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Substance P Regulates PTH Secretion through the Neurokinin-1 Receptor

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The primary regulator of PTH secretion is serum ionized Ca^{2+}; however, neuropeptide-containing nerve fibers have been localized to the parathyroid gland. The purpose of this study was to determine whether or not substance P (SP) regulates PTH secretion. In dispersed porcine parathyroid cells, SP reversibly inhibited 0.5 mM $CaCl_2$-induced PTH secretion (IC_{50} = 0.29 nM) and had no effect at $CaCl_2$ concentrations of 1.5 mM and greater. At 0.5 mM $CaCl_2$, treatment with a NK-1 selective receptor agonist resulted in a concentration-dependent decrease in PTH secretion (IC_{50} = 0.21 nM). In contrast, NK-2 and NK-3 receptor agonists were approximately 100-fold less active than SP or the NK-1 receptor selective agonist. An enantiospecific reversal of the effects of SP on PTH secretion was observed with LY306740, a potent selective NK-1 receptor antagonist (K_i = 0.125 nM). In porcine parathyroid cells, expression of mRNA for the NK-1 receptor was observed using RT-PCR. In summary, a novel neuroendocrine pathway is described whereby the neuropeptide, SP, regulates PTH secretion through NK-1 receptors.

Key Words: **substance P; PTH; neurokinin 1 receptor.**

Extracellular ionized Ca^{2+} concentration is normally regulated by parathyroid hormone (PTH), an 84-amino acid protein. PTH is released from the parathyroid gland in response to hypocalcemic conditions and acts directly on the bone and kidney to normalize extracellular ionized Ca^{2+}. The ionized Ca^{2+} feeds back on the parathyroid gland to decrease PTH secretion. Although, ionized Ca^{2+} appears to be the primary regulator of PTH secretion; serum PTH levels *in vivo* show circadian and hourly pulsatile variations in PTH levels that are not attributable to known regulators of PTH secretion (1). Consequently, there are probably other mechanisms by which PTH is regulated.

Neuroendocrine regulation of hormone secretion has been demonstrated for various endocrine glands. Nerve fibers containing vascular intestinal polypeptide (VIP), peptide histidine iso-leucine, neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP), galanin, and cholecystekinin (CCK) have been localized to the thyroid gland (2). VIP, CGRP, and NPY have been shown to regulate thyroid hormone secretion (2–4). SP, VIP and cholecystokinin (CCK)-4 and CCK-8 have been shown to stimulate calcitonin secretion in rats (5). Other studies have demonstrated that calcium-induced calcitonin secretion is augmented by VIP (6). VIP stimulates insulin and glucagon secretion in the perfused cat pancreas (7). In dispersed parathyroid cells, VIP has been shown to either stimulate PTH secretion (8) or have no effect (9). Since multiple neuroendocrine effects on hormone secretion have been demonstrated it is likely that PTH secretion could be similarly regulated.

Numerous studies have demonstrated neuronal innervation of the parathryoid gland (10–15). Although most of the nerve terminals are located around blood vessels, some were observed in the parenchyma and within the capsule. Galanin, VIP, SP, CGRP, NPY, and pituitary adenylate cyclase activating peptide (PACAP) have been immunolocalized to neurons innervating the parathyroid gland and/or surrounding tissue (16–21). These neuropeptides were identified in chicken, rat guinea pig, cat, dog, and sheep (19), except for galanin which was not identified in guinea pig. The physiologic function of these neurons or the neuropeptides that they contain is not well understood.

The purpose of this study was to evaluate the potential that locally produced SP regulates PTH secretion. SP inhibited 0.5 mM $CaCl_2$-induced PTH secretion, but had no at effect $CaCl_2$ concentrations of 1.5 mM and greater. Furthermore, SP regulated PTH secretion via

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a specific interaction with the parathyroid NK-1 receptors.

METHODS

Source of glands. Porcine parathyroid glands were collected at a slaughterhouse, dipped in 70% ethanol, and then in chilled Ham's F-12 medium (Life Technologies, Grand Island, NY) containing 1.3 mM $MgCl_2$, 1.25 mM $CaCl_2$, and 20 mM Hepes, pH 7.4. The glands were then trimmed of fat and connective tissue and stored on ice for approximately 3 h.

Cell isolation and culture. Parathyroid cells were isolated from porcine glands as described by Zhang *et al.* (22) and seeded into tissue culture dishes at a density of $1.9–2.2 \times 10^5$ viable cells/cm^2 in 1.3 mM $CaCl_2$-supplemented growth medium (Ham's F-12 containing 1 mM $MgCl_2$, 20 mM Hepes, 10% dialyzed FBS, and 1% antibiotic/antimycotic). Trypan blue exclusion was used to evaluate cell viability prior to plating of the cells. The cells were maintained overnight in a 37°C humidified incubator with 5% CO_2. The following day the medium was removed and the cells were treated for 3–6 h with medium containing various $CaCl_2$ concentrations (0.5–3 mM) and/or neuropeptides. The effects of SP, Ac[Arg^6, Sar^9, $Met(O_2)^{11}$]-SP 6-11, [β-Ala^8]-neurokinin A 4-10, or [$MePhe^7$]-neurokinin B (Peninsula Laboratories, Belmont, CA) were evaluated. In some experiments the cells were also treated with the NK-1 receptor antagonist, LY306740, or its less active enantiomer, LY307679, which were synthesized at Lilly Research Laboratories (Eli Lilly and Company, Indianapolis IN) (23). Following the 3–6 h treatment, the culture medium was collected, passed through MultiScreen Filtration plates (Millipore, Marlborough, MA), and the filtrate was analyzed for PTH using the Rat PTH (IRMA) Kit from Nichols Institute Diagnostics (San Juan Capistrano, CA). Following removal of the media, cytotoxicity of the factors was determined using the CyoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) according to the vendor's instructions.

RT-PCR analysis of NK-1 receptor expression. Total RNA was isolated from dissociated pig parathyroid cells and IM9 (human B lymphoblastoid) cells using Trizol reagent (Life Technologies) and 3.5 μg was reverse transcribed with oligo d(T) using the Superscript preamplification system (Bethesda Research Laboratories, Gaithersburg, MD). PCR was performed on a 2 μl aliquot of the synthesized cDNA using a DNA thermal cycler and reagents from GeneAmp (Perkin-Elmer Cetus, Norwalk, CT). The amplification reaction consisted of one cycle of 95°C for 5 min, 56°C for 1 min, and 72°C for 2 min. The remaining amplification consisted of 1 cycle of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, followed by 2 cycles with annealing temperature at 54°C, 2 cycles annealing at 52°C, 2 cycles annealing at 50°C, 2 cycles annealing at 48°C, and, finally, 25 cycles annealing at 46°C. The IM9 sample served as a positive control for NK-1 receptor mRNA. A water and reverse transcriptase negative control was always included to rule out template contamination of PCR reagents. The NK-1 specific primers were synthesized by Genosys Biotechnologies, Inc. (The Woolands, TX) and were as follows: 1NK-1 5′-CTTCTTCCTCCTGCCCTACA-3′ and 5′-GTGGAGATGGTGGTCTC-CAG-3′; 2NK-1 5′-CTTCCTCCTG-CCCTACATCA-3′ and 5′-CTCTCTGTCATGGTCTT-GGA-3′; 3NK-1 5′-GACTGTGCTGATCTACTTCT-3′ and 5′-CTCTCTGTCATGGTCT-TGGA-3′. PCR products were analyzed by agarose gel electrophoresis and transferred by electroblotting onto Hyond-N membrane. The membrane was soaked in 0.4 N NaOH for 7 min, then soaked 3 times in 2× SSC for 2 min each. The membrane was hybridized with an NK-1 specific biotinylated probe, 5′-TCTGGGCTTCAAGCATGCC-TTCCGG-3′, for 1.5 h at 62°C using the Phototope-Star Detection Kit for Nucleic Acids (New England Biolabs, Beverly, MA).

Statistical analysis. Data points represent the mean and standard error of 6 wells. A one-way analysis of variance followed by a least significant difference multiple comparison analysis (Tukey-Kramer) was used to determine the significant differences between individual groups in experiments. The level of significance was set at $p < 0.05$.

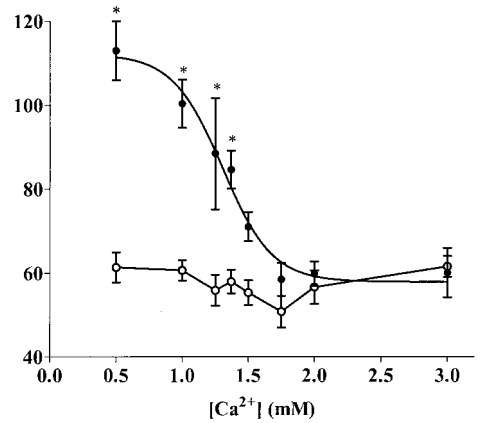

FIG. 1. SP inhibits PTH secretion in a calcium-dependent manner. Dispersed porcine parathyroid cells were treated for 3 h with vehicle (●) or 1 nM SP (○) in the presence of various $CaCl_2$ concentrations. PTH released to the medium was determined using an IRMA. Data are a representative experiment from 4 experiments set up with different cell isolations. Each point represents the mean and standard error of 6 wells. *$p < 0.05$ compared to the control at the same $CaCl_2$ concentration.

RESULTS

In the porcine parathyroid cells, a 1.9–4 fold increase in PTH secretion was observed with 0.5 mM $CaCl_2$ treatment when compared to cells cultured in higher $CaCl_2$ concentrations (Fig. 1). SP (1 nM) treatment resulted in a statistically significant inhibition of PTH secretion at $CaCl_2$ concentrations of 1.37 mM and lower (Fig. 1), but not at higher calcium concentrations. NPY, galanin, CGRP, and VIP had no statistically significant effects on PTH secretion at 0.5 or 1.75 mM $CaCl_2$ (data not shown).

The ability of parathyroid cells to recover from the inhibitory effects of SP was examined. Porcine parathyroid cells were treated for 3 h with medium containing 1.75 mM $CaCl_2$, 0.5 mM $CaCl_2$, or 0.5 mM $CaCl_2$ and 1 nM SP. A 2.4-fold increase in PTH secretion was observed in response to 0.5 mM $CaCl_2$-treatment when compared to the 1.75 mM $CaCl_2$ group (84.23 ± 3.94 and 34.77 ± 1.06 pg PTH/ml, respectively). As expected, SP inhibited the 0.5 mM $CaCl_2$-induced PTH secretion (53.92 ± 1.32 pg PTH/ml). The cells were then cultured for an additional 1–3 h in medium containing 0.5 or 1.75 mM $CaCl_2$, corresponding to the $CaCl_2$ concentrations used during the initial 3 h of treatment. A time-dependent increase in PTH secretion was observed over this subsequent 3 h incubation (Fig. 2). The slopes of the lines for the 0.5 mM $CaCl_2$ control and the group previously treated with SP and

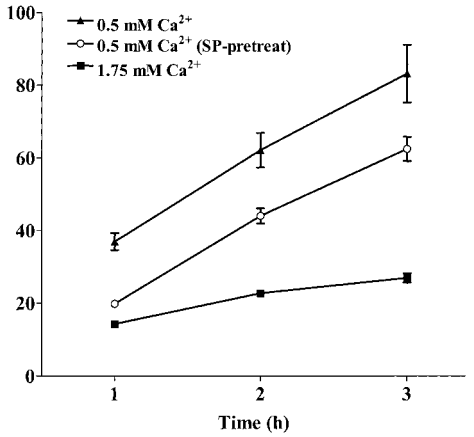

FIG. 2. SP inhibits PTH secretion in a reversible manner. Dispersed porcine parathyroid cells were treated with 1.75 mM $CaCl_2$, 0.5 mM $CaCl_2$, or 0.5 mM $CaCl_2$ and 1 nM SP for 3 h. PTH released to the medium was determined using an IRMA. PTH concentrations of 34.77 ± 1.06, 84.23 ± 3.94, and 53.92 ± 1.32 pg/ml, respectively, were measured at the end of the initial incubation. The cells were then treated for an additional 1–3 h with medium containing the same $CaCl_2$ concentration as the first incubation (1.75 or 0.5 mM $CaCl_2$) in the absence of SP. Each point represents the PTH released to the medium during the second incubation. Data are a representative experiment from 2 experiments set up with different cell isolations. Each point represents the mean and standard error of 6 wells.

0.5 mM $CaCl_2$ were similar suggesting that the cells recovered from the treatment with SP (Fig. 2). The absolute values for PTH secretion from the cells treated with SP never reached the levels of the 0.5 mM $CaCl_2$ control, but this would be expected since the cells were recovering from an inhibited state. Analysis of cell associated lactate dehydrogenase using the Cyto-Tox96 Non-Radioactive Cytotoxicity Assay demonstrated no inhibitory effects of SP (data not shown). These results along with the ability of the cells to recover from SP treatment suggest that SP was not cytotoxic to the parathyroid cells.

Since the actions of SP can be mediated via NK-1, NK-2 or NK-3 receptor subtypes, the effects of receptor selective agonists on PTH secretion were evaluated. SP and the NK-1 receptor selective agonist, Ac[Arg^6, Sar^9, $Met(O_2)^{11}$]-SP 6-11 (24), inhibited 0.5 mM $CaCl_2$-induced PTH secretion in a concentration-dependent manner with IC_{50} values of 0.29 and 0.21 nM, respectively (Fig. 3a). The NK-2 and NK-3 receptor selective agonists, [B-Ala^8]-neurokinin A 4-10 and [$MePhe^7$]-neurokinin B, respectively (24), were approximately 100 fold less potent than SP or the NK-1 receptor agonist (IC_{50} = 24 and 31 nM, respectively, Fig. 3a). These results suggest that the effects of SP are probably mediated through the NK-1 receptor.

The finding that the actions of SP were probably mediated by the NK-1 receptor was confirmed using the selective and specific NK-1 receptor antagonist, LY306740 (23). The inhibitory activity of SP was reversed by LY306740 (K_i = 0.125 nM, Fig. 3b) while the less active enantiomer, LY307679 (K_i = 151 nM, Fig. 3b), was approximately 1000-fold less potent. These results provide additional pharmacologic evidence that the inhibitory effects of SP are specifically mediated through NK-1 receptors.

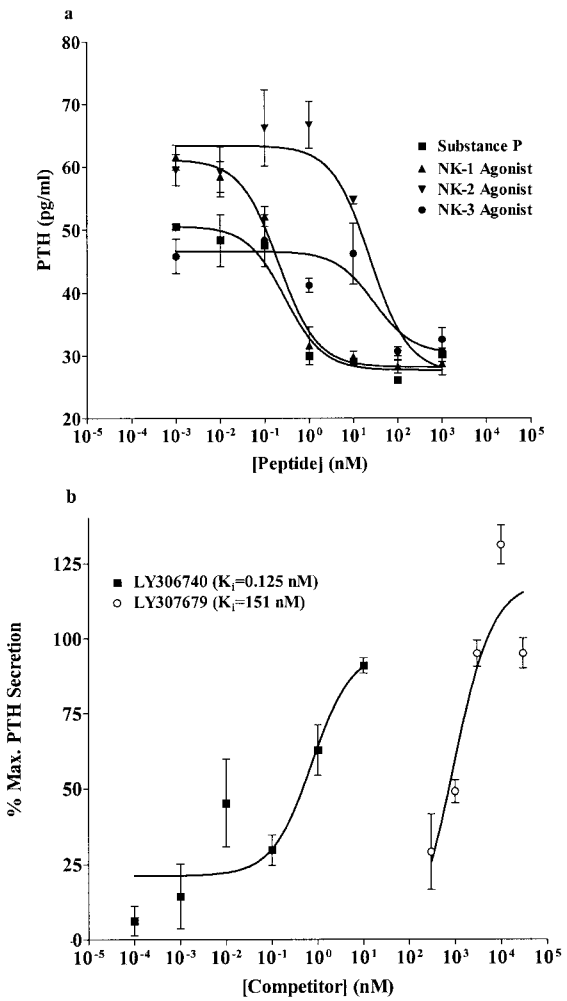

FIG. 3. Pharmacological demonstration of NK-1 receptor-mediated inhibition of PTH secretion. (a) Tachykinins inhibit 0.5 mM $CaCl_2$-induced PTH secretion from porcine parathyroid cells. Dispersed porcine parathyroid cells were treated for 3 h with 10^{-3}–10^3 nM SP (IC_{50} = 0.29 nM), NK-1 selective agonist (IC_{50} = 0.21 nM), NK-2 selective agonist (IC_{50} = 24 nM), NK-3 selective agonist (IC_{50} = 31 nM), or vehicle in the presence of 0.5 mM $CaCl_2$. Secreted PTH was determined using an IRMA. Each point represents the mean and standard error of 6 wells. (b) Inhibition of SP activity by LY306740 and LY307679 in porcine parathyroid cells. Dispersed porcine parathyroid cells were treated for 3 h with 10^{-4}–10^1 nM LY306740 and 3×10^2–3×10^4 nM LY307679, or vehicle in the presence or absence of 1 nM SP in medium containing 0.5 mM $CaCl_2$. Secreted PTH was determined using an IRMA. The data are presented as the % maximal PTH secretion with the minimal secretion being the SP control and the maximal secretion being the 0.5 mM $CaCl_2$ control in the absence of SP and the antagonist. Data are a representative experiment from 2–4 experiments set up with different cell isolations. Each point represents the mean and standard error of 6 wells.

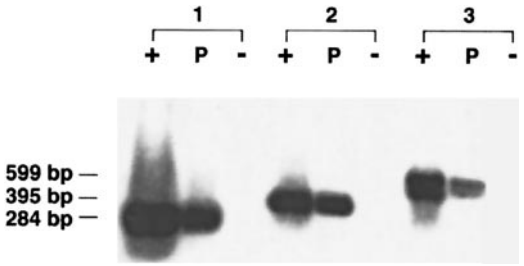

FIG. 4. Porcine parathyroid cells express mRNA for NK-1 receptors. Three sets of NK-1 receptor-specific primers were used to amplify mRNA from porcine parathyroid cells using RT-PCR. The PCR products were then hybridized with an additional NK-1 specific biotinylated probe. The (+) represents the IM9 samples which served as the positive control, (P) represents the porcine parathyroid cells samples, and the (−) represents the no template control PCR reaction. A single band is obtained from 1NK-1 primers (284 bp), 2NK-1 primers (395 bp) and 3NK-1 primers (599 bp) with mRNA from IM9 or porcine parathyroid cells. As expected a band was not observed in the no template control.

Since expression of NK-1 receptor by porcine parathyroid gland cells has not been previously described, RT-PCR experiments were conducted to determine whether or not porcine parathyroid cells express mRNA for the NK-1 receptor. Autoradiographic analysis of PCR products amplified with 3 sets of NK-1 specific primers and hybridized with an additional NK-1 specific biotinylated probe demonstrated a single band in both the IM9 (positive control) and parathyroid cells (Fig. 4). Bands were not observed in the no template control. Thus, expression of NK-1 receptors by dispersed porcine parathyroid cells has been demonstrated by molecular and pharmacological evidence.

DISCUSSION

The neuronal innervation of the parathyroid gland has been documented in numerous studies (10–15); however, the potential role of these neurons in the regulation of PTH secretion has not be extensively evaluated. Therefore, the current study examined the effects of neuropeptides, that have been localized to neurons innervating the parathyroid gland, on PTH secretion. SP was the only neuropeptide evaluated that inhibited 0.5 mM $CaCl_2$-induced PTH secretion in porcine parathyroid cells. Although localization of SP containing nerve fibers has not been studied in porcine tissues, SP containing nerve fibers have been identified in chicken, rat, guinea-pig, cat, dog, sheep, and human parathyroid glands or surrounding tissue (17, 19, 20), suggesting that neural innervation is conserved among species.

SP is an 11-amino acid peptide member of the tachykinin family of neuropeptides which also includes neurokinin (NK)-A and NK-B (25–29). The actions of these tachykinins are mediated through three major receptor subtypes, NK-1, NK-2, and NK-3, which have preferential affinity for SP, NK-A and NK-B, respectively (24, 30, 31). SP functions as a neurotransmitter released from primary sensory nerve fibers (unmyelinated) and its biological activities include mediation of pain in the peripheral and central nervous systems, stimulation of smooth muscle contraction, vasodilatation, plasma extravasation, and the release of inflammatory mediators. The results of this study suggest that SP has a direct effect on PTH secretion. In porcine parathyroid cells, the effects of SP appear to be mediated by the NK-1 receptor since the NK-1 specific agonist mimicked the effects of SP and the NK-1 specific antagonist blocked the effects of SP. The pharmacologic evidence suggesting that SP acts through the NK-1 receptor was further supported by the demonstration of mRNA for the NK-1 receptor.

The inhibitory effects of SP on PTH secretion are in contrast to the findings of Jaborn *et al.* (8) which demonstrated that SP had no effect on PTH release at 1.25 mM extracellular Ca^{2+} in dispersed bovine parathyroid cells. Other extracellular Ca^{2+} concentrations were not examined by Jaborn *et al.* (8) The differences in the findings between the two studies may be due to variation in the ionized calcium concentration, which is the form of calcium that the parathyroid cell senses. Using the present study conditions the $CaCl_2$ concentration is reflective of the ionized Ca^{2+} concentration (data not shown). In the study by Jaborn *et al.* (8), it is not clear whether the extracellular Ca^{2+} concentration is reflective of total or ionized Ca^{2+}. In addition, it is possible that the differences in the response to SP may be due to the different species used in the studies.

It is generally believed that the actions of SP are mediated through changes in inositol phosphate turnover and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (32). Since extracellular Ca^{2+} modulates PTH secretion by regulating the $[Ca^{2+}]_i$ (33), SP may decrease PTH secretion by increasing $[Ca^{2+}]_i$. In the presence of 0.5 mM $CaCl_2$, $[Ca^{2+}]_i$ is low in the parathyroid cell and coincident treatment with SP may lead to an increase in $[Ca^{2+}]_i$ which would result in a decrease in PTH secretion. However, further studies are required to evaluate the effects of SP on $[Ca^{2+}]_i$ in parathyroid cells.

In summary, this study demonstrates that tachykinins, in particular SP, regulate PTH secretion and provides evidence that these effects are mediated through the NK-1 receptor. It is clear that SP is found in nerve fibers localized to the parathyroid gland and these findings demonstrate a direct action of SP on porcine parathyroid cells. This study provides evidence for a SP mediated neuroendocrine mechanism for regulating PTH secretion. Such a mechanism may be involved in decreasing PTH secretion or in inhibiting stress-induced PTH secretion. Further studies are re-

quired to determine if SP regulates PTH secretion *in vivo* and to evaluate the physiologic significance of this *in vitro* observation.

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