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Signaling Pathways Coupled to Melatonin Receptor MT1 in Gastric Smooth Muscle

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Signaling Pathways Coupled to Melatonin Receptor MT1 in Gastric Smooth Muscle

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

By

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Abstract

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The Melatonin, a close derivative of serotonin, is involved in physiological regulation of circadian rhythms. In the gastrointestinal (GI) system, melatonin exhibits endocrine, paracrine and autocrine actions and is implicated in the regulation of GI motility. Generally, melatonin actions oppose the stimulatory actions of serotonin on motility. However, it is not known whether melatonin can also act directly on GI smooth muscle cells. The aim of the present study was to determine the expression of melatonin receptors in smooth muscle and identify their signaling pathways. Muscle cells were isolated from rabbit distal stomach by enzymatic digestion, filtration and centrifugation and cultured in DMEM-10. Expression of melatonin receptors, MT₁ and MT₂, was determined by RT-PCR and Western blot. G protein activity was measured by melatonin-induced increase in Gα binding to [35S]GTPγS. Phosphoinositide (PI)-specific phospholipase C (PLC-β) activity was measured by ion-exchange chromatography. Cytosolic Ca²⁺ was measured in
fura-2 loaded cells and muscle contraction was measured by scanning micrometry. In cultured gastric smooth muscle cells MT₁ was detected by RT-PCR and western blot. Melatonin activated Ga₉, but not Ga₅, Ga₁₁, Ga₁₂, or Ga₁₃. Consistent with activation of Ga₉, melatonin stimulated PLC-β activity (PI hydrolysis), increased cytosolic Ca²⁺, and elicited muscle contraction. Stimulation of PLC-β activity was blocked by the expression of Ga₉ minigene and contraction was blocked by the PLC-β inhibitor, U73122. We conclude that gastric smooth muscle cells express receptors for melatonin (MT₁) coupled to Gq. The receptors mediate stimulation of PLC-β activity and increase in cytosolic Ca²⁺, and elicit muscle contraction.
A-INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine), a derivative of 5-hydroxytryptamine (serotonin) and an endogenous signal of darkness, is an important component of the body’s internal time-keeping system and regulates diverse physiological processes such as diurnal rhythm and gastrointestinal secretions and motility. Melatonin is also an effective antioxidant, which scavenges free radicals and upregulates several antioxidant enzymes, and anti apoptotic signaling molecules. Although melatonin is synthesized in many organs, the major source of circulating melatonin is the pineal gland.

1- Melatonin Synthesis in Pineal Gland

Melatonin synthesis has been well investigated in the pineal gland. The amino acid tryptophan, the primary precursor of melatonin, is absorbed from the circulation into the gland [1]. The uptake process relies on transport mechanisms to cross the blood-brain barrier [1]. Tryptophan then converts to 5-hydroxytryptophan by tryptophan-5-hydroxylase, an enzyme found in mitochondria requiring a pteridine cofactor [1]. The reaction is rate-limiting in the formation of serotonin, and this enzyme is limited to only serotonin-forming tissue. The rate of reaction is directly related to the amount of tryptophan. Manual administration of large amounts tryptophan results in increased formation of serotonin [1]. Decarboxylation of 5-hydroxytryptophan results in the formation of serotonin (5-hydroxytryptamine), and is conducted by aromatic amino acid decarboxylase, a cytoplasmic enzyme [1]. This enzyme has broad specificity and is widely distributed in tissue requiring pyriocoxal phosphate as a cofactor. Interestingly, constant light appears to have a stimulatory effect on this process[1]. The next step in melatonin synthesis is \(N\)-acetylation of serotonin. Arylalkylamine - \(N\)-acetyltransferase (AA-NAT),
the enzyme responsible for this reaction, uses acetyl co-enzyme A as a cofactor. NAT is present in many tissues and is likely a rate limiting step in melatonin synthesis [1]. The final step is O-methylation of N-acetylserotonin by hydroxyindole-O-methyltrasnferase (HIOMT). HIOMT transfers a methyl group from S-adenosyl methionine (SAM). AA-NAT and HIOMT are the key enzymes in melatonin synthesis [2].

Synthesis of melatonin is mainly seen at night and relates to the peak of AA-NAT activity [3]. Recently, studies indicated that the main factor in regulating rhythmic and light-induced changes in AA-NAT activity is the steady-state level of AA-NAT proteins, which in turn reflects the balance of its synthesis and degradation [4]. These processes can be regulated by distinct mechanisms and the significance of each of them is species specific [5]. In humans, nocturnal synthesis of melatonin in the pineal gland is mainly controlled by the central circadian clock, located in the hypothalamic suprachiasmatic nucleus [6]. The circadian clock stimulates norepinephrine release from dense pineal sympathetic fibers. Norepinephrine elevates the intracellular cAMP concentration via β-adrenergic receptors and activates the cAMP-dependent protein kinase A, the essential pathway for the regulation of AA-NAT synthesis and activity. In some mammals, cAMP/protein kinase A protects the enzyme from degradation [7]. Therefore pinealocytes constantly produce AA-NAT from continuously available AA-NAT mRNA. This protein immediately undergoes proteasomal proteolysis during the day, in the absence of noradrenergic stimulation. On the other hand, nocturnal elevation of the cAMP level causes phosphorylation of AA-NAT via protein kinase protecting the enzyme from degradation. Hence, increases of intracellular concentrations of AA-NAT are incongruence with increases in enzyme activity. In rodents, the cAMP/protein kinase pathway induces
transcriptional activation of the AA-NAT gene, the primary mechanism initiating melatonin biosynthesis. In contrast, the importance of a transcriptional regulation of the AA-NAT gene for melatonin production in humans is questionable [8]. Thus, analysis of pineal tissue, taken from regular autopsies, showed significant rhythm for the correlation between time of death and both AA-NAT activity and melatonin content, while AA-NAT and HIOMT mRNA, as well as HIOMT activity showed no diurnal rhythm [8]. Although dominant mechanisms for the generation of rhythmic melatonin synthesis in the human pineal gland remain to be clarified, it is generally accepted that AA-NAT is alternating melatonin synthesis with photoperiodic variation in duration, while the level of HIOMT activity may tune the seasonal magnitude of hormone production [9, 10].

The rhythmical production of melatonin in the pineal gland accurately reflects environmental light conditions; light exposure can significantly suppress nocturnal melatonin secretion [11]. It has been reported that these non-visual effects of light are mediated by the retinohypothalamic tract, a distinct neural pathway mediating the regulation of melatonin production by light [12, 13], which is initiated in the photoreceptor system. The mammalian eyes perceive light and dark cycles which entrain the neural activity of the suprachiasmatic nucleus, and alter the rhythmic secretion of melatonin from the pineal gland [14]. However, it has been shown recently that rod and cone photoreceptors of the retina do not participate in this pathway [15]. In effect, light at a wavelength of 555 nm, providing a peak effect on the visual system [16], suppresses melatonin synthesis in healthy humans approximately four times less than monochromatic light at 505 nm [14]. The visual photoreceptors conducting these effects remain unknown [14]. Moreover, light-induced melatonin suppression and circadian entrainment have been
demonstrated in humans with specific color vision deficiencies [17] or complete visual blindness [18]. These studies suggest that melatonin biosynthesis is controlled, at least in part, by photoreceptors that differ from the known visual photoreceptors [14]. Recent studies on various vertebrate species identified several new molecules that may serve as circadian photopigments. These pigments include opsin-based molecules (e.g. vertebrate ancient opsin [19], melanopsin [20] and peropsin [21]) as well as non-opsin molecules (bilirubin [22] and cryptochrome [14, 23]). Among these new photopigments, only melanopsin has been discovered in the human neural retina [24]. Consequently, the diurnal rhythmical production of melatonin is controlled by diverse mechanisms characterized by highly adaptive plasticity regulatory mechanisms, most of which need to be further studied and researched.

2- Melatonin Synthesis in Gastrointestinal Tract

In 1976, Raikhlin and Kvetnoy were one of the first to illustrate melatonin production by serotonin-rich enterochromaffine (EC) cells in the gastrointestinal tract acting as a paracrine molecule and hormone released into the portal vein [25]. Several studies revealed that gastrointestinal tract substantially contributes to the peripheral blood concentrations of melatonin. Considering the size of the gastrointestinal tract relative to the pineal gland, it has been estimated that there is 400 times more melatonin in the gut than in the pineal gland. Melatonin concentrations in the gut surpass levels in the blood 10-100 fold. Melatonin does not seem to be uniformly distributed and concentration varies in various regions of the gut. In the digestive tract, melatonin has been identified using a variety of methods including immunocytochemical, chromatographic and
radioimmunomethods. Additionally, the melatonin synthesizing enzyme is reportedly present in the gut as well as its synthesis from its precursor, serotonin, in EC cells of the intestinal mucosa. Oral application of tryptophan (150 mg/kg), a known precursor to melatonin, in rats causes a rapid elevation of circulating melatonin that was higher than that acquired after administration of this amino acid [26]. Ligation of the portal vein led to elimination of the increment in plasma level of melatonin following the tryptophan application. On the other hand, plasma level of melatonin remained unaffected upon pinealectomy, suggesting the gastrointestinal tract by itself is the major source of circulating melatonin after oral tryptophan application [26]. Melatonin is present in gastrointestinal tract mucosa even under fasting conditions, yet this melatonin content in the gastrointestinal tract increases exponentially following food intake and tryptophan administration [26]. A number of other studies using immunohistochemistry and radioimmunoassay techniques, validated by HPLC [27-30] confirmed the presence of melatonin in gastrointestinal tract mucosa and indicated that enterochromaffine cells are the main source of melatonin in human gastrointestinal tract aside from the pineal gland. Similarly, Messner, who studied the distribution of melatonin in human hepatobiliary-gastrointestinal tract, verified high concentration of melatonin in gastric and duodenal mucosa with considerable amounts of this indole excreted into the bile [31].

Furthermore, extrapineal melatonin synthesis was more or less accepted when melatonin-synthesizing enzymes, Nacetyltransferase [35] and hydroxyindole-O-methyltransferase [36] were detected in the gastrointestinal tract. Through the use of transcriotionpolymerase chain reaction, the presence of both enzymes was confirmed [37]. Additional research confirmed there are fundamental differences between pineal-produced
and GIT-produced melatonin. While pineal-produced melatonin acts mostly as an endocrine substance, extrapineal-derived melatonin functions not only in endocrine, but also in autocrine, paracrine and luminal capacity [38, 39]. There are also substantial differences in the mode of secretion and response to food intake. Whereas nighttime levels of melatonin in blood are mostly of pineal origin, day-time melatonin concentrations in blood are produced mostly in the GIT [40]. Because of high levels of melatonin in the GIT tissues and the large size of the GIT, the amount of melatonin in GIT is quite high. In 1994, Gerald Huether calculated that at any moment of the day or night, GIT contains at least 400 times more melatonin than the pineal gland [41].

3- The Role of Melatonin in Circadian Rhythm

Melatonin signals time of day and time of year in mammals based on its pattern of secretion, which defines ‘biological night.’ Animal studies revealed the fundamental role of melatonin as a photoneuroendocrine transducer of information on day length. Melatonin, by its changing duration of secretion at different day lengths (the longer the night the greater the duration of hormone secretion), is probably a universal biological signal indicating darkness [1, 42, 43]. Imposition of different day lengths in humans shows a conserved photoperiodic response, and the evidence indicates that melatonin acts as a biological signal for dawn and dusk in humans, as well as other species [44-47]. There is evidence that the timing of melatonin secretion is closely associated with the timing of sleep tendency [45, 48]. The timing of peak melatonin concentration coincides closely to the nadirs of core body temperature, alertness and performance, clearance of plasma triglycerides, and other night-time phenomena [49]. There is also evidence of melatonin’s
influence on glucose homeostasis [50], the immune system [51], and cardiovascular function [52].

Certainly humans sleep better during the appropriate period of melatonin secretion; yet, is this due to melatonin itself, other factors, or to the whole circadian system working in orchestra? In 1997 a study showed if melatonin is present at an abnormal phase during the day, it is strongly associated with daytime naps [53]. The significance of melatonin presence in sleep cycle can be seen by pharmacological suppression of daytime melatonin production which diminishes daytime sleepiness, and treatment with exogenous melatonin at night which restores night-time sleep [54, 55]. Pharmacological inhibition of endogenous melatonin by atenolol heightens the human phase-shifting response to light [56]. If given at an appropriate circadian phase, exogenous melatonin can act in congruence with light and can also partially counteract light for phase-shifting [57, 58]. Therefore, melatonin’s presence modifies circadian responses. Based on these few observations, it appears the presence of melatonin hinders rapid adaptation to phase-shift as originally observed in rats [1] and that persons treated with b1 antagonists are likely to suffer less jet-lag and to adapt more readily to night work than untreated individuals. It is also reasonable to state that appropriate combinations of light and melatonin treatment should be more efficient in the changing phase than either treatment alone; however, there is little information on this subject. Although the evidence is not compelling, it has been suggested that melatonin from the digestive tract may be secreted into the blood in a circadian manner, as it is from the pineal gland. The circadian rhythm of gastrointestinal tract melatonin depends on the type of digestion each species exhibits (e.g., diurnal vs. nocturnal mon-gastric vs. ruminants).
4- Melatonin receptor signaling

Two mammalian subtypes of G-protein coupled melatonin receptors have been cloned and identified, MT1 and MT2 [59, 60]. While both share generally similar binding characteristics for 125I-melatonin, the human MT2 receptor has a lower affinity (Kd=160pmol/l) compared to the human MT1 receptor (Kd = 20 – 40 pmol/l) [61]. However both are of high affinity and the agonist binding is guanosine triphosphate (GTP)-sensitive [60].

There has been discussion of a third melatonin binding site, “MT3,” yet it was later characterized as the enzyme quinine reductase 2 [61]. Belonging to a group of reductastases that participate in the protection against oxidative stress, enzyme quinine reductase prevents electron transfer reactions of quinones. Additionally, functional and ligand binding studies have shown the existence of low affinity (Kd in the 10 nm range) melatonin membrane binding sites in the preoptic area of the hypothalamus and in the medulla-pons in rats and hamsters [62-64]. Furthermore, melatonin interacts with intracellular proteins such as calmodulin, calreticulin, or tubulin and antagonizes the binding of Ca2+ to calmodulin [65-69]. These interactions are most likely related to some of the physiological effects of melatonin but critical data regarding this point have yet to be obtained.

MT1 and MT2 receptors are expressed both singly and together in various tissues of the body [70, 71, 60]. Functional MT1 receptors have been localized in the SCN [72, 60], cerebellum [73], hippocampus [74], central dopaminergic pathways [75], ovary [76], retina [77], coronary blood vessels and aorta [78, 79], liver and kidney [80], and the gallbladder [81]. Melatonin MT2 receptors are more restrictively expressed, being found
mainly in the brain, although their presence has also been detected in the lung, cardiac, aortic and coronary tissue, myometrium and granulosa cells, immune cells, duodenum and adipocytes [60]. The “MT3 receptor” is expressed in the liver, kidney, brain, heart, lung, intestine, muscle and brown adipose tissue [82].

After binding to its membrane-bound receptors, melatonin changes the conformation of the Alpha-subunit of specific intracellular G proteins. It regulates cell function via intracellular second messengers such as cAMP, calcium ions, cGMP, DAG, PKC, and Arachidonic acid; yet, the signal transduction pathways for melatonin receptors appear to differ among varying tissues and cell types [83, 84]. Activation of G-Alpha I through the binding of melatonin to MTR1 inhibits cAMP formation, PKA activity, resulting in a decrease in CREB phosphorylation. Aside from the cAMP-dependent cascade, MTR1 can mediate a PLC-dependent signal transduction cascade directly or indirectly via G-BetaGamma subunits for phosphoinositide turnover and activate PKC. Activation of MTR1 by melatonin also increases phosphorylation of MAPK or MEK1 and MEK2 and ERK1/2. Interestingly, receptors themselves can modulate formation of arachidonic acid and JNKs. Comprehensive regulation of activator protein-1a, a transcription factor produced by the early gene products of c-Fos and c-Jun, has been observed by the MAPK pathways, specifically ERK1, ERK2, and JNKs.

Furthermore, melatonin also has regulatory effects on several ion channels. Binding of melatonin to MTR1 receptors potentiates vasoconstriction via blocking calcium-activated potassium channels in smooth muscle cells. The obstruction of this channel may be due to a decrease in cAMP and in phosphorylation of the calcium-activated potassium channel by PKA. In addition, melatonin directly vasoconstricts cerebral arteries through
inhibition of calcium-activated potassium channels. MTNR1As also couple to the GIRK (G-protein-activated Inward Rectifier Potassium)/Kir 3 channels.

Activation of MTNR1Bs modulates levels of cAMP and cGMP. Like the case with MTNR1A, activation of the MTNR1B by melatonin inhibits cAMP formation. Additionally, MTNR1B activation leads to the inhibition of cGMP formation. Inhibition occurs through proteins upstream of the Guanylyl Cyclase such as NOS. In the SCN, melatonin increases PKC activity through activation of GN-AlphaQ, which activates the PLC and DAG pathway. MT1 activation of Gq proteins has been shown to mediate calcium mobilization via activation of the inositol-specific phospholipase C (PLC) [85] and activation of protein kinase C [86]. The melatonin receptors also serve as the mediators of several other physiological responses to melatonin. These responses include (i) phase advance of circadian rhythms in the isolated SCN, which involves PKC activation; (ii) enhancement of cell-mediated and humoral immunity; (iii) inhibition of leukocyte rolling in the microvasculature; and (iv) inhibition of proliferation of human choriocarcinoma cells, probably by delay of the G1 to S cell cycle transition. Furthermore, activation of MTR2 decreases the expression of the glucose transporter GLUT4 that in turn decreases glucose uptake in human brown adipocytes, modulates neuronal activity in the hippocampus, and mediates vasodilation in arterial beds.

5- Effects of Melatonin in Gastrointestinal Tract

Melatonin has a special importance in the gastrointestinal tract because the enterocromaffin cells of the gut are the main source of extrapineal melatonin. Melatonin modulates gastrointestinal motility, transport of ion and electrolytes and postprandial
sequential activation of gastrointestinal tract. Numerous studies demonstrated that melatonin exerts protective and healing effects in gastric ulcers and experimental colitis. Direct antioxidative effect and release of endogenous prostaglandins are associated with these effects [115]. Melatonin administration at pharmacological doses decreases free radical formation and leads to substantial recovery of the major antioxidative enzymes [39]. In addition, melatonin has pleiotropic effects on the immune system causing an enhancement of both innate and cellular immunity, which underlies its immunomodulatory role in inflammation [80]. Therapeutic potential of melatonin in the recovery of gallbladder neuromuscular function during acute cholecystitis and aging was also reported, but the exact cellular mechanism through which melatonin exerts its effects in the gallbladder are not known [116, 117].

Many factors released in the gut modulate motility and secretion. Melatonin plays a particularly important role in modulation of gastrointestinal functions reflecting the cyclic pattern of food intake. Binding to selective receptors expressed on the smooth muscles and myenteric plexus cells of gastrointestinal tract allows melatonin to have a significant influence on gastrointestinal motility. The basal mechanism underlying the gastrointestinal smooth muscle contraction is the cyclic generation of the electrical current.

The myoelectric activity of gastrointestinal tract is comprised of two kinds of potentials – slow wave and spiked activity coordinated in the myoelectric migrating complex (MMC). Internal and external autonomic nerves modulate the Interstitial Cajal’s pacesetter cells (ICC) [87]. ICC produces the basic electrical rhythm (BER) which is simply the generation of slow waves determining bowel motility.
Frequency of the slow wave determines the contraction/relaxation activity of the gastrointestinal smooth muscle. The intestinal pacemaker is located in the duodenal bulb, near the pylorus, and is independent from the gastric electrical activity pacemaker. The duodenal region is where the frequency and velocity of slow waves are greatest and decreases caudally [88, 89]. In the rat, the duodenal slow wave frequency ranges from 20 to 40 cycles per minute and has greater amplitude and plateau than the jejunum. Furthermore, the BER amplitude ranges from -55 to -35 mV [90]. This electrical activity can be used as an indicator of gastro duodenal coordination because it is greatly influenced by a number of paracrine and neurocrine factors. Melatonin in the fasted state induces dramatic reduction of the irregular spike activity (ISA) on the entire intestine including phase III of the MMC. Periodicity of MMC length decreased by 50% [91]. This study revealed effects of melatonin on myoelectric activity provide strong evidence that melatonin relaxes the bowel by acting mostly on phasic contractions.

The gastrointestinal tract motility depends on a dark-light cycle. Namely, motility is activated more during daylight than during the dark period. The latest reports provided data that melatonin plays an important role in regulation of the gastrointestinal motility. In fact, it has been documented that postprandial motor response is shorter in the dark phase than in the light phase and this effect can be reversed by melatonin antagonist [92].

There is belief that endogenous melatonin act on pre and postprandial intestinal motility at night and this effect has also been observed with exogenously administered hormone. Additionally, there are findings that melatonin reinforce cyclic pattern of MMC but inhibit occurrence of irregular spike activity [91]. There is also apparent interaction between melatonin and CCK in terms of motility. Melatonin was found to reduce the
duration of CCK stimulatory effects on gastrointestinal smooth muscle [92]. There is speculation that melatonin is released to the lumen of proximal bowel in response to food intake and travels to the distal part. This may in part explain why the MMC pattern first reappears in the distal part of the bowel in the end period of satiety.

However, present reports are inconsistent in the matter of the melatonin’s role on gut motility. Martín at al. suggests that melatonin release induced by lipid infusion alleviate inhibitory effect of lipid related ileal break suggesting that melatonin plays a modulatory role on gastric emptying [93]. These results correspond partially with reports of Drago at al. who observed that small doses of melatonin accelerate the intestinal transit in rats. High doses reversed stimulatory effects of lower doses of melatonin indicating that intestinal melatonin receptors might mediate melatonin effects. Moreover, administered intraperitoneally melatonin increased intestinal myoelectrical activity. This effect was reversed by the competitive receptor antagonist, Luzindole, and seems to be mediated by peripheral receptors [94].

6- Gastric Motility

Gastric smooth muscle activity is responsible for two fundamental patterns of motility, mixing and propulsion. The formation of chyme, a liquefied state of partly digested food, is formed through a process of mixing, crushing, and grinding of ingested food. Subsequently, smooth muscle activity forces the chyme through the pyloric canal, into the small intestines, a process known as gastric emptying. Based on motility pattern, the stomach can be divided into two regions harboring either tonic contraction or phasic contraction.
The proximal stomach, composed of primarily the fundus, shows low frequency, sustained contractions that are responsible for generating a basal pressure within the stomach. Importantly, these tonic contractions also generate a pressure gradient from the stomach to small intestine and are therefore responsible for gastric emptying. Swallowing of food and consequent gastric distention inhibits contraction of this region of the stomach, allowing it to increase in volume and form a large reservoir without a significant increase in pressure. This phenomenon is called "adaptive relaxation."

The distal stomach, comprised of primarily the antrum, develops strong peristaltic waves of contraction that increase in amplitude as they propagate toward the pylorus. These powerful contractions constitute a very effective gastric grinder; they occur about 3 times per minute in people. There is a pacemaker in the smooth muscle of the greater curvature that generates rhythmic slow waves from which action potentials and hence peristaltic contractions propagate. Gastric distention strongly stimulates this type of contraction, accelerating liquefaction and gastric emptying. The pylorus is functionally part of this region of the stomach. When the peristaltic contraction reaches the pylorus, its lumen is effectively degraded; chyme is thus delivered to the small intestine.

A study by Kasimay et al. [95] indicated that melatonin inhibits gastric motility by interacting with serotonin receptors present on the vagal afferent fibers via vago-vagal inhibitory reflexes. Storr et al. [96, 97] has demonstrated the expression of MT1, but not MT2 receptors using RNA isolated from the muscle layers of rat stomach and intestine. Addition of melatonin to isolated muscle strips inhibited non-adrenergic and non-cholinergic mediated relaxations, but had an effect on exogenous nitric oxide (NO)-mediated relaxation. In synaptosomal preparations melatonin inhibited basal nNOS
activity. These results suggest that melatonin actions are mediated by inhibition of nNOS activity via MT1 receptors. In addition to its effect on basal nNOS activity, melatonin was shown to suppress nNOS express in rat hypoglassal nucleus following peripheral nerve injury [98], nNOS activity in rat striatum [99] and eNOS activity in rat cerebellum [100].

These studies indicate the difficulties in identifying the signaling pathways activated by melatonin in vivo and in innervated in vitro preparations. The effect of melatonin may vary in different specific regions of the gut depending on whether the activated receptor is present predominantly on smooth muscle cells or enteric neurons. Transmitters released from the enteric neurons, in turn, modulate the intrinsic electrical and mechanical activity of the gastrointestinal smooth muscle. To avoid the confounding effects of neural activation by melatonin, the present study has characterized the receptors for melatonin and the signaling pathways to which these receptors are coupled in freshly dispersed and cultured smooth muscle cells of the gut.
B- METHODS

1- Collection of tissue

Rabbits were sacrificed by injection of Euthasol (100 mg/kg body weight) into the ear vein. The stomach was rapidly removed, emptied of its contents and placed in a cold smooth muscle buffer with the following composition: NaCl 120 mM, KCl 4 mM, KH$_2$PO$_4$ 2.6 mM, CaCl$_2$ 2.0 mM, MgCl$_2$ 0.6 mM, HEPES (N-2-hydroxyethylpiperazine-N’ 2-ethanesulfonic acid) 25 mM, glucose 14 mM, and essential amino mixture 2.1% (pH 7.4)

2- Preparation of dispersed gastric smooth muscle cells

The antrum was separated from the rest of the stomach and the mucosal layer was removed by sharp dissection. Smooth muscle cells from the circular muscle layer of the antrum were isolated by sequential enzymatic digestion of muscle strips, filtration, and centrifugation as described previously [101-108]. The antrum was cut into thin slices using a Stadie-Riggs tissue slicer and then the slices were incubated for 30 min in a smooth muscle buffer at 31°C containing 0.1% collagenase (300 U/ml) and 0.01% soybean trypsin inhibitor (w/v). The tissue was continuously gassed with 100% oxygen during the entire isolation procedure. The partly digested tissues were washed twice with 50-ml of collagenase-free smooth muscle buffer and the muscle cells were allowed to disperse spontaneously for 30 min in collagenase-free medium. Cells were harvested by filtration through 500 µm Nitex and centrifuged twice at 350 g for 10 min to eliminate broken cells and organelles. The cells were counted in a hemocytometer and it is
estimated that 95% of the cells excluded trypan blue. The experiments were done within 2-3 h of cell dispersion.

3- Preparation of cultured gastric smooth muscle cells

Dispersed muscle cells isolated from the antrum were resuspended in DMEM containing penicillin (200 U/ml), streptomycin (200 µg/ml), gentamycin (100 µg/ml), amphotericin B (2.5 µg/ml) and 10% fetal bovine serum (DMEM-10). The muscle cells were plated at a concentration of 5 X 10^5 cells/ml and incubated at 37°C in a CO₂ incubator. DMEM-10 medium was replaced every three days for 2-3 weeks until confluence was attained. The muscle cells in confluent primary cultures were trypsinized (0.5 mg trypsin/ml), re-plated at a concentration of 2.5 X 10^5 cells/ml and cultured under the same conditions. All experiments were done on cells in the first passage. Previous studies have determined the purity of cultured muscle cells with smooth muscle-specific γ-actin [109]. Cultured muscle cells were starved in serum-free medium for 24 hours before each use.

4- Expression of MT1 in smooth muscle cells by RT-PCR

Total RNA was isolated from smooth muscle cells with TRIzol® reagent (Invitrogen) and treated with TURBO DNase (Ambion). RNA from each preparation was reversely transcribed using the SuperScript™ II system containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM deoxynucleoside triphosphates (dNTP), 2.5 µM random hexamers and 200 units of reverse transcriptase in a 20 µl reaction volume. The reactions were carried out at room temperature for 10 min
and at 42°C for 50 min, and terminated by heating at 70°C for 15 min. Three µl of the reversely transcribed cDNA was amplified in a final volume of 50 µl by PCR in standard conditions (2 mM MgCl₂, 200 µM dNTP, 2.5 units Taq polymerase) with specific primers designed based on conserved sequences in human, rat and mouse cDNAs: MT1 (Forward: TTCATTGGTCTTGCAGTGGCCTCAG; Reverse: GAACATCCTTGCTGTA CACATTGAG) and MT2: (Forward: TTGTCCACCGTGCTCATCGTCACCAC; Reverse: TGGCCTTGCAATGCCTCTCCAG). PCR for MT1 and MT2 receptors was performed for 30 cycles. For each experiment, a parallel control without reverse transcriptase was processed. The amplified PCR products were analyzed on 1.5% agarose gel containing 0.1 µg/ml ethidium bromide [109].

5- Expression of MT1 in smooth muscle cells by Western Blot

Muscle cells were solubilized in Triton X-100-based lysis buffer plus protease and phosphatase inhibitors (100 µg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 30 mM sodium fluoride and 3 mM sodium vanadate). After centrifugation of the lysates at 20000 g for 10 min at 4 °C, the protein concentrations of the supernatant were determined with a Dc protein assay kit from Bio-Rad. Equal amount of proteins were fractionated by SDS/PAGE, and transferred on to nitrocellulose membrane. Blots were blocked in 5% (w/v) non-fat dried milk/TBS-T [tris-buffered saline (pH 7.6) plus 0.1% Tween-20] for 1 h and then incubated overnight at 4 °C with various primary antibodies in TBS-T plus 1% (w/v) non-fat dried milk. After incubation for 1 h with horseradish-peroxidase-conjugated corresponding secondary antibody (1:2000; 10 µg/ml, Pierce) in TBS-T plus 1% (w/v) non-fat dried milk, immunoreactive proteins were visualized using SuperSignal Femto
maximum sensitivity substrate kit (Pierce). All washing steps were performed with TBS-T. The protein bands were identified by enhanced chemiluminescence reagent [110-112].

6- Identification of G proteins coupled to MT1

G proteins selectively activated by melatonin was identified from the increase in $G_\alpha$ binding to the [$^{35}$S]GTP$_\gamma$S (5'-O-3-thiotriphosphate) as described previously [106-108]. Ten ml of muscle cell suspension (3 X 10$^6$ cells/ml) were homogenized in 20 mM HPES medium (pH 7.4) containing 2 mM MgCl$_2$, 1 mM EDTA and 2 mM DTT. After centrifugation at 30,000 g for 15 min, the crude membranes were solubilized for 60 min at 4 $^\circ$C in 20 mM HEPEES medium (pH 7.4) containing 2 mM EDTA, 240 mM NaCl, 0.5% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 2 mM PMSF, 20 µg/ml aprotinin, and 20 µM leupetin. The membrane were incubated for 20 min at 37°C with 60 nM [$^{35}$S]GTP$_\gamma$S in the presence or absence of melatonin (1 µM) in a solution containing 10 mM HEPES (pH 7.4), 100 µM EDTA and 10 mM MgCl$_2$. The reaction was terminated with 10 volumes of 100 mM of Tris-HCl medium (pH 8.0) containing 10 mM MgCl$_2$, 10 mM NaCl and 10 µM GTP, and the mixture was placed in wells precoated with specific antibodies to $G_\alpha_q$, $G_\alpha_{i1}$, $G_\alpha_{i2}$, $G_\alpha_{i3}$, and $G_\alpha_s$. Coating with G protein antibodies (1:1000) was done after the wells were first coated with anti-rabbit IgG (1:1000) for 2 h on ice. After incubation for 2 h on ice, the wells were washed three times with phosphate buffer saline solution (PBS) containing 0.05% Tween-20 and the radioactivity from each well was counted by liquid scintillation. The amount of [$^{35}$S]GTP$_\gamma$S bound to the activated $G_\alpha$ subunit was expressed as counts per minute (cpm) per milligram of protein.
7- Phosphoinositide(PI)-specific phospholipase C (PLC-β) activity

PI hydrolysis (PLC-β activity) was determined in freshly dispersed or cultured smooth muscle cells by measuring the formation of inositol phosphates using ion-exchange chromatography as previously described [101-103]. Ten ml of cell suspension (2 x 10^6 cells/ml) were labeled with myo-[³H] inositol (15 µCi/ml) for 90 min at 31 °C. Then cells were centrifuged at 350 g for 10 min to move excess [³H]inositol and resuspended in 10 ml of fresh medium. Lithium was added to a final concentration of 10 mM and the suspension was incubated for another 10 min. Melatonin was added to 0.5 ml of cell suspension and the mixture was incubated in a shaking water bath for 1 min. Cultured smooth muscle cells were labeled with [³H]myo-inositols (0.5 µCi/ml) for 24 h in inositol-free DMEM medium. The cultures were washed with phosphate-buffered saline (PBS) and treated with anandamide (1 µl) for 1 min in HEPES medium (pH 7.4). The reaction was terminated by the addition of chloroform:methanol:HCl (50:100:1 v/v/v). After chloroform (310 µl) and water (310 µl) were added, the samples were vortexed and the phases were separated by centrifugation at 1000 g for 15 min. The upper aqueous phase was applied to a column containing 1 ml of 1:1 slurry of Dowex AG-1 X8 resin (100-200 mesh in formate form) and distilled water.

The column was washed with 10 ml of water followed by 10 ml of 5 ml sodium tetraborate-60 mM ammonium formate to remove [³H]glycerophosphoinositol. Total inositol phosphates were eluted with 6 ml of 0.8 M ammonium formate-0.1 M formic acid. The eluates were collected into scintillation vials and counted in gel phase after addition of 10 ml of scintillant. The results were expressed as counts per minute per mg protein.
8- Measurement of Ca\textsuperscript{2+} release

Melatonin-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} was measured by fluorescence in single smooth muscle cell loaded with fluorescent Ca\textsuperscript{2+} dye fura 2 [113]. Dispersed muscle cells were plated on coverslips for 12 h in DMEM. After being washed with PBS, the cells were loaded with 5 µM fura 2-AM for 1 h at room temperature. The cells were visualized through a x40 objective (ZEISS; 0.9 NA) with a Zeiss Axioskop 2 plus upright fluorescence microscope and imaged with a setup consisting of a charge coupled device camera (Imago, TILL Photonics, Applied Scientific Instrumentation, Eugene, OR) attached to an image intensifier. The cells were alternately excited at 380 and 340 nm. The background and autofluorescence were corrected from images of a cell without the fura 2. Results are expressed as increased in 340/380 ratio.

9- Measurement of contraction in dispersed smooth muscle cells

Contraction in freshly dispersed gastric circular smooth muscle cells was determined by scanning micrometry as previously described [101-113]. An aliquot (0.4 ml) of cells containing approximately 10\textsuperscript{4} cells/ml was treated with 1 µM melatonin in the presence and absence of the PLC β inhibitor U73122 (10 µM) for 30 s and the reaction was terminated with 1% acrolein at a final concentration of 0.1%. Acrolein kills and fixes cells without affecting the cell length. The resting cell length was determined in control experiments in which muscle cells were incubated with 100 µl of 0.1% bovine serum albumin without the agonists. The mean lengths of 50 muscle cells treated with various agonists was measured by scanning micrometry and compared with the mean lengths of
untreated cells. The contractile response was expressed as the percent decrease in mean cell length from control cell length.

10- Materials

Melatonin was purchased from Bachem (Torrance, CA). [3H]myo-inositol were obtained from PerkinElmer Life Sciences, (Boston, MA). Polyclonal antibodies to MT1, MT2, Galpha1, Galpha2, Galpha3, Galpha, and Galphaq were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western blotting, Dowex AG-1 X 8 resin (100-200 mesh in formate form), chromatography material and protein assay kit, 15% Tris-HCl Ready Gels were obtained from Bio-Rad Laboratories (Hercules, CA); collagenase and soybean trypsin inhibitor were obtained from Worthington Biochemical (Freehold, NJ). RT- PCR primers were purchased from Integrated DNA technologies, Inc (Coralville, IA). Fura-2/AM was obtained from Molecular Probes (Carlsbad, CA). Effectene Transfection Reagent, QIAEX®II Gel extraction Kit and QIAprep®Spin Miniprep Kit were obtained from QIAGEN Sciences, Maryland; PCR reagents were obtained from Applied Biosystems, Roche; SuperScript™ II Reverse Transcriptase and TOPO TA Cloning® Kit Dual Promoter were obtained from Invitrogen, CA; EcoRI was obtained from New England Bio Labs; Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Fisher Scientific. All other chemicals were obtained from Sigma, St. Louis, MO.

New Zealand white rabbits (weight: 4-5 lbs) were purchased from RSI Biotechnology, Clemmons, NC and were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University. Animals were killed by sodium pentobarbital overdose (100 mg/kg), as approved by the Institutional
Animal Care and Use Committee of the Virginia Commonwealth University. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

11- Statistics Analysis

The results were expressed as means ± S.E. of n experiments and analyzed for statistical significance using Student’s t-test for paired and unpaired values. Each experiment was done on cells obtained from different animals. Differences among multiple groups were tested by using ANOVA and checked for significance using Fisher’s protected least significant difference test. A probability of P< 0.05 was considered significant.
C- RESULTS

1- Expression of MT1 in gastric smooth muscle

Specific primers for MT1 and MT2 were designed based on the conserved sequences in human, rat and mouse cDNAs. MT1, but not MT2 receptors were detected by RT-PCR on RNA extracted from cultures of gastric smooth muscle cells in first passage (Fig. 1). PCR product of the expected size (225 bp) was obtained. The isolated partial nucleotide sequence of rabbit MT1 was similar to the corresponding amino acid sequences of human (86%) and rat (83%) (Fig. 2). As shown previously [109], the use of confluent cultures of smooth muscle in first passage ensured the absence of neural, endothelial, or interstitial cell contaminants and the presence of PCR product in cultured muscle cells demonstrate the expression of MT1 mRNA in smooth muscle cells.

Expression of MT1 and MT2 receptor protein was examined by western blot analysis using selective antibody to MT1 or MT2. The results demonstrate the expression of MT1 of predicted size (36 kDa) in the homogenates of isolated smooth muscle cells (Fig 1). Expression of MT2 was not detected in the homogenates of dispersed gastric muscle cells.

2- Identification of G proteins coupled to MT1 receptors in smooth muscle

Studies in various tissues and cell lines suggest that MT1 receptors are coupled to activation of G proteins, but the specific G proteins coupled to MT1 receptors in smooth muscle has not been identified. Muscle cells membranes were incubated with $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ (60 nM) in the presence or absence of melatonin (1 µM) and the aliquots were added to
wells precoated with different Gα antibodies; an increase in the binding of [35S]GTPγS complexes to a specific Gα antibody reflected the activation of the corresponding G protein. In some experiments low concentrations of GTPγS stimulated binding of [35S]GTPγS to Gαi2, Gαq and Gαs. However, incubation of muscle membranes with melatonin in the presence of low concentrations GTPγS caused a significant increase in the binding of [35S]GTPγS selectively to Gαq (392±45% increase above basal levels, p<0.001, n=8), but not to Gαi1, Gαi2, Gαi3, or Gαs (Figs. 3-7). These results suggest that MT1 receptors are preferentially coupled to activation of Gq in gastric smooth muscle.

Previous studies in gastric smooth muscle disclosed the presence of Gαi1, Gαi2, Gαi3, Gαs, and Gαq and activation of G proteins in smooth muscles is receptor specific: somatostatin sstr3 receptors are coupled to Gi1, opioid δ receptors are to Gi2, muscarinic m2 receptors are coupled to activation of Gi3, muscarinic m3 receptors are coupled to expression of Gq and VPAC2 receptors are coupled to Gs [106, 107, 111, 114].

3- **Signaling pathways activated by MT1 in gastric smooth muscle**

Previous studies in smooth muscle have shown that activation of Gq by excitatory neurotransmitters such as acetylcholine and substance P results in the stimulation of phosphoinositide(PI)-specific phospholipase C (PLC-β) activity, generation of inositol 1,4,5-trisphosphate (IP3) and IP3-dependent Ca2+ release leading to smooth muscle contraction. The effector enzyme stimulated by Gq proteins coupled to MT1 receptors was examined by measurements of inositol formation in response to melatonin in cells labeled with [3H]myo-inositol. As expected from the activation of Gq, incubation of muscle cells with melatonin for 60 s caused an increased in inositol formation in a
concentration-dependent fashion with an IC\textsubscript{50} of 4±1 nM (Fig. 8). The increase was significant at 10 nM (1473±104 cpm/mg protein above basal levels of 896±101 cpm/mg protein, p<0.01, n=4) and a maximal increase was obtained at 1 µM (7142±351 cpm/mg protein/mg protein above basal levels, p<0.001, n=4). The extent of stimulation of PLC-β activity with melatonin was similar to that obtained with other contractile agonists such as acetylcholine or substance P in gastric smooth muscle cells [111].

The G protein involved in the activation of PLC-β activity in response to melatonin was obtained by expression of G\textsubscript{α} minigenes in cultured smooth muscle cells. The synthetic peptide corresponding to the COOH terminus of G\textsubscript{α} subunits selectively antagonized G protein activation by blocking receptor-G protein interaction [112, 113]. Minigene plasmid constructs that encode COOH-terminal peptide sequence of G\textsubscript{i} and G\textsubscript{q} were expressed to selectively block G\textsubscript{i} and G\textsubscript{q} activation, respectively. Treatment of cultured muscle cells with melatonin (1 µM) caused a significant increase in PLC-β activity and the extent of stimulation was closely similar to that obtained in freshly dispersed smooth muscle cells (5867±980 cpm/mg protein above basal levels in cultured smooth muscle cells and 6245±1005 cpm/mg protein above basal levels in freshly dispersed smooth muscle cells). Expression of G\textsubscript{α}q minigene blocked PLC-β activity in response to melatonin (Fig. 9). In contrast, expression of G\textsubscript{i} minigene had no effect on PLC-β activity (Fig. 9). The results suggest that MT1 receptor coupled to activation of PLC-β activity via G\textsubscript{α}q and is consistent with the selective activation of G\textsubscript{α}q by melatonin.
Activation of PLC-β results in the generation of inositol 14,5-trisphosphate (IP3) and IP3-dependent Ca\(^{2+}\) release from intracellular sarcoplasmic reticulum stores [114]. Consistent with the activation of G\(_q\) and stimulation of PLC-β activity, addition of melatonin to cells loaded with fura-2 resulted in an increase in cytosolic Ca\(^{2+}\) (Fig. 10). The increase in Ca\(^{2+}\) was not affected by removal of extracellular Ca\(^{2+}\) suggesting that the increase is due to release of Ca\(^{2+}\) from intracellular stores.

Stimulation of PLC-β activity and increase in intracellular Ca\(^{2+}\) by contractile agonists in smooth muscle leads to muscle contraction. The functional significance of MT1 receptor-mediated stimulation of PLC-β activity and increase in cytosolic Ca\(^{2+}\) was examined by measurements of muscle contraction by scanning micrometry. Contraction was measured as decrease in muscle cell length in response to melatonin compared to control cell length. Treatment of muscle cells with 1 µM melatonin caused 25±42% (p<0.001, n=6) decrease in cell length (control cell length 125±4 µm) (Fig. 11). The extent of contraction induced by melatonin is similar to that obtained with other contractile agonists such as acetylcholine, substance P, ATP, sphingosine 1-phosphate (27±3% to 32±4% decrease in cell length) in smooth muscle cells [111, 112, 114]. Melatonin-induced contraction was blocked by the selective PLC-β inhibitor, U-73122 (10 µM) (5±3% contraction in the presence of U73122) (Fig. 11). These results suggest that contraction in response to melatonin was mediated via activation of PLC-β and generation of IP3 and IP3-dependent Ca\(^{2+}\) release.
In summary,

1. MT1, but not MT2 receptors are expressed in gastric smooth muscle cells.

2. MT1 receptors are coupled to activation of $G_q$, but not $G_s$, $G_{i1}$, $G_{i2}$ and $G_{i3}$

3. Melatonin induced activation of PI-specific PLC-β activity in a concentration-dependent manner in freshly dispersed muscle cells.

4. Melatonin also stimulated PLC-β activity in cultured smooth muscle cells and the stimulation was blocked in cells expressing $G_\alpha_q$ minigene, but not $G_\alpha_i$ minigene

5. Melatonin induced an increase in cytosolic Ca$^{2+}$ in the absence of extracellular Ca$^{2+}$.

6. Melatonin elicited muscle contraction and the response is similar to other contractile agonists such as acetylcholine, as shown previously. Contraction was inhibited by the PLC-β inhibitor U73122 consistent the stimulation of PLC-β activity and increase in intracellular Ca$^{2+}$ by melatonin.
Figure 1: Expression of MT1 receptors in gastric smooth muscle cells. A). Total RNA isolated from cultured (first passage) rabbit gastric muscle cells was reverse transcribed, and cDNA was amplified with specific primers for MT1 or MT2. Experiments were done in the presence or absence of RT. PCR product with predicted size (226) was obtained in the presence of RT only with primers for MT1. B) Western blot analysis of lysates prepared from dispersed smooth muscle cells (lane 1) and cultured gastric smooth muscle cells (lane 2) of rabbit and cultured gastric smooth muscle cells of rat (lane 3). Proteins were probed with polyclonal antibodies to MT1 (1:1000) or MT2 (1:1000). A protein band corresponding to 36 kDa was obtained with only MT1 antibody.
Figure 1

RT-PCR

Western Blot

MW  + RT  - RT

1  2  3
Figure 2. Partial nucleotide sequence of MT1-R receptors from rabbit gastric muscle cells. The RT-PCR product from cultured rabbit gastric smooth muscle cells was sequenced.
Figure 2

TGC CAT TTG CTG GGC TCC TCT AAA CTT CAT TGG TCT TGC AGT GGC
CTC AGA CCC TGT CAG CAT GGT ACC CAG AAT CCC AGA GTG GCT GTT
TGT GGC TAG TTA TTA CAT GGC AAT ATT TCA ACA GCT GTC TCA ATG
CAA TCA TAT ATG GAC TAC TGA ACC AGA ATT TCC GGG AGG AAT ATA
GAA GAA TTA TAA TCT CAA TGT GTA CAG CAA GGA TGT TCT TTG TGG
A
Figure 3. Activation of $G_{i1}$ proteins by melatonin in dispersed muscle cells.

Membranes were isolated from dispersed gastric muscle cells and incubated with $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ for 20 min in the presence or absence of melatonin (1 µM). Aliquots were added to wells coated with $G\alpha_{i1}$ antibody for 2 h and bound radioactivity from each well was counted by liquid scintillation. The amount of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ bound to the activated $G\alpha$ subunit was expressed as counts per minute (cpm) per milligram of protein. Melatonin did not induce significant increase in the binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}.G\alpha$ complexes to wells coated with $G\alpha_{i1}$ antibody. Values are mean±SEM of 4 experiments.
G<sub>i1</sub> Activation

[Superscript 35]S-GTP<sub>γ</sub>S.G<sub>α</sub> binding (cpm/mg protein)

- Basal
- GTP<sub>γ</sub>S
- GTP<sub>γ</sub>S + Melatonin
Figure 4. Activation of G_{i2} proteins by melatonin in dispersed muscle cells. Membranes were isolated from dispersed gastric muscle cells and incubated with [^{35}S]GTP\gamma S for 20 min in the presence or absence of melatonin (1 \mu M). Aliquots were added to wells coated with G\alpha_{i2} antibody for 2 h and bound radioactivity from each well was counted by liquid scintillation. The amount of [^{35}S]GTP\gamma S bound to the activated G\alpha subunit was expressed as counts per minute (cpm) per milligram of protein. Melatonin did not induce significant increase in the binding of [^{35}S]GTP\gamma S.G\alpha complexes to wells coated with G\alpha_{i2} antibody. Values are mean±SEM of 4 experiments.
Figure 4

$[\text{GTP}^\gamma S, \text{G}^\alpha]$ binding (cpm/mg protein)

$G_i^2$ Activation

Basal | GTP$_\gamma$S | GTP$_\gamma$S + Melatonin

0 200 400 600 800 1000 1200
Figure 5. Activation of G\textsubscript{i3} proteins by melatonin in dispersed muscle cells. Membranes were isolated from dispersed gastric muscle cells and incubated with [\textsuperscript{35}S]GTP\textsubscript{γ}S for 20 min in the presence or absence of melatonin (1 µM). Aliquots were added to wells coated with G\textalpha{}\textsubscript{i3} antibody for 2 h and bound radioactivity from each well was counted by liquid scintillation. The amount of [\textsuperscript{35}S]GTP\textsubscript{γ}S bound to the activated G\textalpha{} subunit was expressed as counts per minute (cpm) per milligram of protein. Melatonin did not induce significant increase in the binding of [\textsuperscript{35}S]GTP\textsubscript{γ}S.G\textalpha{} complexes to wells coated with G\textalpha{}\textsubscript{i3} antibody. Values are mean±SEM of 4 experiments.
Figure 5

G\textsubscript{i3} Activation

\[\text{[^{35}S]GTP\gamma S.G\alpha binding (cpm/mg protein)}\]

Basal  GTP\gamma S  GTP\gamma S + Melatonin

0  200  400  600  800  1000  1200
Figure 6. Activation of G\textsubscript{s} proteins by melatonin in dispersed muscle cells. Membranes were isolated from dispersed gastric muscle cells and incubated with [\textsuperscript{35}S]GTP\textgamma S for 20 min in the presence or absence of melatonin (1 \(\mu\)M). Aliquots were added to wells coated with G\textalpha\textsubscript{s} antibody for 2 h and bound radioactivity from each well was counted by liquid scintillation. The amount of [\textsuperscript{35}S]GTP\textgamma S bound to the activated G\textalpha subunit was expressed as counts per minute (cpm) per milligram of protein. Melatonin did not induce significant increase in the binding of [\textsuperscript{35}S]GTP\textgamma S.G\textalpha complexes to wells coated with G\textalpha\textsubscript{s} antibody. Values are mean±SEM of 4 experiments.
Figure 6

### Gs Activation

- **[35S]GTPγS: Gα binding (cpm/mg protein)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>[35S]GTPγS Binding (cpm/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>Basal</td>
<td>600</td>
</tr>
<tr>
<td>GTPγS</td>
<td>800</td>
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<tr>
<td>GTPγS + Melatonin</td>
<td>1000</td>
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</table>
Figure 7. Activation of Gq proteins by melatonin in dispersed muscle cells.
Membranes were isolated from dispersed gastric muscle cells and incubated with \(^{35}\text{S} \text{GTP}\gamma\text{S}\) for 20 min in the presence or absence of melatonin (1 µM). Aliquots were added to wells coated with G\(\alpha_q\) antibody for 2 h and bound radioactivity from each well was counted by liquid scintillation. The amount of \(^{35}\text{S} \text{GTP}\gamma\text{S}\) bound to the activated G\(\alpha\) subunit was expressed as counts per minute (cpm) per milligram of protein. Melatonin induced significant increase in the binding of \(^{35}\text{S} \text{GTP}\gamma\text{S}.G\alpha\) complexes to wells coated with G\(\alpha_q\) antibody. Values are mean±SEM of 4 experiments. ** \(P< 0.001\)
Figure 7

Gq Activation

[^35S]GTPγS.Gα binding (cpm/mg protein)

- Basal
- GTPγS
- GTPγS + Melatonin

0 1000 2000 3000 4000 5000 6000
Figure 8. Concentration-response curve for the stimulation of melatonin-induced
PLC-β activity in dispersed gastric muscle cells. Phosphoinositide-specific (PI)
hydrolysis (PLC-β activity) in response to melatonin was measured in muscle cells
labeled with myo-[³H]inositol. Freshly dispersed muscle cells were treated for 60 s with
different concentrations of melatonin and PLC-β activity was measured as increase in
water-soluble [³H]inositol formation. The results are expressed as [³H]inositol phosphate
formation in counts per minute (cpm) per mg protein above basal levels (cpm/mg
protein). Values are means±SEM of 4 experiments. **P< 0.01
Figure 8

Stimulation of PI Hydrolysis

PI Hydrolysis ([3H]inositol)
(cpm/mg protein)

Melatonin (log M)

-12 -11 -10 -9 -8 -7 -6 -5

0 1000 2000 3000 4000 5000 6000 7000 8000 9000
Figure 9. Inhibition of melatonin-stimulated PLC-β activity by Gαq minigene

Cultured gastric muscle cells labeled with myo-[3H]inositol and expressing Gαq minigene, Gαi minigene, or control vector were treated with melatonin (1 µM) for 60 s. Total [3H]inositol phosphates were separated by ion-exchange chromatography. PLC-β activity stimulated by melatonin was abolished in cells expressing Gαq minigene, but was not affected in cells expressing Gαi minigene. Results are expressed as total [3H]inositol phosphate formation in cpm/mg protein. Values are means ± S.E. of four experiments.

** Significant inhibition from control response (P<0.01)
Figure 9

PI Hydrolysis ([³H]inositol) (cpm/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Melatonin</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gαq</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gαi minigene</td>
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</tbody>
</table>

Values for Gαq minigene and Gαi minigene are significantly lower compared to the control and basal values.

Melatonin treatment shows a marked increase in PI hydrolysis compared to the basal and control conditions.

Legend:
- Basal
- Melatonin
Figure 10. Melatonin-induced increase in cytosolic Ca\(^{2+}\) in single muscle cells.

Muscle cells were loaded with 5 µM fura-2 and treated with 1 µM melatonin in the absence of extracellular Ca\(^{2+}\). The cells were alternately excited at 380 nm and 340 nm. The background and autofluorescence were corrected from images of a cell without the fura 2. Results are expressed as 340/380 ratio and an increase in ratio reflects an increase in cytosolic Ca\(^{2+}\). The figure shows results obtained from 38 cells.
Figure 10

Increase in Cytosolic Ca$^{2+}$

340/380 Ratio vs Milli seconds

1 µM Melatonin
Figure 11. Melatonin-induced muscle contraction in dispersed gastric muscle cells.

Contraction of muscle cells was measured as decrease in basal cell length in response to melatonin (1 µM). Muscle cells (0.5 ml cell suspension) were treated with melatonin (1 µM) in the presence or absence of U73122 (10 µM). The mean length of 50 muscle cells was measured by scanning micrometry and was compared with the length of untreated muscle cells. The contractile response was expressed as the percent decrease in the mean cell length from control cell length. Melatonin induced a significant decrease in cell muscle length that was blocked by the PLC-β inhibitor, U73122 (10 µM). Values are means±SEM of 6-8 experiments.
Figure 11

Contraction (% decrease in cell length)

Muscle contraction

Melatonin + U73122

Contractions

0 5 10 15 20 25 30 35
Melatonin release from pineal gland displays a circadian rhythmic pattern with the increased release during nighttime and decreased release during the daytime. Melatonin is also released from serotonin-rich enterochromaffin cells in the gut, but the release does not exhibit a pronounced circadian rhythm but seems to depend on the nutritional state. Melatonin regulates rhythmic changes in gastrointestinal motility. Several in vitro studies in isolated muscle strips demonstrated that the actions of melatonin are complex and involve both direct and indirect effects on smooth muscle including antagonistic relationship between serotonin and melatonin [95-100, 115-121].

The present study characterized the signaling pathways mediated by melatonin receptors in gastric smooth muscle cells using biochemical, molecular and functional methods. The results demonstrate the expression of MT1, but not MT2 receptors in gastric smooth muscle cells and their ability to stimulate PLC-β activity, increase intracellular Ca^{2+} and induce smooth muscle contraction.

The evidence for the coupling of MT1 receptors to G_{q}-dependent stimulation of PLC-β activity and to elicit muscle contraction was based on a combination of experimental strategies.

(i) mRNA and protein expression of MT1 was demonstrated in cultured muscle cells by RT-PCR and in isolated muscle cells by western blot. MT2 receptors are not detected by RT-PCR or western blot analysis, raising the possibility that the expression of these proteins is either absent or not abundant in these cells. The selective expression of MT1 receptors in smooth muscle cells of the gut is consistent with their expression in
other cells types of the gut. Receptors for melatonin have been identified in the gut of several species. Storr et al [96, 97] has demonstrated the expression of MT1, but not MT2 receptors using RNA isolated from the muscle layers of stomach and intestine. The distribution of melatonin receptors through the gut seems to vary with species [122, 123] and their density as well as their affinity for the ligand may vary over the photoperiod [124].

(ii) The MT1 receptors are coupled to activation of Gq. Selective activation of Gq was demonstrated using [35S]GTPγS and subtype-selective G protein antibodies. [35S]GTPγS-Gα complexes activated by melatonin bound selectively to Gαq antibodies reflecting activation of Gq proteins. No melatonin induced increase in the binding to Gαi1, Gαi2, Gαi3, or Gαs antibodies to [35S]GTPγS could be detected. Studies in various cell lines suggest that MT1 are coupled to both PTx-sensitive and PTx–insensitive G proteins [83, 115]. Our studies demonstrate that MT1 receptors are coupled to PTX-insensitive Gq protein. The coupling of MT1 to G proteins likely depends on the cell type and the relative abundance of G protein subtype.

(iii) Melatonin caused an increase in PLC-β activity (PI hydrolysis) in a concentration-dependent fashion. The extent of increase was similar to that obtained with other Gq-coupled receptors (e.g., muscarinic m3 receptors) in gastric smooth muscle cells. Previous studies in these muscle cells have shown that receptors coupled to both Gq and Gi proteins stimulate PI hydrolysis via distinct PLC-β isoforms. Gq coupled receptors such as muscarinic m3, sphingosine-1-phosphate 2 (S1P2), endothelin ETA, purinergic P2Y2, and NPY2 are coupled to stimulation of PI hydrolysis via Gαq-dependent PLC-β1 isozyme, whereas Gi3-coupled m2, P2Y2, and adenosine A1
receptors, \( G_{i1} \)-coupled somatostatin \( sst3 \) receptors, \( G_{i2} \)-coupled opioid, \( \mu \), \( \kappa \), \( \delta \) receptors, and \( G_{i1/2} \)-coupled S1P2 receptors are coupled to stimulation of PI hydrolysis via \( G\beta\gamma i \)-dependent PLC-\( \beta3 \) isozyme [106-108, 112]. Thus, P2Y2 and S1P2 receptors are coupled to stimulation of PI hydrolysis via both \( G\alpha q \)-dependent PLC-\( \beta1 \) isozyme and \( G\beta\gamma i \)-dependent PLC-\( \beta3 \) isozyme. Although all four PLC-\( \beta \) isozymes are expressed in the smooth muscle of the gut, neither PLC-\( \beta2 \) nor PLC-\( \beta4 \) is activated [104]. PLC-\( \beta1 \) is activated via binding of its COOH-terminal tail, a characteristic feature of PLC-\( \beta \) isozymes, to \( G\alpha q \), whereas PLC-\( \beta3 \) is activated via binding to its NH2-terminal pleckstrin homology (PH) domain to \( G\beta\gamma i \). Phosphatidylinositol 4,5-bisphosphate (PIP2) is the predominant phosphoinositide substrate hydrolyzed by both PLC-\( \beta1 \) and PLC-\( \beta3 \) isozyme, resulting in the generation of diacylglycerol, an activator of protein kinase C (PKC) and inositol 1,4,5-trisphosphate (IP3), a diffusible \( Ca^{2+} \)-mobilizing messenger.

The specific G proteins involved in the stimulation of PLC-\( \beta \) activity by melatonin was examined using minigene approach. Previous studies have shown that the COOH-terminus of \( G \) protein \( \alpha \) subunits is critical in mediating receptor-G protein interaction and peptides corresponding to COOH-terminus serve as competitive inhibitors of receptor-G protein interaction [112, 113]. The minigene plasmid vectors were designed to express the COOH-terminal peptide sequences of various \( G\alpha \) subunits after transfection into cells. In gastric muscle cells transfection of minigene plasmid constructs that encode oligonucleotide sequences corresponding to \( G\alpha q \) completely blocked the activation of PLC-\( \beta \) activity by melatonin. The results provide evidence that
MT1 receptors are coupled to stimulation of PLC-β activity via \( G_q \) and this is consistent with the selective activation of \( G_q \).

(iv) Melatonin, as other Gq-coupled receptors, induced an increase intracellular \( \text{Ca}^{2+} \) in single muscle cells. The increase in \( \text{Ca}^{2+} \) was not affected by removal extracellular \( \text{Ca}^{2+} \) suggesting that the source of \( \text{Ca}^{2+} \) was intracellular. This is consistent with the activation of PLC-β activity which results in the generation of \( \text{Ca}^{2+} \) mobilizing messenger IP3.

(v) Consistent with the stimulation of PLC-β activity and increase in intracellular \( \text{Ca}^{2+} \) in response to melatonin, addition of melatonin to dispersed gastric muscle cells elicited rapid (30 s) muscle contraction. Contraction was blocked by the PLC-β inhibitor U73122. The extent of muscle contraction was similar to the other contractile agonists such as acetylcholine and substance P [108, 111]. In smooth muscle of the gut, various Gq protein-coupled receptor agonists initiate contraction by increasing cytosolic \( \text{Ca}^{2+} \) via IP3-dependent \( \text{Ca}^{2+} \) release from sarcoplasmic \( \text{Ca}^{2+} \) stores. The sarcoplasmic \( \text{Ca}^{2+} \) stores contain high-affinity IP3 receptors and release \( \text{Ca}^{2+} \) in response to increase in cytosolic IP3 levels. Three types IP3 receptors, IP3R-I, IP3 R-II and IP3 R-III, have been cloned. Although smooth muscle of the gut expresses both IP3 R-I and IP3 R-II, only IP3 R-I is involved in \( \text{Ca}^{2+} \)-release in response to receptor activation [110]. The increase \( \text{Ca}^{2+} \) and the binding of \( \text{Ca}^{2+} \) to calmodulin results in the stimulation of \( \text{Ca}^{2+}/\text{calmodulin-dependent myosin light chain} \) (MLC) kinase activity and phosphorylation of MLC\(_{20}\) at Ser\(^{19}\), a prerequisite for initiation of actomyosin interaction and muscle contraction [114].

Studies by Lucchelli et al [118] also demonstrated direct contractile effect of melatonin in gastrointestinal smooth muscle. These studies demonstrated that melatonin
and its analogues induced contractile responses in guinea pig isolated proximal colon in a concentration-dependent manner. The rank of agonist potency was: 2-indomelatonin>6-chloromelatonin>N-acetyl-5-HT>5-MCA-NAT>melatonin, an order typical for MT2 receptors. However, prazosin, an α-adrenoreceptor antagonist possessing moderate/high affinity for melatonin MT2 sites had no effect on melatonin-induced contractions. While the rank order of agonist potencies would suggest the participation of MT2 receptors, the ineffectiveness of prazosin on melatonin-induced contractions suggests to the contrary. The lack of MT1 selective antagonists precluded the conclusion of implicating MT1 receptors. Melatonin responses were also resistant to atropine and tetrodotoxin suggesting a direct effect on muscle cells.

In contrast to contractile effect, studies by Storr et al [96, 97] have demonstrated that addition of melatonin to isolated gastric and intestine muscle strips caused inhibition of NANC-mediated muscle relaxation via MT1 receptors. These studies indicate the difficulties in identifying the signaling pathways activated by melatonin in innervated muscle strips. The effect of melatonin may vary in different species and different regions of the gut depending on whether the activated receptor is present predominantly on smooth muscle cells or enteric neurons. Our studies provide evidence for the involvement of MT1 receptors in melatonin-induced contraction is isolated muscle cell devoid of neural elements.

In summary, the present study demonstrated that gastric smooth muscle cells express receptors (MT1) for melatonin preferentially coupled to Gq. These receptors are coupled to stimulation of PLC-β activity and Ca^{2+} release from intracellular stores resulting in muscle contraction (Fig. 12).
Figure 12. **Pathway mediating muscle contraction by melatonin.** In gastric smooth muscle, melatonin interacts with MT1 receptors, which is coupled to stimulation of phosphoinositide-specific phospholipase C (PLC-β) via Gq. Stimulation of PLC-β activity results in the generation of inositol 1,4,5-trisphosphate (IP3) and IP3-dependent Ca^{2+} release leading to muscle contraction.
Signaling by MT1 in Smooth Muscle

Melatonin

MT1

\( \alpha_q \cdot GTP \)

\( \gamma \)

\( \beta \)

PLC-\( \beta \)

\( \text{IP}_3 : \text{Ca}^{2+} \)

Contraction
E- References


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