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Serotonin interferes with Ca^{2+} and PKC signaling to reduce gonadotropin-releasing hormone-stimulated GH secretion in goldfish pituitary cells

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ABSTRACT

In goldfish, two endogenous gonadotropin-releasing hormones (GnRH), salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), are thought to stimulate growth hormone (GH) release via protein kinase C (PKC) and subsequent increases in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). In contrast, the signaling mechanism for serotonin (5-HT) inhibition of GH secretion is still unknown. In this study, whether 5-HT inhibits GH release by actions at sites along the PKC and Ca^{2+} signal transduction pathways leading to hormone release were examined in primary cultures of goldfish pituitary cells. Under static incubation and column perifusion conditions, 5-HT reduced basal, as well as sGnRH- and cGnRH-II-stimulated, GH secretion. 5-HT also suppressed GH responses to two PKC activators but had no effect on the GH-releasing action of the Ca^{2+} ionophore ionomycin. Ca^{2+}-imaging studies with identified somatotropes revealed that 5-HT did not alter basal $[Ca^{2+}]_i$ but attenuated the magnitude of the $[Ca^{2+}]_i$ responses to the two GnRHs. Prior treatment with 5-HT and cGnRH-II reduced the magnitude of the $[Ca^{2+}]_i$ responses induced by depolarizing levels of K^+. Similar inhibition, however, was not observed with prior treatment of 5-HT and sGnRH. These results suggest that 5-HT, by direct actions at the somatotrope level, interferes with PKC and Ca^{2+} signaling pathways to reduce the GH-releasing effect of GnRH. 5-HT action may occur at the level of PKC activation or its downstream signaling events prior to the subsequent rise in $[Ca^{2+}]_i$. The differential Ca^{2+} responses by depolarizing doses of K^+ is consistent with our previous findings that sGnRH and cGnRH-II are coupled to overlapping and yet distinct Ca^{2+}-dependent mechanisms.

1. Introduction

Since its identification as a neurotransmitter, serotonin (5-HT) has been implicated as a regulator of hypothalamic functions in mammals, including stress, satiation, mood, sleep and body temperature (Struder and Weicker, 2001). Among the actions of 5-HT on the hypothalamo-hypophysial axis, the dynamic interplay between 5-HT and the hypothalamus-pituitary-adrenal axis has been extensively studied because of the importance of 5-HT in regulating stress (Lowry, 2002). In addition, 5-HT has also been shown to regulate growth hormone (GH) release although its effects are controversial. Early studies with in vivo treatment of 5-HT or 5-HT receptor agonists and antagonists suggested a stimulatory effect of 5-HT on GH release in mammals (Arnold and Fernstrom, 1978, 1981; Collu et al., 1979); however, Spencer et al. (1991) and Muller et al. (1976) reported that 5-HT inhibits GH release in sheep and dog, respectively. In birds, evidence indicates that 5-HT inhibits GH secretion by reducing hypothalamic GH-releasing activity (Hall, 1982; Carew et al., 1983). The lack of direct 5-HT effects at the level of the pituitary in most mammalian studies supports the view that 5-HT indirectly affects GH release via actions on GH-releasing hormone (GHRH), pituitary adenylate cyclase-activating polypeptide (PACAP) and somatostatin neurons in the hypothalamus (Willoughby et al., 1987; Conway et al., 1990; Radcliff et al., 2003). However, recent findings with rat pituitary cell aggregates suggest that direct action at the level of the pituitary is also possible (Papageorgiou and Denef, 2007).

In teleosts such as goldfish, rainbow trout and Atlantic croaker, 5-HT immunoreactive fibers have been detected in the pars distalis of the pituitary gland (Kah and Chambolle, 1983; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Khan and Thomas, 1993), suggesting that 5-HT can directly act at the level of the pituitary in bony fishes. In support of the hypothesis of direct action at the level of the pituitary, 5-HT reduced GH release from goldfish pituitary fragments and cultured pituitary cells in vitro (Somoza and Peter, 1991; Wong et al., 1998). This ability of 5-HT to inhibit GH release was not confined to basal secretion but was also observed with stimulated GH secretion in goldfish (Wong et al., 1998). The exact physiological condition(s) under which 5-HT influence on GH release is important has not been elucidated. However, sex steroids such as estradiol are known to elevate serum GH levels in

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several fish species, including the goldfish and rainbow trout (Canosa et al., 2007). In rainbow, estradiol induced an increase in pituitary 5-HT level and decreased 5HIAA/5-HT ratio, suggesting that 5-HT turnover is suppressed (Hernandez-Rauda and Aldegunde, 2002). Whether estradiol feedback stimulation of pituitary GH release involves the removal of a 5-HT inhibitory influence is a possibility that is worth investigating.

Extensive information is available on how 5-HT acts in mammals. To date, at least seven distinct serotonergic receptor subtypes (5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, 5-HT6 and 5-HT7) have been identified or cloned in mammals. In addition, the 5-HT1, 5-HT2 and 5-HT5 have been further subclassified (Bockaert et al., 2006). In mammals, the stimulation role of 5-HT on the GH regulation has been attributed to 5-HT1B/D and 5-HT2 receptors (Mota et al., 1995; Katz et al., 1996; Valverde et al., 2000; Papageorgiou and Denef, 2007). Using pharmacological manipulation of 5-HT receptors, it was reported that 5-HT2-like receptors may be involved in inhibition of 5-HT on basal GH release in goldfish (Wong et al., 1998). However, very little is known with regard to the molecular mechanisms responsible for this inhibitory action of 5-HT on GH release in goldfish.

In teleosts, hypothalamic neurons directly innervate the pars distalis. In goldfish, GH release is stimulated by many hypothalamic neuroendocrine factors, including gonadotropin-releasing hormone (GnRH), GHRH, dopamine and PACAP (reviewed in Canosa et al., 2007). Two GnRHs are found in, and released from, the pituitaries of goldfish, these being GnRH forms initially identified in salmon and chicken, namely, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II). Among these neuroendocrine stimulators, the mechanisms of action for the two GnRHs, are the best characterized. Both sGnRH and cGnRH-II stimulate GH release via activation of protein kinase C (PKC), increases in intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$), and activation of voltage-sensitive Ca^{2+} channels (VSCC); however, the involvement of intracellular Ca^{2+} stores in their GH-releasing actions are not identical. Although a caffeine-sensitive Ca^{2+} store participates in both sGnRH and cGnRH-II stimulation of GH release, only sGnRH uses an IP3-sensitive mechanism, whereas cGnRH-II utilizes a ryanodine-sensitive Ca^{2+} signaling pathway. In addition, only sGnRH-induced GH release is attenuated by mitochondrial Ca^{2+} buffering. These and other results not only indicate that multiple pharmacologically distinct intracellular Ca^{2+} stores are present in goldfish somatotropes but that the two GnRHs mobilizes Ca^{2+} from dissimilar intracellular stores (Canosa et al., 2007). In this study, we examined the signal transduction mechanisms mediating 5-HT inhibition of GH release from primary cultures of goldfish pituitary cells under basal condition and GnRH stimulation. The possible involvement of PKC was examined by studying 5-HT action on the GH-releasing effects by PKC activators including the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Liu and Heckman, 1998) and the synthetic diacylglycerol dioctanoylglycerol (DiC8; Nishizuka, 1986). Involvement of $[Ca^{2+}]_i$ was also examined in Ca^{2+}-imaging experiments with morphologically identified goldfish somatotropes (Johnson et al., 2002) and in hormone release experiments with the Ca^{2+} ionophore ionomycin (Kwong and Chang, 1997). Our results suggest that 5-HT can modulate GnRH-stimulated increase in $[Ca^{2+}]_i$ and inhibit GH release responses to PKC activation in goldfish somatotropes.

2. Materials and methods

2.1. Animals

All animal maintenance and experimental protocols used in this study have been approved by the Biological Sciences Animal Care Committee, University of Alberta in accordance with national guidelines. Common goldfish (*Carassius auratus*, 8–13 cm in length) were purchased from Aquatic Imports (Calgary, Alberta, Canada) and kept in flow-through aquaria (1800 liters) with simulated (Edmonton, Alberta, Canada) photoperiod at 18 °C. Fish were generally used within a month of purchase. Both male and female goldfish were used. Goldfish were killed by cervical transection after deep anaesthesia with 0.05% tricaine methanesulfonate (Syndel Lab., Vancouver, BC). Pituitaries were then excised and dispersed pituitary cells were prepared by a trypsin/DNAse protocol (Chang et al., 1990a).

2.2. Chemicals and drugs

Serotonin (5-HT), TPA, DiC8 were purchased from Sigma Chemical Co (St Louis, MO). Ionomycin was obtained from Calbiochem (San Diego, CA). Fura-2/AM and its solvent, Pluronic F-127, were purchased from Invitrogen (Carlsbad, CA). Synthetic sGnRH ([Trp^7, Leu^8]GnRH) and cGnRH-II ([His^5, Trp^7, Tyr^8]GnRH) were purchased from Peninsula Laboratories (Belmont, CA). 5-HT was freshly made prior to use by dissolving directly in the perifusion medium. Distilled, deionised water was used to prepare stock solutions of sGnRH and cGnRH-II. TPA, DiC8 were dissolved in dimethyl sulphoxide (DMSO). Ionomycin was dissolved in ethanol. Aliquots of stock solutions were stored at −20 °C. Final concentrations of DMSO and ethanol were always less than 0.1% and had no effect on basal GH release and $[Ca^{2+}]_i$ in identified goldfish somatotropes. Stock solutions of 10 mM fura-2/AM was made up in DMSO with 20% Pluronic F-127 and sonicated for 10 min prior to use. Depolarizing media (30 mM KCl) for imaging experiments was made by equimolar substitution of NaCl with KCl.

2.3. Column perifusion of goldfish pituitary cells

Perifusion experiments were carried out using an Accusyst system as described in Chang et al. (1990b). Briefly, after dispersion, pituitary cells were cultured overnight with preswollen Cytodex beads (Cytodex I, Sigma) in plating medium (Medium M199 with Earles' salts, 26 mM $NaHCO_3$, 25 mM Hepes, 100 mg/l streptomycin, 100,000 units/l penicillin and 1% horse serum; pH 7.2) at 28 °C, under 5% CO_2 and saturated humidity. Cells on Cytodex beads were then transferred into temperature controlled columns (1.5 million cells/column, 18 °C) and perifused with testing medium (Medium 199 with Hank's salts, 26 mM $NaHCO_3$, 25 mM Hepes, 100 mg/l streptomycin, 100,000 units/l penicillin and 0.1% BSA; pH 7.2) at a flow rate of 13 ml/h. Fractions of perifusate were collected every 5 min and stored at −26 °C until hormone levels were assayed by a radioimmunoassay (RIA) previously validated for goldfish GH (Marchant et al., 1987). Treatments were performed in duplicate in each experiment and all experiments were repeated a minimum of three times. Cell column perifusion experiments are suitable for studies on acute release responses and have the advantage that hormone release kinetics can be revealed. In addition, the flow-through nature of this system means that cells are not exposed to products released from neighbouring cells for extended periods of time (calculated volume replacement time for the cell chamber $\leqslant$ 1.2 min). However, the large number of samples generated is a drawback.

2.4. Static incubation experiments assessing GH release

Static incubation studies were carried out according to Chang et al. (1990a). Briefly, after dispersion, pituitary cells were plated at a density of 0.25 million cells/well in 24-well plates (Falcon Primaria, Franklin lakes, NJ) and cultured overnight at 28 °C, and under 5% CO_2 and saturated humidity in culture media. Following a rinse with testing medium on the next day, cells were cultured in the presence of 5-HT and/or pharmacological agents for 2 h. At