, is comparable not only with our previous results [4] but also with those observed by Scott et al. [29] in breast cancer MCF-7 cell cultures. However, Scott and co-workers [29] showed that Mel addition diminished the cell number but had no effect on [^{3}H] – thymidine incorporation, suggesting that it induces apoptosis rather than an arrest of the cell cycle. In our experiments we did not assess the effect of Mel on apoptosis and it requires further studies.

According to the pharmacological properties, there are at least two types of membrane-bound Mel receptors: - mt_1 and MT_2 [30]. In chicken splenocytes mainly the sensitive to selective antagonist, luzindole, MT_2 receptors were demonstrated [Olszanska, personal information], and their involvement in Mel effects on proliferation in vitro was shown in the present study (Fig. 2a and b). Membrane-bound Mel receptors in chicken brain [31] and retina [32] as well as in the avian immune system [33] has been demonstrated to be coupled with G_i proteins. To our knowledge the mechanism(s) involved in Mel signal transduction in chicken lymphocytes has been not examined to date, therefore in this present study we have assessed the effect of Mel on cAMP concentration in those cells in different culture conditions. We have found that in chicken splenocytes, like in mammalian lymphoid cells [18], Mel alone did not alter the cAMP level, whereas it inhibited forskolin and VIP-induced increase in cAMP formation (Fig. 3). Interestingly, cAMP content was not modified by higher dose of VIP (10^{-8} M, Fig. 3) alone, but it increased after Mel addition. This apparent discrepancy with our previous supposition that in chicken lymphocytes Mel acts via G_i-coupled receptors may be explained by the well known fact that many G_i-coupled receptors have the ability to stimulate AC II isoform and the formation of cAMP by releasing βγ subunit [34]. Yung and co-workers [19] have described the inhibitory and stimulatory effects of Mel on cAMP formation in human embryonic kidney 293 cells transfected with cloned *Xenopus* Mel receptor. In those cells Mel significantly inhibited stimulated previouslyAC activity, but when the cells where co-transfected with cDNAs encoding the AC II, Mel increased the basal cAMP concentration. Both effects were abolished by preincubation of the cells with PTX. On the other hand VIP receptors are coupled with G_s proteins and their activation leads to release of αs subunits prerequisite to reveal a stimulatory effect of βγ on AC II. Mel has been shown to potenciate the VIP-stimulated cAMP formation in human lymphocytes [35]. Because the involvement of Mel receptors may inhibit or stimulate different isoforms of AC, the net change in cAMP will depend on the relative abundance of the various isozymes of AC. Whether the stimulatory Mel effect on cAMP in chicken splenocytes is due to βγ-mediated stimulation of AC II remains to be determined.

Involvement of cyclic nucleotides in the regulation of cell growth is well documented as an inhibition of ligand-induced lymphocyte proliferation by sustained high level of intracellular cAMP has been demonstrated in different experimental approaches [36]. The results of the present study have demonstrated the existence of the same interdependence in chicken lymphoid cells. Namely, both factors increasing the content of the intracellular cAMP used in the experiment - forskolin and dbcAMP- caused an inhibition of proliferation of splenocytes and PBMCs, respectively.

Finally, a negative correlation between cell proliferation and cAMP level in chicken splenocytes cultured for 24 h with PHA and/or Mel was demonstrated (Fig.5). Mel alone neither influenced the proliferation nor modified cAMP content in unstimulated otherwise splenocytes. Mitogen caused a concentration-dependent increase in the cell proliferation accompanied by a significant decrease in cAMP formation, inversely proportional to the increase of proliferation. Mel addition to the cell cultures stimulated with higher PHA dose caused, as in experiment indicated in Fig. 1b, a significant decrease in cell proliferation, negatively correlated ($r = -0.9$) with an increase in cAMP content. Surprisingly, Mel added to the cell cultured with lower mitogen concentration, thus not fully stimulated, caused an additional increase in proliferation, accompanied by a decrease in cAMP content, but again, this relationship was strongly negatively correlated ($r = -0.9$). For a while it is difficult to explain this stimulatory effect of Mel on chicken proliferation, especially that the negative correlation with cAMP content was still observed.

Above mentioned effects of Mel on cAMP content seemed to be in partial disagreement with results obtained in experiment in which Mel addition to the splenocytes stimulated with forskolin or lower dose of VIP caused a decrease in cAMP formation. It has to be underline, however, that in both experiments the different ways of cell activation and time of exposition to Mel were used. When the AC was directly activated (for 30 min.) by forskolin or by VIP, a well-known inhibitory effect of Mel on cAMP concentration was observed (Fig. 3). In 24 h cultures, however, when the splenocytes were activated by PHA, Mel addition increased the level of cAMP like in the presence of ineffective dose of VIP. It has been demonstrated that PHA similarly as βγ subunit of Gi-coupled receptors could activate the phospholipase C β leading to the phosphatidylinositol turnover and finally to the activation of protein kinase C (PKC), which in turn stimulate the AC II isoform, at least in mammals [37, 38]. It is possible that the stimulatory effect of Mel on cAMP concentration in chicken splenic cells cultured with a mitogen for 24 h is mediated by its synergic action with PHA on PKC activity.

In conclusion, it was demonstrated that Mel influences early steps of spontaneous and mitogen-stimulated chicken splenocyte proliferation possibly via membrane-bound Mel receptors as its effects were antagonized by luzindole. In chicken lymphoid cells cAMP is involved in Mel signal transduction: Mel-induced changes in splenocyte proliferation are

negatively correlated with the intracellular cAMP content.

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