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Melatonin Inhibits Release of Luteinizing Hormone (LH) via Decrease of $[Ca^{2+}]_i$ and Cyclic AMP

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Summary

The role of $[Ca^{2+}]_i$ and cAMP in transduction of the melatonin inhibitory effect on GnRH-induced LH release from neonatal rat gonadotrophs has been studied, because melatonin inhibits the increase of both intracellular messengers. Treatments increasing Ca^{2+} influx (S(−) Bay K8644 or KCl) or cAMP concentration (8-bromo-cAMP or 3-isobutyl-1-methylxanthine) potentiated the GnRH-induced LH release and partially diminished the inhibitory effect of melatonin. Combination of the treatments increasing cAMP and calcium concentrations blocked completely the melatonin inhibition of LH release. The combined treatment with 8-bromo-cAMP and S(−) Bay K8644 also blocked the melatonin inhibition of GnRH-induced $[Ca^{2+}]_i$ increase in 89 % of the gonadotrophs, while any of the treatments alone blocked the melatonin effect in about 25 % of these cells. These observations suggest that a cAMP-dependent pathway is involved in regulation of Ca^{2+} influx by melatonin and melatonin inhibition of LH release may be mediated by the decrease of both messengers.

Key words
Melatonin – GnRH – Luteinizing hormone – Cyclic AMP – Calcium – Gonadotrophs

Introduction

Gonadotrophs are target cells of melatonin in the rat during early development. Melatonin inhibits GnRH-induced LH-release from neonatal rat gonadotrophs through a pertussis toxin-sensitive action involving a high-affinity melatonin receptor (Martin and Klein 1976, Vaněček 1988a,b, Vaněček and Klein 1992a). The effect of melatonin is age-dependent, it is abolished in prepubertal and adult pituitary, in parallel with more than 10-fold decrease of the melatonin receptor concentration (Martin *et al.* 1982, Vaněček 1988a). Because melatonin acts through membrane-bound receptors its intracellular effect has to involve second messengers.

Melatonin has been shown to decrease $[Ca^{2+}]_i$ and inhibit cAMP accumulation induced by GnRH in neonatal rat gonadotrophs (Vaněček and Vollrath 1989, Vaněček and Klein 1992a). The mechanism of melatonin inhibition of LH release involves inhibition of GnRH-induced increase of $[Ca^{2+}]_i$, because it is mimicked by inhibitors of Ca^{2+}-influx and is prevented by drugs that increase $[Ca^{2+}]_i$ (Vaněček and Klein 1995). However, we have recently found that the melatonin effect on LH release in cultured neonatal hemipituitaries is blocked by a combination of both (±)-Bay K8644 and 8-bromo-cAMP, while neither of the drugs alone is able to prevent the melatonin effect completely (Vaněček 1995b). This finding suggests that melatonin may act *via* a decrease of both messengers, cAMP and $[Ca^{2+}]_i$, to inhibit LH release from the gonadotrophs.

We have therefore examined this question in detail using dispersed pituitary cells, which allow better reproducibility of the results. To promote the Ca^{2+} influx we have used either KCl-induced depolarization or a selective agonist of L-type calcium channels S(−) Bay K8644.

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To increase the cAMP concentration, either 8-bromo-cAMP, forskolin (FSK) or 3-isobutyl-1-methylxanthine (IBMX) have been used. In order to understand better the mechanisms involved, we have measured not only the release of LH but also the changes of $[Ca^{2+}]_i$ induced by these treatments.

Materials and Methods

Materials and animals

The following were purchased: GnRH, melatonin, ethylenediaminetetraacetic acid (EDTA), L-cysteine, trypsin inhibitor, bovine serum albumin, glucose, N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid] (HEPES), KCl, $CaCl_2$, 8-bromo-cAMP, forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX), S(−) Bay K8644, calcium ionophore A23187, iodogen (Sigma Chemical Co., St. Louis, MO); papain (Worthington Biochemical Corporation, Freehold, NJ); and Minimal Essential Medium (MEM) (ÚSOL, Prague, Czech Republic). [^{125}I]NaI was purchased from Amersham.

Litters of two-day-old female Wistar rats were kept in our facilities in an automatically regulated light:dark cycle (12:12 L:D), with lights on at 06:00 h. Unless otherwise stated, 40 pups (6- to 8-day-old, approx. 16 g) were killed by decapitation between 08:30 and 10:00. Anterior pituitary glands were then rapidly removed under sterile conditions.

Cell cultures

Anterior pituitaries were gently disrupted and dispersed by papain treatment and dispersed cells were collected using a discontinuous albumin gradient and resuspended in MEM (Mandler *et al.* 1990). This method yielded about $8x10^5$ cells per neonatal pituitary gland. The cells were cultured by either of the two methods. For measurements of LH release, the cells were diluted to $4x10^5$ cells/ml MEM containing 5 % foetal bovine serum and 5 % neonatal rat serum. Samples of $2x10^5$ cells in 0.5 ml were plated on 24-well-plates (diameter 16 mm) and incubated overnight at 37 °C in 5 % CO_2/95 % air. The following day, the medium was changed and the drugs were added as 100 x concentrated solutions after 2.5 h preincubation. Cells were incubated for 3 h at 37 °C, and medium was collected for LH assay. For calcium analysis, samples of 10^6 cells were plated on 25 mm cover slips coated with poly-L-lysine, incubated in 5 % CO_2/95 % air, 37 °C and used the following day.

$[Ca^{2+}]_i$ determination

Single cell $[Ca^{2+}]_i$ was determined using the fluorescent dye Fura-2AM (Grynkiewicz *et al.* 1985). The cells were attached to coverslips, washed and loaded with Fura-2AM (1 μM) for 40 min at 3 °C. Cells were then washed twice with buffer A (10 mM HEPES-buffered salt solution containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, pH 7.35). Individual coverslips were transferred into a Leiden coverslip dish with 1 ml of buffer A, and mounted on the stage of an inverted microscope (Nikon Diaphot) attached to a CCD camera. Temperature of the dish was maintained at 31 °C. After 10 to 15 min preincubation, the cells were excited with a light beam from a 100 W xenon lamp filtered through alternating 340 or 380 nm interference filters and their fluorescence was examined under a 40x oil immersion objective and directed to the camera through 510 nm interference filter. Pairs of images were taken at intervals ranging from 3 to 7 s. GnRH and melatonin were added as 5-fold concentrated solutions and changes in $[Ca^{2+}]_i$ were recorded. After the first treatment series the medium was carefully aspirated and cells washed 4 times. The second treatment series was started after about a 10 min recovery interval. All cells showing an increase of $[Ca^{2+}]_i$ after GnRH treatment were classified as gonadotrophs, because they are the only pituitary cells bearing the GnRH receptors.

The ratio of intensities was determined by computer and $[Ca^{2+}]_i$ values were calculated, after background correction, from the equation described by Grynkiewicz *et al.* (1985). Maximal and minimal ratio values (R_{max} and R_{min}) were determined after addition of ionomycine (10 μM) and 5 mM $CaCl_2$ or EGTA, respectively.

LH-RIA

LH was assayed by RIA, using an antiserum and standards (RP-3) provided by the National Hormone and Pituitary Program, NIDDK. Inter- and intra-assay coefficients of variation were 9.2 % and 2.1 %, respectively; the sensitivity of the assay was 8 pg. The data are presented as means ± S.E.M. of released LH (nanograms per well). Statistical evaluations of significance of the melatonin effect were performed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls test (Snedecor and Cochran 1968).

Results

LH release

In neonatal rat pituitary cells, GnRH induced a 4- to 6-fold increase of LH release (Fig. 1). Melatonin had no effect on basal LH release, but decreased the GnRH-induced LH release by 50−60 %. In agreement with the published results (Vaněček and Klein 1995), S(−) Bay K8644 had no effect on basal but potentiated the GnRH-induced LH release from neonatal rat gonadotrophs (Fig. 1, upper part). The inhibitory effect of melatonin on GnRH-induced LH release was

partially reduced at 1 and 10 μM S(−) Bay K8644, as melatonin decreased the GnRH-induced LH release in its presence by 38 and 33 %, respectively. 8-bromo-cAMP (1 mM) or IBMX (1 mM) alone also potentiated the stimulatory effect of GnRH on LH release and partially diminished the inhibitory effect of melatonin (Fig. 1, middle and bottom parts). However, when added together with S(−) Bay K8644, the treatments increasing cAMP completely prevented the melatonin inhibition of LH release. The combination of the cAMP-increasing treatment with S(−) Bay K8644 also increased the basal LH release, and this effect was significantly inhibited by melatonin (Fig. 1, middle and bottom sections).

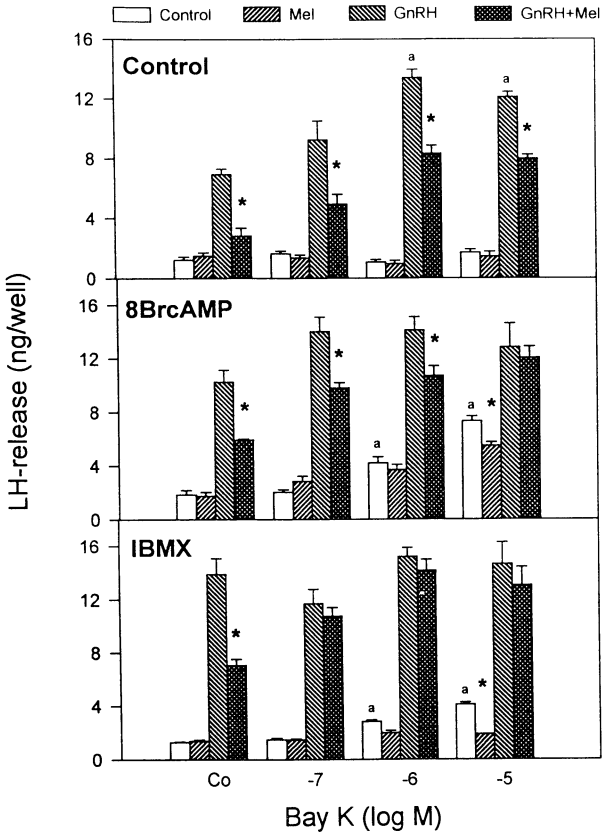

Fig. 1. *Effect of increasing concentration of S(−) Bay K8644 on melatonin inhibition of GnRH-induced LH release in the absence or presence of 8-bromo-cAMP (1 mM) or isobutyl-1-methylxanthine (IBMX; 1 mM). Anterior pituitary cells, prepared and cultured as described, were exposed to one of the following conditions for 3 h: control, melatonin (100 nM), GnRH (2 nM), GnRH (2 nM) + melatonin (100 nM). Each bar represents the mean ± S.E.M. LH release from 3 samples. * Significant effect of melatonin ($p<0.05$) vs control or GnRH alone in the same group. [a] Significant difference ($p<0.05$) vs equal treatment in the absence of S(−) Bay K8644.*

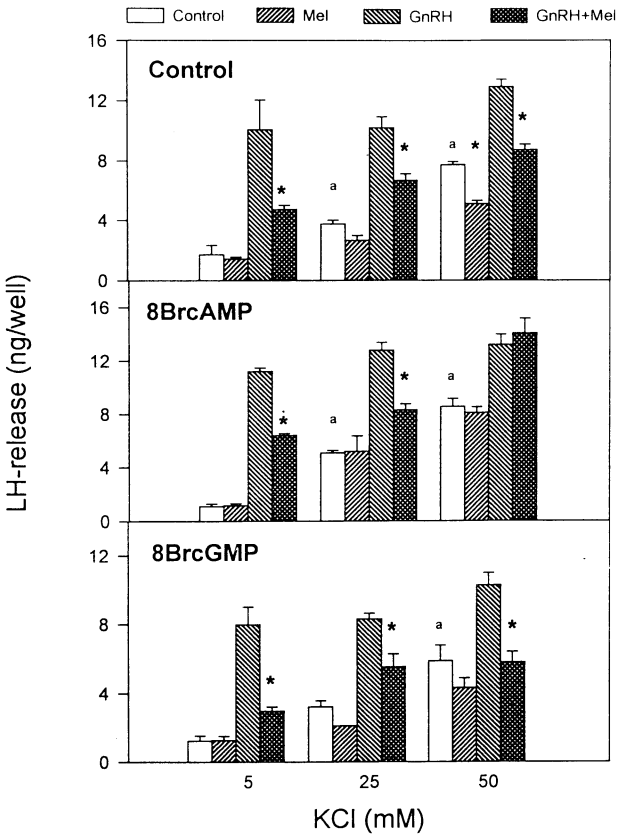

Fig. 2. *Effect of KCl on melatonin inhibition of GnRH-induced LH release in the absence or presence of 8-bromo-cAMP (1 mM) or 8-bromo-cGMP (1 mM). See the legend to Fig. 1 for details. Each bar represents the mean ± S.E.M. LH release from 3 samples. * Significant effect of melatonin ($p<0.05$) vs control or GnRH alone in the same group. [a] Significant difference ($p<0.05$) vs equal treatment in the presence of 5 mM KCl.*

High extracellular K^+ concentration increased $[Ca^{2+}]_i$ in gonadotrophs *via* depolarization-induced Ca^{2+} influx through voltage-sensitive channels (Chang *et al.* 1988, Izumi *et al.* 1993). KCl alone enhanced LH release, a 25 mM concentration to about 2-fold values, 50 mM to about 4-fold values (Fig. 2, upper part). The increase of LH release induced by KCl was significantly inhibited by melatonin. KCl did not potentiate the GnRH-induced LH release, but it diminished the melatonin inhibition of GnRH-induced LH release. In the presence of 50 mM KCl and 8-bromo-cAMP (1 mM), the melatonin inhibition was completely prevented (Fig. 2, middle part). 8-bromo-cGMP had no effect either alone or in combination with KCl (Fig. 2, bottom part).

$[Ca^{2+}]_i$ measurements

An increased intracellular calcium concentration ($[Ca^{2+}]_i$) is of primary importance for releasing LH from the gonadotrophs (Chang *et al.*

1988, Conn *et al.* 1987). Therefore, we have studied the effects of melatonin on the GnRH-induced increase of $[Ca^{2+}]_i$ in the presence of drugs increasing intracellular concentrations of cAMP and Ca^{2+}. The magnitude and shape of the $[Ca^{2+}]_i$ response to GnRH differs considerably among individual cells (Fig. 3), similarly as was seen in primary cultures of pituitary cells by other authors (Rawlings *et al.* 1993, Tomic *et al.* 1994). However, a similar type of response has usually been observed in each cell treated repeatedly with GnRH, although desensitization had sometimes occurred. Based on this observation, we have always compared the GnRH-induced $[Ca^{2+}]_i$ response in the absence and in the presence of drugs increasing the concentrations of cAMP and Ca^{2+} in the same cell.

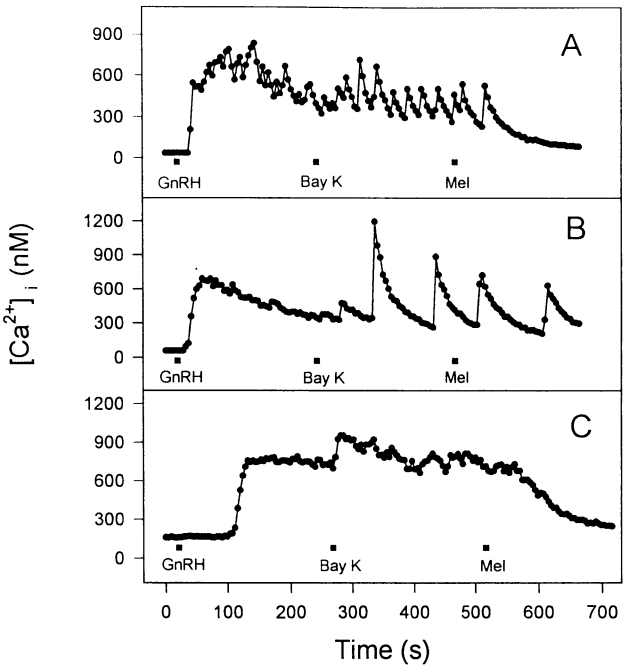

Fig. 3. *Effect of melatonin (Mel; 100 nM) and S(−) Bay K8644 (1 μM) on GnRH (2 nM)-induced increase in neonatal rat gonadotrophs $[Ca^{2+}]_i$. The drugs were added at the indicated times in five-fold concentration and remained present till the end of recording. Three different types of the gonadotroph response are shown. The responses shown in each panel were observed in more than five individual cells.*

S(−) Bay K8644 alone had a small or no effect on resting $[Ca^{2+}]_i$ in most of the cells (data not shown), but it potentiated GnRH-induced $[Ca^{2+}]_i$ increase (Fig. 3). While in the absence of S(−) Bay K8644, GnRH-induced $[Ca^{2+}]_i$ increase in neonatal gonadotrophs was seldom oscillatory in character, oscillations were often observed in its presence (Figs 3A, 3B). In most of the gonadotrophs, S(−) Bay K8644 did not prevent melatonin-induced $[Ca^{2+}]_i$ decrease; in the presence of S(−) Bay K8644 melatonin decreased GnRH-induced $[Ca^{2+}]_i$ in 42 % of gonadotrophs while in its absence in 51 % of gonadotrophs. However, in the presence of S(−) Bay K8644 the lag interval between melatonin administration and its effect was prolonged, as the $[Ca^{2+}]_i$ decrease usually occurred after a longer delay than in control cells.

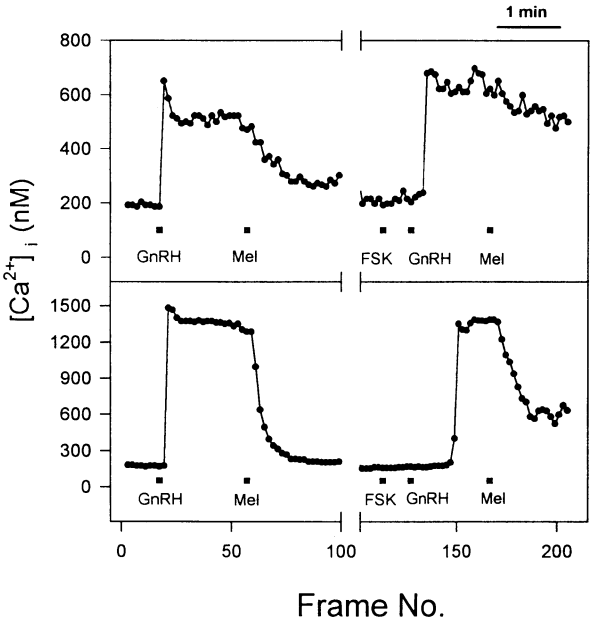

Fig. 4. *Effect of melatonin (100 nM) on the GnRH (2 nM)-induced $[Ca^{2+}]_i$ increase in neonatal rat gonadotrophs in the absence or presence of forskolin (FSK; 10 μM). The drugs were added at the indicated times in five-fold concentration and remained present until the end of the first treatment series marked by the break sign. Then the medium was changed four times and the cells were treated for the second time after about a 10 min recovery period. Forskolin blocked completely (upper panel) or partially (lower panel) the inhibitory effect of melatonin in 28 % of the cells. The responses shown in each panel were observed in more than four individual cells.*

Treatment with 8-bromo-cAMP or FSK had no effect on the resting or GnRH-induced increase of $[Ca^{2+}]_i$ in most of the gonadotrophs. The increase of $[Ca^{2+}]_i$ after administration of 8-bromo-cAMP (Fig. 5A) or FSK (data not shown) was observed in about 20 % of gonadotrophs. Forskolin prevented or reduced the inhibitory effect of melatonin on $[Ca^{2+}]_i$ in 28 % of the melatonin-sensitive gonadotrophs (Fig. 4). Nevertheless, in agreement with our previously published data (Vaněček 1995a), the elevated cAMP did not interfere in most of the cells, with the melatonin inhibition of GnRH-induced $[Ca^{2+}]_i$. In contrast, the combined treatment with S(−) Bay K8644

and 8-bromo-cAMP or FSK increased basal $[Ca^{2+}]_i$ in 39 % of gonadotrophs (Fig. 5B) and prevented the decrease of GnRH-induced $[Ca^{2+}]_i$ by melatonin in 89 % of the melatonin-sensitive gonadotrophs (Fig. 5C). In some gonadotrophs, the combination of 8-bromo-cAMP and S(−) Bay K8644 added after melatonin reversed the melatonin-induced decrease of $[Ca^{2+}]_i$ (Fig. 5D). These observations indicate that melatonin may inhibit the $[Ca^{2+}]_i$ increase through a cAMP-sensitive mechanism.

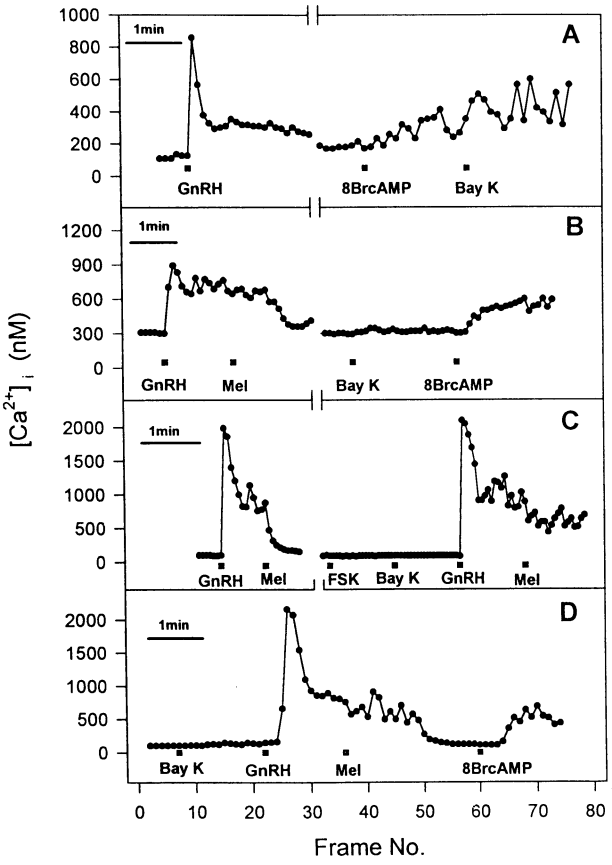

Fig. 5. *Effect of 8-bromo-cAMP (8BrcAMP; 1 mM) or forskolin (10 μM; FSK) alone or in combination with S(−) Bay K8644 (1 μM) on $[Ca^{2+}]_i$ in neonatal rat gonadotrophs. The drugs were added at the indicated times in five-fold concentration and remained present until the end of the first treatment series marked by the break sign. Then the medium was changed four times and the cells were treated for the second time after about 10 min recovery period. Four different types of the response are shown. The responses shown in each panel were observed in more than five individual cells. For details see the legend to Fig. 3.*

Discussion

Melatonin receptors in neonatal rat gonadotrophs are coupled to at least two different intracellular messengers. Melatonin has been shown to inhibit the GnRH-induced increase of intracellular concentrations of cAMP and calcium (Vaněček and Vollrath 1989, Vaněček and Klein 1992a,b). The present data indicate that both intracellular messengers may be involved in the melatonin inhibition of GnRH-induced LH release, because the effect of melatonin is completely blocked by combined treatments increasing cAMP and Ca^{2+} concentrations in the cells, while either of the treatments alone has only a partial effect. The mechanism of the melatonin effect on LH release may thus involve the decrease of both messengers – $[Ca^{2+}]_i$ and cAMP.

The melatonin-induced decrease of cAMP may affect the LH release *via* inhibition of GnRH-induced $[Ca^{2+}]_i$ increase. LH release is driven mainly by the increase of $[Ca^{2+}]_i$ (Chang *et al.* 1988, Conn *et al.* 1987), and melatonin decreases $[Ca^{2+}]_i$ *via* a cAMP-dependent mechanism at least in some gonadotrophs. This conclusion is based on the finding that the melatonin effect on $[Ca^{2+}]_i$ is blocked by treatments increasing cAMP in 28 % of the melatonin-sensitive gonadotrophs and by a combination of the cAMP-increasing treatment and S(−) Bay K8644 in 89 % of the cells.

The mechanism by which cAMP regulates $[Ca^{2+}]_i$ in neonatal rat gonadotrophs is not clear. Two cAMP-dependent pathways have been described in other cells: i) cAMP-dependent kinase may phosphorylate L-type Ca^{2+} channels and prolong their opening (Ammala *et al.* 1993, Inukai *et al.* 1993), or ii) cAMP may open the cyclic nucleotide-gated cationic channels, permeable for Ca^{2+} (Nakamura and Gold 1987, Zufall and Firestein 1993). At present it is not clear which of these mechanisms is involved.

Although the cAMP-dependent mechanism is involved in the melatonin-induced inhibition of Ca^{2+} influx in some gonadotrophs, the decrease of cAMP is not the main mechanism by which melatonin inhibits Ca^{2+} influx. In most of the cells, the drugs increasing cAMP alone did not prevent the melatonin-induced decrease of $[Ca^{2+}]_i$, in agreement with our previously published findings (Vaněček 1995b). However, the mechanism, by which melatonin inhibits the GnRH-induced $[Ca^{2+}]_i$ increase in most of the gonadotrophs, is not quite clear. Melatonin decreases $[Ca^{2+}]_i$ obviously by blocking the influx through voltage-sensitive channels, because the effect is dependent on the presence of Ca^{2+} in the extracellular medium and may be mimicked by calcium channel blocker verapamil (Vaněček and Klein 1992a). The melatonin-induced inhibition of Ca^{2+} influx through voltage-sensitive channels may involve the membrane potential, because melatonin hyperpolarizes the plasma membrane of neonatal rat pituitary cells (Vaněček and Klein 1992b), and hyperpolarization blocks Ca^{2+}-influx through voltage sensitive channels. The mechanism of the melatonin effect may involve Na^+ fluxes, because melatonin does not affect the membrane potential or

$[Ca^{2+}]_i$ in a sodium-free medium (Vaněček and Klein 1992b). Nevertheless, the detailed mechanism of the melatonin effect is unknown.

In our previous paper, high concentrations of Bay K8644 blocked the inhibitory effect of melatonin on LH release almost completely (Vaněček and Klein 1995). However, in these experiments (±)-Bay K8644 has been used, which has combined effects as a Ca^{2+} channel agonist and antagonist. Therefore, some of the channels may have been inhibited by (±)-Bay K8644 and thus became non-responsive to the melatonin treatment, while other channels may have been opened for a prolonged period, letting more Ca^{2+} to enter into the cells. The resulting increase of $[Ca^{2+}]_i$ and decrease in the number of melatonin-responsive channels may have been responsible for the observed disappearance of the melatonin effect on LH release. In fact, the observed biphasic dose-response curve of (±)-Bay K8644 potentiation of GnRH-induced LH release is in agreement with the above hypothesis (Vaněček and Klein 1995).

In conclusion, our data indicate that the mechanism of the melatonin inhibitory effect on LH release involves a decrease of both second messengers $[Ca^{2+}]_i$ and cAMP. The cyclic AMP-dependent pathway seems to be involved in the regulation of Ca^{2+} influx in some of the gonadotrophs and the melatonin-induced decrease of cAMP thus contributes to the decrease of $[Ca^{2+}]_i$ in synchrony with other melatonin transduction pathway(s) inhibiting Ca^{2+} influx.

Acknowledgments

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