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Heterogeneous expression of SNARE proteins SNAP-23, SNAP-25, Syntaxin1 and VAMP in human parathyroid tissue

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Abstract

In regulated exocytosis synaptosomal-associated protein of 25 kDa (SNAP-25) is one of the key-players in the formation of SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor) complex and membrane fusion. SNARE proteins are essentially expressed in neurons, neuroendocrine and endocrine cells. Whether parathyroid cells express these proteins is not known. In this study, we have examined the expression of the SNARE protein SNAP-25 and its cellular homologue SNAP-23, as well as syntaxin1 and VAMP (vesicle-associated membrane protein) in samples of normal parathyroid tissue, chief cell adenoma, and parathyroid carcinoma, using immunohistochemistry and Western blot analysis. SNAP-23 and VAMP were evenly expressed in all studied parathyroid tissues using immunohistochemistry and/or Western blot analysis. SNAP-25 (and Syntaxin1) was not expressed in normal parathyroid tissue, but in approximately 20% of chief cell adenomas, and in ∼45% of parathyroid carcinoma samples. It is likely that the SNARE proteins SNAP-23 and VAMP play a role in the stimulus-secretion coupling and exocytosis of parathyroid hormone as these proteins were expressed in all of the parathyroid samples we studied. In particular, preferential expression of SNAP-23 rather than SNAP-25 provides an explanation of the high level of PTH secretion that occurs under conditions of low cytoplasmic free Ca^{2+} concentration (around 0.1 μmol/l). SNAP-25 (and Syntaxin1) appears to be a tumour-specific protein(s) in parathyroid tissues since its expression was restricted to pathological tissues.

Keywords: SNAP-23; SNAP-25; Syntaxin1; VAMP; SNARE; Parathyroid and human

1. Introduction

Intracellular membrane trafficking and fusion is essential for many cellular processes and requires a wide range of proteins. In regulated exocytosis, secretion vesicles fuse with the plasma membrane after appropriate stimuli and release their contents into the extracellular space. Central to the process of membrane fusion are soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors (SNAREs) localized in various intracellular organelles and membranes (Chen and Scheller, 2001). Specific SNARE proteins are present both on membraneous vesicles (v-SNARE, such as vesicle-associated membrane protein (VAMP) and Synaptotagmin) and the target membranes (t-SNARE, such as Syntaxin and synaptosomal-associated proteins (SNAP)), and the interaction forms a highly stable complex which is tightly coupled to membrane fusion. SNARE proteins have been studied extensively in neurons and neuroendocrine tissues, but, in contrast, relatively little information is currently available on the expression and roles of SNAREs in endocrine tissues (Wollheim and Maechler, 2002). In most endocrine cells elevated intracellular free Ca^{2+} concentration

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(Ca_i^{2+}) stimulates hormone secretion. In parathyroid cells, however, elevated levels of Ca_i^{2+} occur under conditions in which PTH secretion is markedly suppressed (Nemeth and Scarpa, 1986), which could indicate a different arrangement of exocytotic proteins. We have therefore focused on the SNARE proteins well known to be involved in Ca_i^{2+}-regulated exocytosis, namely SNAP, Syntaxin and VAMP. For example, parathyroid cells might express the Ca^{2+}-independent SNARE, SNAP-23 rather than SNAP-25 which works at higher Ca_i^{2+} levels (Chieregatti et al., 2004). In this study we have characterized the expression of SNAP-23, SNAP-25, Syntaxin1, and VAMP in human normal, adenomatous and carcinoma parathyroid tissues.

2. Materials and methods

2.1. Clinical cases

The study material included 51 parathyroid glands (Tables 1 and 2), all classified according to the World Health Organization classification (DeLellis et al., 2004). All samples except 7 of 9 carcinoma cases were collected at the Karolinska University Hospital, Stockholm, Sweden, the Mater Private Hospital, North Sydney, NSW, Australia or the Royal North Shore Hospital, St. Leonards, NSW, Australia. Fifteen of the glands (Case no. 28–42) were diagnosed as normal parathyroid tissue (obtained from patients undergoing major thyroid surgery and the normal parathyroid being localized under the fibrous capsule of the removed thyroid), 27 as chief cell adenomas (No. 1–27), and two as parathyroid carcinomas (No. 44–45). In addition, a pooled sample from biopsies of three different normal parathyroid glands was used in Western blot analysis. The informed consent of each patient was obtained and the local ethics committees of the Karolinska University Hospital, St Vincents Hospital, Darlinghurst, NSW, Australia (covering the Mater Private Hospital, North Sydney) and Northern Sydney Area Health Service covering the Royal North Shore Hospital approved the study. Seven additional samples of parathyroid carcinomas (No. 43, 46–51) had been previously collected world wide for a molecular genetics study (Bondeson et al., 1993). The patients had previously consented to an investigation of the causes of carcinogenesis and the present study was considered to be an extension of the original study.

2.2. Antibodies and controls

Following primary antibodies were used: anti-SNAP-25:SMI 81, a monoclonal antibody (mAb) directed against the C-terminal of the protein (Cat. No. SMI-81R, Sternberger Monoclonals, USA), anti-SNAP-25 polyclonal antibody (Cat. No. 111 002, Synaptic System, Germany), anti-SNAP-23, a polyclonal antibody against the N-terminal (Cat. No. 111 203, Synaptic System, Germany), anti-Syntaxin1 (mAb) (Prod. No. S0664, Sigma–Aldrich, USA), anti-PTH (mAb) (Cat. No. NCL-PTH-488, Novocastra, UK) and anti-VAMP (Cat. No. 104 001, Synaptic System, Germany) which is a monoclonal antibody directed against the N-terminal and binds to VAMP-1, VAMP-2 and VAMP-3 (synonymous with Cellubrevin). In fluorescent immunocytochemistry experiments, the following secondary antibodies were used: tetramethylrhodamine isothiocyanate (TRITC)-conjugated Donkey anti-mouse IgG (Code No. 715-025-150, Jackson ImmunoResearch Incorporated Inc, PA, USA) and cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG (Code No. 111-165-045, Jackson ImmunoResearch).

Protein extracts from human Uterus and mouse Brain (C57BL/6NCrl) served as positive control in Western blot. Mouse brain was homogenized in (mM): 20 HEPES, 1 $MgCl_2$, 250 D-sucrose, 2 EDTA with a protease inhibitor cocktail (Roche Diagnostics GmbH) (pH 7.4). The animal experiments in this study were approved by the local ethics committee for animal experimentation. Omission of the primary antibody served as a negative control in immunohistochemistry experiments, and human brain and/or phaeochromocytoma tissue sections were used as positive controls for the expression of all SNARE proteins investigated (data not shown).

2.3. Western blot

Total protein samples were extracted from frozen human parathyroid adenomas and a pool of normal parathyroid biopsies according to standard procedures and were quantified using a dye-binding assay (Bradford, 1976). Before loading the protein samples (75 μg/lane), the protein suspensions were preheated at 70 °C for 10 min in Novex-Tricine SDS sample buffer (Invitrogen, Cat. No.: LC 1676) with 5% (v/v) beta 2-mercaptoethanol. Samples were subsequently size-fractionated by electrophoresis in 10% Tricine gels (Invitrogen, Carlsbad, CA, USA) at 125 V for 1.5 h, followed by blotting onto 0.2 μm nitrocellulose membranes (Invitrogen) at 25 V for 2 h. Membranes were stained with Ponceau red to confirm equivalent protein loading between lanes and successful protein transfer in Western blots. The membranes were then blocked in milk for 1 h, incubated overnight at 4 °C with the respective primary antibody (with dilutions in parenthesis): anti-SNAP-25:SMI 81 ($1{:}2 \times 10^6$), anti-SNAP-23 (1:1000), anti-Syntaxin1 (1:5000) or anti-VAMP (1:800). Incubation with appropriate horseradish peroxide-conjugated secondary antibody (goat anti-rabbit 1:25,000 or goat anti-mouse 1:25,000) then followed for 1.5 h at room temperature. The signals were subsequently revealed using enhanced chemiluminescence (ECL, Amersham Biotech, Little Chalfont, UK) for 1 min and exposure to Hyperfilm (Amersham Biotech). The western standard used was MagicMark™Xp (Cat. No. LC5602, Invitrogen).

2.4. Immunohistochemistry

Paraffin sections of parathyroid tissues and control samples were all cut at 4 μm, deparaffinized, rehydrated and immersed in preheated citrate buffer, pH 6 (Dako, Glostrup, Denmark) at 95 °C for 20 min in order to unmask the antigens. The sections were incubated in 0.5% hydrogen peroxide for 30 min, blocked in 1% BSA with Na-azide for 1 h and subsequently incubated overnight with primary antibody diluted in 1% BSA. The following primary antibodies were applied: anti-SNAP-25:SMI 81 (dilution 1:750), anti-PTH (1:200), anti-SNAP-23 (1:200). The slides were then incubated with appropriate biotinylated secondary antibody (1:200) for 45 min at room temperature. The antigen-antibody complex was visualized using the avidin-biotin-peroxidase complex (ABC) method (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 45 min. Diaminobenzidine tetrahydrochloride was used as a chromogen, followed by counterstaining for 3 min in haematoxylin. Sections were washed five times in Tris-buffered saline (pH 7.6) between all steps.

2.5. Fluorescent immunocytochemistry

Paraffin sections of parathyroid tissues were deparaffinized, rehydrated and retrieved as described in immunohistochemistry section above. The slides were incubated with mouse anti-SNAP-25 SMI 81 (dilution 1:750) or rabbit anti-SNAP-23(1:200) overnight at 4 °C and thereafter with tetramethylrhodamine isothiocyanate (TRITC)-conjugated Donkey anti-mouse IgG (1:200) or cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG (1:800) for 1 h at room temperature. Prior to the last wash the sections were incubated with DAPI (4,6-Diamidino-2-Phenylindole) in order to detect cell nuclei. The slides were then mounted with Glycerol-gelatin and examined in Confocal Laser Scanning Microscopy (CLSM). CLSM images were obtained using the Axiovert 200 M inverted microscope equipped with Ar/ArKr (458, 477, 488 and 514 nm), HeNe 543 nm and HeNe 633 nm lasers, and the scanning module LSM 510 META (Carl Zeiss, Jena, Germany). Images were recorded at a 512×512 pixels resolution. The C-Apochromat 40×, NA = 1.2, water immersion UV–vis-IR objective was used. Further magnification was obtained electronically, by decreasing the scanned area. Fluorescent dyes, Cy3 and TRITC were excited using the HeNe 543 nm laser. Images were recorded by averaging more than eight scans of each image line (line average); scanning rate 6.4 μs/pixel; main dichroic beam splitter HFT 488/543/633 and long pass 560 nm collecting filter. TRITC and Cy3 fluorescence was visualized using green and red pseudo-colors, respectively.