Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6200–6203, August 1990
Neurobiology

Expression of melatonin receptors in arteries involved in thermoregulation

(receptor autoradiography/circle of Willis/caudal artery/arterial tone/second messengers)

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Communicated by Irvine H. Page, May 21, 1990

ABSTRACT **Melatonin binding sites were localized and characterized in the vasculature of the rat by using the melatonin analogue 2-[^{125}I]iodomelatonin (^{125}I-melatonin) and quantitative *in vitro* autoradiography. The expression of these sites was restricted to the caudal artery and to the arteries that form the circle of Willis at the base of the brain. The arterial ^{125}I-melatonin binding was stable, saturable, and reversible. Saturation studies revealed that the binding represented a single class of high-affinity binding sites with a dissociation constant (K_d) of 3.4×10^{-11} M in the anterior cerebral artery and 1.05×10^{-10} M in the caudal artery. The binding capacities (B_{max}) in these arteries were 19 and 15 fmol/mg of protein, respectively. The relative order of potency of indoles for inhibition of ^{125}I-melatonin binding at these sites was typical of a melatonin receptor: 2-iodomelatonin > melatonin > *N*-acetylserotonin >>> 5-hydroxytryptamine. Norepinephrine-induced contraction of the caudal artery *in vitro* was significantly prolonged and potentiated by melatonin in a concentration-dependent manner, suggesting that these arterial binding sites are functional melatonin receptors. Neither primary steps in smooth muscle contraction (inositol phospholipid hydrolysis) nor relaxation (adenylate cyclase activation) were affected by melatonin. Melatonin, through its action on the tone of these arteries, may cause circulatory adjustments in these arteries, which are believed to be involved in thermoregulation.**

Melatonin, a hormone produced by the pineal gland of vertebrates, is thought to serve as a neuroendocrine transducer of photoperiodic information (1, 2). Through use of the radioiodinated melatonin analogue 2-[^{125}I]iodomelatonin (^{125}I-melatonin) putative melatonin receptors have been described in specific areas of the brain (3–12) and retina (13) of several vertebrates.

Until now, only neuronal tissues have revealed specific, high-affinity ^{125}I-melatonin binding sites. During our studies on the characterization of ^{125}I-melatonin binding in the suprachiasmatic nuclei of the rat (7, 14), we noticed specific binding sites in the anterior cerebral artery that prompted us to examine various blood vessels for the presence of ^{125}I-melatonin binding sites. In this report, we describe the localization and characterization of high-affinity binding sites for melatonin in the vasculature of the rat. We also demonstrate that these binding sites are functional melatonin receptors.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats weighing 250–300 g were obtained from Zivic–Miller and maintained on a 12-hr light/12-hr dark cycle. Food and water were provided ad libitum. Rats were killed by decapitation. Brain and arteries to be used for binding assays were quickly dissected out and frozen in isopentane kept at −70°C.

Horizontal or coronal sections of the brain and cross sections of the caudal artery (16 μm) were cut in a cryostat at −17°C, thaw-mounted onto gelatin-coated slides, and dried in a desiccator at 4°C overnight.

Binding Assays. The binding conditions used have been described (14). Tissue sections were incubated for 60 min in 50 mM Tris·HCl buffer (pH 7.4) containing 4 mM $CaCl_2$ and ^{125}I-melatonin (specific activity, 1800–2100 Ci/mmol; Amersham; 1 Ci = 37 GBq). Nonspecific binding was determined in the presence of 1 μM melatonin. After incubation, the sections were washed twice for 5 min in 50 mM Tris·HCl buffer followed by 30 sec in distilled water at 0°C. There was no loss of high-affinity binding when tissue sections were washed in Tris·HCl for up to 30 min at 0°C.

In the anterior cerebral artery, ^{125}I-melatonin binding reached equilibrium within 60 min at 22°C and was stable for at least 120 min. Excess of melatonin (1 μM), added at equilibrium, was able to compete with ^{125}I-melatonin for binding to the arterial sites, demonstrating reversibility of the binding. The apparent dissociation constant (K_d) was 1.27×10^{-11} M, calculated as k_2/k_1 (k_1 = association rate constant = 7.5×10^8 $M^{-1} \cdot min^{-1}$, k_2 = dissociation rate constant = 9.5×10^{-3} min^{-1}). Thin-layer chromatography (14) confirmed that the integrity of bound and free ligand was maintained during incubation (not shown).

Autoradiography. Sections were dried after washing and exposed to Hyperfilm-^{3}H (Amersham) for 18–21 days along with 16-μm sections of ^{125}I-labeled Micro-scale standards (Amersham). ^{125}I-melatonin binding was quantified as described (15) by using computerized RAS 1000 research analysis system (Loats Associates, Westminster, MD).

***In Vitro* Contraction.** Helical strips of caudal artery from rats killed by decapitation were maintained in 20-ml muscle baths containing Krebs–Henseleit solution (KHS) of the following composition: 118.0 mM NaCl, 4.7 mM KCl, 1.4 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 11.0 mM glucose (pH 7.4), aerated with 95% O_2/5% CO_2, and kept at 37°C. The development of tension was measured with a Grass FT.03 force displacement transducer and recorded on a Grass model 7 polygraph. Isolated artery strips were equilibrated for 60–90 min under a basal tension of 0.7 g before the addition of norepinephrine (NE). Contractile responses of the strips to 60 mM KCl were determined three times before each experiment. To accurately quantify the effect of melatonin on NE-induced contraction, melatonin was added immediately after the contractile response of the artery had stabilized. All data were expressed as the percentage of the contractile response to KCl (60 mM), which was considered to be 100%.

Inositol Phospholipid Turnover and cAMP Assay. After animals were decapitated, caudal artery was removed and

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Abbreviations: KHS, Krebs–Henseleit solution; NE, norepinephrine.

cleaned in ice-cold KHS. All incubations were performed at 37°C in the presence of 95% O_2/5% CO_2. The inositol phospholipid hydrolysis in the caudal artery was assessed as the accumulation of inositol monophosphates in the presence of 10 mM LiCl (16, 17). The segments (10 mm long) were prelabeled for 2 hr with *myo*-[^{3}H]inositol at 37°C, after preincubation for 30 min in KHS. The artery segments (n = 5) then were washed in KHS containing 10 mM LiCl and incubated for 30 min in KHS with LiCl at 37°C in the presence and absence of NE (10 μM) and melatonin (10 nM). The treatment was terminated by adding ice-cold CCl_3COOH (final concentration, 6%) and transferring the vials to ice. The details of the rest of the procedure were as described before (18). To standardize the variation due to size differences in the segments, inositol monophosphate accumulation was expressed for individual arterial segments by measuring the radioactivity in the inositol monophosphate fraction as a percentage of that associated with membranes.

To determine the effect of melatonin on forskolin-stimulated cAMP formation, caudal artery segments (n = 7) were preincubated for 90 min in KHS at 37°C and then incubated individually in a total volume of 500 μl per segment for 20 min at 37°C with and without forskolin (1 μM) in the presence and absence of melatonin (10 nM). Incubation was stopped by the addition of CCl_3COOH (final concentration, 6%), and the samples were homogenized and centrifuged. The supernatants were washed four times with water-saturated diethyl ether. After lyophilization of the aqueous extract, cAMP was determined by using ^{125}I-labeled cAMP radioimmunoassay kits (Amersham). Protein was determined by a modified Bradford assay (19).

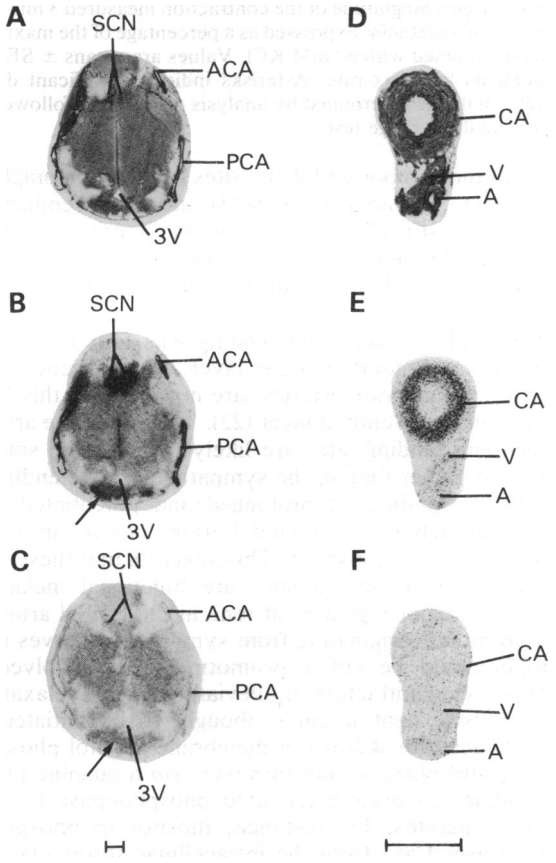

FIG. 1. Autoradiographic analysis of ^{125}I-melatonin binding in horizontal sections of the rat brain showing the anterior cerebral artery (ACA) and posterior communicating artery (PCA), which form part of the circle of Willis (*A–C*), and cross sections of the ventral caudal artery (CA) (*D–F*). (*A* and *D*) Sections stained with hematoxylin and eosin after producing autoradiographic images of ^{125}I-melatonin binding, shown in *B* and *E*, respectively (^{125}I-melatonin at 119–150 pM). (*C* and *F*) Autoradiographic images of sections adjacent to *B* and *E*, incubated with ^{125}I-melatonin in the presence of 1 μM melatonin (nonspecific binding). In cross sections containing the caudal artery, vein (V), and small artery (A), specific binding was seen only in the smooth muscle layer of the caudal artery. SCN, suprachiasmatic nuclei; arrow, pars tuberalis; 3V, third ventricle. (Bars = 0.5 mm.)

RESULTS

Autoradiography revealed specific ^{125}I-melatonin binding in the rostral region of the basilar artery, posterior communicating, middle cerebral, and anterior cerebral arteries (Fig. 1 *A–C*). Horizontal sections through the base of the hypothalamus showed specific binding also in the suprachiasmatic nuclei and pars tuberalis—areas known to contain putative melatonin receptors (6, 10, 14). Several blood vessels were examined for the presence of ^{125}I-melatonin binding. Specific binding was detected in the caudal artery (Fig. 1 *D–F*) but not in the aorta, carotid, coronary (including myocardium), mesenteric, or renal arteries. In the caudal artery, the binding sites were restricted to the smooth muscle layer.

Saturation studies and Scatchard analysis of the data using the LIGAND program (20) revealed a single class of high-affinity binding sites with a K_d of 3.4×10^{-11} M in the anterior cerebral artery and 1.05×10^{-10} M in the caudal artery (Fig. 2). The binding capacities (B_{max}) in these arteries were 19 and 15 fmol/mg of protein, respectively.

2-Iodomelatonin and melatonin had much higher potency than *N*-acetylserotonin in displacing ^{125}I-melatonin binding (Fig. 3). α-Adrenergic compounds (prazosin and yohimbine) and serotoninergic compounds [8-hydroxy-2-(di-*n*-propylamino)tetraline and 5-hydroxytryptamine] had only low po-

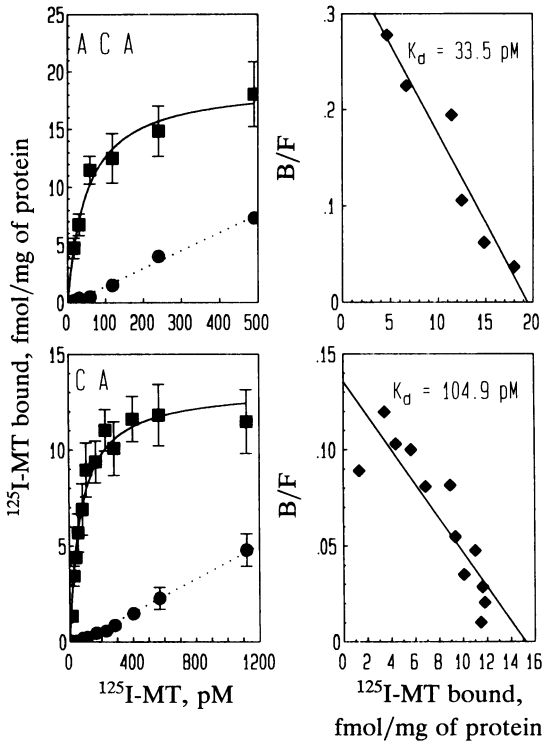

FIG. 2. Saturation isotherms (*Left Top* and *Left Bottom*) and Scatchard transformation (*Right Top* and *Right Bottom*) of specific ^{125}I-melatonin (^{125}I-MT) binding in the anterior cerebral and caudal arteries (ACA and CA, respectively). ——, Specific binding; · · · ·, nonspecific binding.

tency. 5-Hydroxytryptamine and yohimbine did not affect ^{125}I-melatonin binding.

To study whether these arterial binding sites are functional melatonin receptors, the effect of melatonin on contractions induced by NE, a major regulator of tone in the caudal artery, was examined. Contractions of the caudal artery induced by NE were significantly prolonged and potentiated by melatonin (Fig. 4). These effects of melatonin were concentration dependent. Melatonin (0.1–100 nM) by itself did not affect the resting tension of this artery. NE-induced contraction of the rat aorta, which does not display specific ^{125}I-melatonin binding, was not affected by melatonin, even at a concentration of 0.1 μM (data not shown).

In an effort to understand the mechanism of the action of melatonin on arterial contraction, the effect of melatonin on NE-induced breakdown of inositol phospholipids was studied. While NE induced a significant increase in caudal artery inositol phospholipid turnover, assessed as inositol monophosphate accumulation (basal, 2.1 ± 0.6%; NE, 86.0 ± 2.1%), melatonin alone or in combination with NE failed to significantly affect this increase (melatonin, 6.8 ± 3.2%; NE and melatonin, 85.9 ± 2.0%).

We also tested the possibility that melatonin may inhibit cAMP formation and thereby potentiate contraction induced by NE. Forskolin caused a significant increase in caudal artery cAMP formation (basal, 157.2 ± 12.9 pmol/mg of protein; forskolin, 470.7 ± 63.2 pmol/mg of protein). However, melatonin alone or in combination with forskolin did not affect cAMP levels (melatonin, 196.9 ± 16.1 pmol/mg of protein; forskolin and melatonin, 482.0 ± 31.8 pmol/mg of protein).

DISCUSSION

Our results demonstrate a unique expression of melatonin binding sites in the vasculature of the rat: in the arteries that form the circle of Willis at the base of the brain and the caudal artery. Arterial ^{125}I-melatonin binding was stable, saturable, and reversible and represented a single class of high-affinity binding sites. The affinities of these binding sites closely

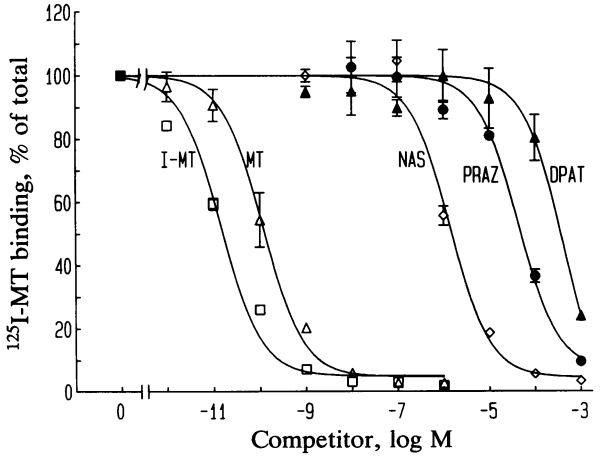

FIG. 3. Specificity of ^{125}I-melatonin (^{125}I-MT) binding in sections of rat caudal artery. Sections were incubated with a fixed concentration of ^{125}I-melatonin (122 pM) in the absence or presence of increasing concentrations of 2-iodomelatonin (I-MT), melatonin (MT), *N*-acetylserotonin (NAS), prazosin (PRAZ), 8-hydroxy-2-(di-*n*-propylamino)tetraline (DPAT), 5-hydroxytryptamine, and yohimbine. IC_{50} values, obtained from competition curves prepared with the computer program GRAPHPAD, were used to calculate (21) inhibition constants (K_i): I-MT, 6.70×10^{-12} M; MT, 5.36×10^{-11} M; NAS, 6.37×10^{-7} M; PRAZ, 1.97×10^{-5} M; DPAT, $>2 \times 10^{-4}$ M; 5-hydroxytryptamine and yohimbine, $>1 \times 10^{-3}$ M (not shown). Each point represents the mean ± SEM of caudal arteries from three rats (six to eight sections from each).

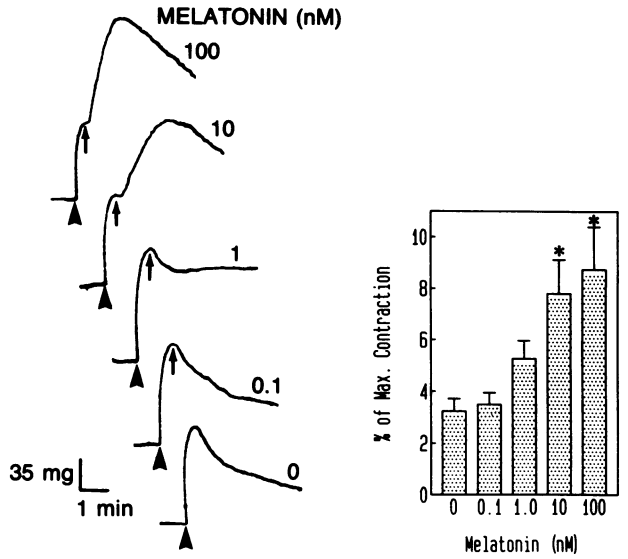

FIG. 4. (*Left*) Typical effect of melatonin on NE-induced contraction of the rat caudal artery. Arrowheads indicate addition of NE (3.2 nM). Arrows indicate addition of melatonin at concentrations indicated. (*Right*) Magnitude of the contraction measured 3 min after the addition of melatonin, expressed as a percentage of the maximum contraction attained with 60 mM KCl. Values are means ± SEM of measurements from six rats. Asterisks indicate significant differences ($P < 0.05$) as determined by analysis of variance followed by Duncan's multiple range test.

agree with those reported for the sites in the rat suprachiasmatic nuclei (7, 14), area postrema (8), and median eminence/pars tuberalis (10). Pharmacological characterization of the caudal artery binding sites showed high specificity for melatonin as do other ^{125}I-melatonin binding sites in the rat (8, 10, 14).

The arterial ^{125}I-melatonin binding was found to be restricted to the smooth muscle layer. Sympathetic nerve axons, which innervate arteries, are not found in this layer but in the outer adventitial layer (22). Therefore, the arterial ^{125}I-melatonin binding sites are likely to be in the smooth muscle cells rather than in the sympathetic nerve endings.

Melatonin significantly prolonged and potentiated NE-induced contraction of the caudal artery *in vitro* in a concentration-dependent manner. This suggests that these arterial ^{125}I-melatonin binding sites are functional melatonin receptors. A major regulator of tone in the caudal artery is known to be NE originating from sympathetic nerves (23). Melatonin could be either promoting steps involved in smooth muscle contraction or alleviating muscle relaxation. Smooth muscle contraction is thought to be initiated by agonist-induced breakdown of membrane inositol phospholipids (inositol phospholipid turnover) *via* a guanine nucleotide-binding (G) protein-activated phospholipase C. This pathway generates, for instance, inositol trisphosphate, which releases Ca^{2+} from the intracellular stores (24, 25), giving rise to a transient contraction followed by a sustained response, presumably due to Ca^{2+} influx (23). Therefore, we tested whether melatonin affected inositol phospholipid turnover in the caudal artery *in vitro*. In the presence of LiCl, while NE induced a 40-fold increase in caudal artery inositol phospholipid turnover, melatonin alone or in combination with NE failed to significantly affect this increase.

Relaxation of smooth muscle is believed to be mediated via adenylate cyclase-mediated cAMP formation and is mimicked by the diterpene forskolin (23). Moreover, melatonin has been found to inhibit forskolin-stimulated cAMP formation in pars tuberalis of sheep (26) and hamster (27) and luteinizing hormone-releasing hormone-stimulated cAMP

production in the rat pituitary (28). Therefore, we tested the possibility that melatonin might inhibit forskolin-stimulated cAMP formation and thereby potentiate NE-induced contraction. Melatonin alone or in combination with forskolin did not affect cAMP levels. Thus, melatonin does not seem to act directly on the primary steps involved in smooth muscle contraction or relaxation. Arterial contraction can occur without the mediation of intracellular messengers such as inositol trisphosphate and cAMP (29), and melatonin may use such a mechanism for its action. We have found that potassium-induced (24 mM) caudal artery contraction was also potentiated by nanomolar concentrations of melatonin (unpublished data), raising the possibility that melatonin may exert its action by regulating the influx of Ca^{2+}.

The above data revealed expression of functional melatonin receptors in specific arteries of the rat. The physiological significance of these receptors may be deduced from their highly restricted distribution in the rat vascular system. Most of the metabolic heat from the brain is eliminated by cerebral arterial circulation (30), of which the circle of Willis is an integral part. Similarly, blood flow to the tail of the rat as well as heat dissipation from the tail skin are important in thermoregulatory homeostasis (31, 32). Chronic heat exposure increases plasma melatonin levels in the rat and cutaneous evaporative water loss is lower in pinealectomized rats (33). These findings, when considered in conjunction with the present results, suggest that the arterial melatonin receptors, by modulating tone and thereby blood flow in these arteries, may work in concert with other thermoregulatory mechanisms.

We thank Dr. Jorge Pinto for his helpful discussion of the manuscript.

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