Brain Research, 294 (1984) 166–168
Elsevier

BRE 20077

Pineal methoxyindoles depress calcium uptake by rat brain synaptosomes

MARÍA I. VACAS*, MARÍA I. KELLER SARMIENTO and D. P. CARDINALI*

Centro de Estudios Farmacológicos y de Principios Naturales (CEFAPRIN), Serrano 665/669, (1414) Buenos Aires (Argentina)

(Accepted November 15th, 1983)

Key words: pineal gland — methoxyindoles — melatonin — calcium uptake — synaptosomes

The effect of several pineal methoxyindoles on $^{45}Ca^{2+}$-uptake was examined in a crude synaptosome preparation from adult rat brains. 5-Methoxytryptol, 5-methoxytryptamine, melatonin and 6-chloromelatonin (10^{-8}–10^{-6} M) depressed significantly the K^+-stimulated increase in Ca^{2+}-uptake, without affecting the basal, unstimulated Ca^{2+}-uptake by synaptosomes. At 10^{-6} M concentration the following order of potency was found: 6-chloromelatonin ≥ 5-methoxytryptamine > 5-methoxytryptophol ≥ melatonin. Serotonin did not affect significantly either basal or stimulated Ca^{2+}-uptake.

Significant progress has been made in the knowledge on the mammalian pineal physiology during the past few years. Much of this progress lies on the evidence that pineal melatonin is a hormone involved in the regulation of annual reproductive cycles in rodents like the hamster[9] and ungulates like the ram[6]. The sites and mode of action of melatonin are, however, less known. Most experimental evidence points out the brain as the primary locus where melatonin acts[2,14], and high affinity binding sites for the hormone are found either in membranes[4] or in cytosol[8] of several brain regions, particularly the medial basal hypothalamus. At the concentrations known to saturate its binding sites melatonin depresses hypothalamic cyclic AMP[13] and prostaglandin E_2 synthesis[3], and enhances cyclic GMP synthesis[13]. Additionally melatonin impairs the β-adrenoceptor-induced activation of cyclic AMP synthesis in rat astroglial cell cultures[12]. Since in a number of cell preparations prostaglandins and/or cyclic nucleotides appear to be linked to Ca^{2+} metabolism[10,11,15], we considered it worthwhile to examine the effect of a number of pineal indoles on $^{45}Ca^{2+}$-uptake in a crude synaptosomal preparation from adult rat brain.

Female Wistar rats (180–220 g) were kept under light from 07.00 to 21.00 h daily and were given access to food and water ad libitum. The animals were killed by decapitation, and the brains were quickly removed, homogenized in 0.32 M sucrose (1:9 w/v), and centrifuged at 900 *g* for 10 min at 0 °C. Nuclei-free homogenates were further centrifuged at 30,000 *g* for 20 min. The pellet was resuspended in the incubation medium (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM Na_2HPO_4, 0.1 mM $CaCl_2$, 10 mM glucose, and 30 mM Tris-HCl, pH 7.4 at 37 °C) and aliquots (0.2–0.3 mg protein in 450 μl) were incubated for 10 min at 37 °C after adding the different indoles, dissolved in 50 μl of buffer. At the end of the 10-min preincubation period, $^{45}CaCl_2$ (about 0.8 μCi, New England Nuclear, Boston, MA, 5–20 mCi/mg calcium) was added dissolved in 500 μl of incubation or depolarizing buffer (i.e. that in which K^+ concentration was increased up to a final concentration of 65 mM; the increase in K^+ was always compensated for by an equimolar decrease in Na^+). To keep drug's concentration unaltered the $^{45}Ca^{2+}$ medium contained the same indole concentration as used for the preincubations. Incubation with $^{45}Ca^{2+}$ was stopped after 2 min or as indicated by adding 4 ml of iced wash medium (i.e. incubation medium without $CaCl_2$ or glucose but supplemented with 3 mM EGTA) and the mixture was immediately poured onto a Whatman GFB filter under vacuum. The filter was washed thrice with 4 ml of medium.

* Established Investigator, CONICET.
Correspondence: M. I. Vacas, CEFAPRIN, Serrano 665/669, (14 14) Buenos Aires, Argentina.

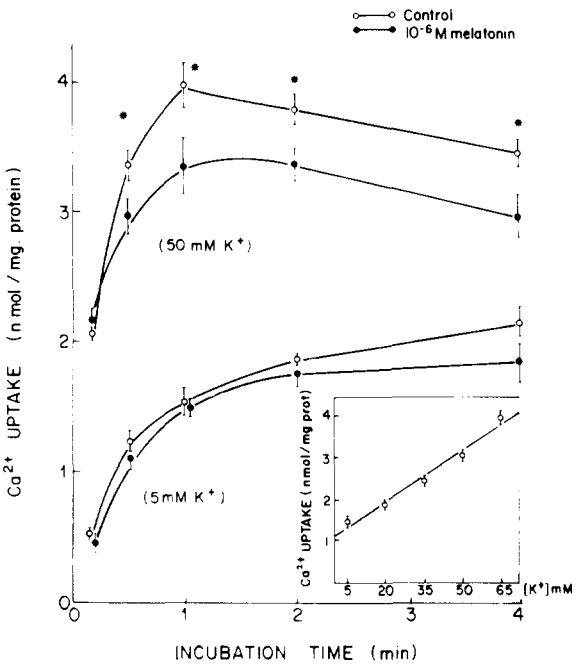

Fig. 1. Time course of $^{45}Ca^{2+}$-uptake by rat brain synaptosomes in the presence (●) or absence (○) of 10^{-6} M melatonin. Ca^{2+}-uptake was determined at 37 °C in an assay system containing 5 mM KCl or 50 mM KCl. The reaction was initiated at 0 min by addition of 0.5 ml media containing 0.8 μCi $^{45}Ca^{2+}$ and 5 or 95 mM KCl to 0.5 ml of preincubated synaptosomes (0.25–0.35 mg protein) in 5 mM. KCl medium. Shown are the means ± S.E.M. (n = 6 in each group). Statistical analysis of results was carried out by a Student's *t*-test. * $P < 0.05$ as compared with respective control without melatonin. Inset: KCl concentration effect curve. Synaptosomes were incubated for 2 min in media containing different KCl concentrations as indicated on the abscissa (n = 6).

and $^{45}Ca^{2+}$ radioactivity on the filter was counted in 10 ml of a toluene–phosphor solution containing 30% Triton X-100 (v/v). K^+-stimulated $^{45}Ca^{2+}$-uptake was calculated as the difference between the uptake in high potassium (50 mM) and low potassium (5 mM). Medium protein was measured by the method of Lowry et al.[7] with bovine serum albumin as a standard. The different indoles were purchased from Sigma Chemicals (St. Louis, MO) and 6-chloromelatonin was kindly supplied by Dr. Michael F. Flaugh (Lilly Research Lab., Indianapolis, IN).

Fig. 1 shows the time course of $^{45}Ca^{2+}$-uptake into rat brain synaptosomes. After a rapid influx during the first 30 s the Ca^{2+}-uptake reached equilibrium at about 1 min. The concentration–effect curve for K^+ ions, measured after 2 min of $^{45}Ca^{2+}$-uptake, is shown in the Fig. 1 inset. Melatonin up to 10^{-6} M failed to affect Ca^{2+}-uptake in low K^+ medium but depressed significantly the uptake stimulated by 50 mM K^+ (Fig. 1).

The effect of several indoles on $^{45}Ca^{2+}$-uptake is shown in Table I. 5-Methoxytryptamine, 6-chloromelatonin, 5-methoxytryptophol and melatonin depressed the uptake stimulated by 50 mM K^+. At 10^{-6} M concentrations 5-methoxytryptamine or 6-chloromelatonin were more potent than melatonin or 5-methoxytryptophol to induce the effect. Serotonin failed to modify $^{45}Ca^{2+}$-uptake by brain synaptosomes. Neither compound affected the basal, unstimulated radionuclide uptake (results not shown).

In synaptosomes voltage-dependent Ca^{2+} channels are activated during stimulation which allows the diffusion of Ca^{2+} into the intracellular space[1]. To evoke neurotransmitter release intracellular Ca^{2+} concentrations must increase transiently about 2 orders of magnitude to a peak of 10^{-6}–10^{-5} M. Subsequently intracellular organelles like the endoplasmic reticulum or the mitochondria provide the buffering system to sequester free Ca^{2+}, thus restoring the resting state[1,15]. Our present results demonstrate that

TABLE I

Effect of pineal indoles on K^+-stimulated $^{45}Ca^{2+}$-uptake by crude synaptosomal preparations

$^{45}Ca^{2+}$-uptake was determined at 37 °C in an assay system containing 5 mM or 50 mM KCl. Incubations with $^{45}Ca^{2+}$ were for 2 min. K^+-stimulated uptake was calculated as the difference between the uptake in 50 mM KCl and 5 mM KCl. Data are expressed as percent of controls (mean ± S.E.M.) with n given in parentheses.

Concentration (nM)	*Melatonin*	*6-Chloro-melatonin*	*5-Methoxy-tryptamine*	*5-Methoxy-tryptophol*	*Serotonin*
—	100.0±0.32 (14)	100.0±1.56 (9)	100.0±0.94 (10)	100.0±0.53 (11)	100.0±0.81 (9)
10^{-8}	92.8±1.26* (14)	87.1±3.04* (8)	92.8±3.90 (8)	89.2±1.76* (10)	99.8±2.47 (6)
10^{-7}	92.76±0.95* (14)	88.2±5.42* (9)	81.8±2.08* (10)	90.3±1.97* (8)	104.4±6.31 (8)
10^{-6}	91.74±1.43* (14)	80.9±2.05* (9)	82.8±3.98* (8)	88.8±2.23* (10)	105.8±6.08 (7)
10^{-5}	94.50±1.12* (14)	93.7±1.20 (10)	94.7±6.68 (11)	106.9±2.24 (9)	101.9±4.79 (9)

* $P < 0.05$ Dunnet's *t*-test.

several methoxyindoles impair the Ca^{2+}-uptake elicited by a depolarizing solution of KCl in rat brain synaptosomes whereas the resting uptake remained unaffected to methoxyindole exposure. Serotonin, whose indole ring is devoid of a methoxy moiety, was essentially inactive on the parameter examined.

In a recent publication[16] melatonin was found to inhibit at nanomolar concentrations dopamine release in slices prepared from several regions of the rat brain. Such an effect was linked to the inhibition by melatonin of maximal Ca^{2+} entry in the same preparation[17]. Our results are compatible with those observations and extend them to other methoxyindoles. Within this context pineal methoxyindoles could be considered typical modulators, that is, substances that set the 'tonus' of target cells to the principal message (in this case the action potential arriving at the terminals), being otherwise incapable of inducing the main effect of the pathway. Observations that melatonin impairs in brain the norepinephrine-stimulated prostaglandin E_2[3], and cyclic nucleotide[12] synthesis in vitro as well as the release of PGE_2 into the CSF evoked in vivo by peripheral stimuli[5] are compatible with this hypothesis. Further experiments are necessary to assess whether the changes by melatonin in cyclic nucleotide or prostaglandin E_2 production are involved in the modification of $^{45}Ca^{2+}$-uptake described herein.

This study was supported by grants from Fundación 'Alberto J. Roemmers', Buenos Aires, and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

1 Borle, A. B., Control, modulation and regulation of cell calcium, *Rev. Physiol. Biochem. Pharmacol.*, 90 (1981) 13–153.
2 Cardinali, D. P., Melatonin. A mammalian pineal hormone, *Endocrine Rev.*, 2 (1981) 327–346.
3 Cardinali, D. P., Ritta, M. N., Fuentes, A. M., Gimeno, M. F. and Gimeno, A. L., Prostaglandin E release by rat medial basal hypothalamus in vitro. Inhibition by melatonin at submicromolar amounts, *Europ. J. Pharmacol.*, 67 (1980) 151–153.
4 Cardinali, D. P., Vacas, M. I. and Boyer, E. E., Specific binding of melatonin in bovine brain, *Endocrinology*, 105 (1979) 437–441.
5 Leach, C. M., Reynoldson, J. A. and Thornburn, G. D., Release of E prostaglandins into the cerebrospinal fluid and its inhibition by melatonin after cervical stimulation in the rabbit, *Endocrinology*, 110 (1982) 1320–1324.
6 Lincoln, G. A. and Almeida, O. F. X., Inhibition of reproduction in rams by long day lengths and the acute effect of superior cervical ganglionectomy, *J. Reprod. Fertil.*, 66 (1982) 417–423.
7 Lowry, O. H., Rosebrough, N. R., Farr, A. L. and Randall, R. J., Protein measurement with the Folin phenol reagent, *J. biol. Chem.*, 193 (1951) 265–275.
8 Niles, L. P., Wong, Y. W., Mishra, R. K. and Brown, G. M., Melatonin receptors in brain, *Europ. J. Pharmacol.*, 55 (1979) 219–220.
9 Reiter, R. J., The pineal and its hormones in the control of reproduction, *Endocrine Rev.*, 1 (1980) 109–131.
10 Rubin, R. P., Calcium–phospholipid interactions in secretory cells: a new perspective on stimulus-secretion coupling, *Fed. Proc.*, 41 (1982) 2181–2187.
11 Rubin, R. P. and Laychock, S. G., Prostaglandins, calcium, and cyclic nucleotides in stimulus-response coupling. In G. B. Weiss (Ed.), *Calcium in Drug Action*, Plenum Press, New York, 1978, pp. 135–155.
12 Vacas, M. I., Berría, M. I., Cardinali, D. P. and Lascano, E. F., Melatonin inhibits β-adrenoceptor-stimulated cyclic AMP accumulation in rat astroglial cell cultures, *Neuroendocrinology*, in press.
13 Vacas, M. I., Keller Sarmiento, M. I. and Cardinali, D. P., Melatonin increases cGMP and decreases cAMP levels in rat medial basal hypothalamus in vitro, *Brain Research*, 225 (1981) 207–211.
14 Waldhauser, F. and Wurtman, R. J., The secretion and actions of melatonin. In G. Litwack (Ed.), *Biochemical Actions of Hormones, Vol. 10*, Academic Press, New York, 1983, pp. 187–225.
15 Westfall, T. S., Neuroeffector mechanisms, *Ann. Rev. Physiol.*, 42 (1980) 383–397.
16 Zisapel, N., Egozi, Y. and Laudon, M., Inhibition by melatonin of dopamine release from rat hypothalamus in vitro: variations with sex and the estrous cycle, *Neuroendocrinology*, 37 (1983) 41–48.
17 Zisapel, N., Laudon, M., Inhibition by melatonin of dopamine release from rat hypothalamus: regulation of calcium entry, *Brain Research*, 272 (1983) 378–381.