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Rapid increase in enzyme and peptide mRNA in sympathetic ganglia after electrical stimulation in humans

(catecholamines/neuropeptide tyrosine/tyrosine hydroxylase/dopamine β-hydroxylase/gene regulation)

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ABSTRACT **Thoracic ganglia in humans were studied after electrical, preganglionic stimulation using *in situ* hybridization with synthetic oligonucleotide probes against the catecholamine-synthesizing enzymes tyrosine hydroxylase (EC 1.14.16.2) and dopamine β-hydroxylase (EC 1.14.17.1) and neuropeptide tyrosine. Immunohistochemical analysis was also performed. Following short peroperative stimulation a severalfold increase in all three mRNAs was found in principal ganglion cells, whereas no definite changes could be detected in enzyme or peptide levels with immunohistochemistry. The results suggest a very rapid and sensitive regulation of genes involved in signal transmission in the sympathetic nervous system of humans. Moreover, they indicate that electrical stimulation of neurons and/or pathways combined with *in situ* hybridization may be used as a method to define neuronal projections by visualizing increases in mRNAs for transmitter enzymes and/or peptide in target cells.**

Several enzymes are involved in the synthesis of catecholamines in neurons and endocrine cells (1). Histochemical and immunohistochemical studies have revealed that many sympathetic ganglion cells contain, in addition to catecholamines (2, 3) and their synthesizing enzymes (4), several coexisting peptides, including neuropeptide tyrosine (NPY) (5–7). The cloning of these enzymes (8, 9) and of NPY (10) has made it possible to study their localization and expression with *in situ* hybridization techniques (11) in rodents under normal and experimental conditions (12, 13). In the present study this technique is applied to analysis of sympathetic ganglia in humans. The regulation of gene expression of the enzymes tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase, L-tyrosine, tetrahydropteridine: oxygen oxireductase (3-hydroxylating), EC 1.14.16.2] and dopamine β-hydroxylase [DBHase; dopamine β-monooxygenase; 3,4,-dihydroxyphenyl-ethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] as well as of NPY was studied in thoracic sympathetic ganglia of patients undergoing microsurgical sympathectomy aided by electrical stimulation of the sympathetic chain (14). The results demonstrate that mRNAs for all three markers, TyrOHase, DBHase, and NPY, increase severalfold after <20 min of stimulation.

MATERIALS AND METHODS

Three patients with the diagnosis of essential palmar hyperhidrosis underwent bilateral high thoracic sympathectomy. The procedure had been approved by the Regional Ethical Committee of the Karolinska Hospital and was carried out with informed consent from the patients. The patients were of Scandinavian ancestry and did not describe a familial history. They had undergone multiple conservative treatment regimens that did not provide relief of symptoms. The standard dorsal midline approach to the sympathetic chain was used. The second and third thoracic ganglia (T_2 and T_3 ganglia) were identified and isolated with the help of the operating microscope after removal of the head of the second rib. A bipolar silver electrode connected to a constant current stimulator unit (Grass) was placed on the freed sympathetic chain. Square-wave stimulation was used to ascertain that the proper ganglia had been localized (14). Various parameters, including sudomotor function and blood flow, were monitored as described in detail (14). Then surgical clips were placed on the sympathetic chain rostrally and caudally, and one side was stimulated for 15–19 min with 10 Hz, 3 mA at 0.5-msec duration. Following this stimulation, ganglia from both sides were excised between surgical clips, and alternate slices were processed for immunohistochemistry and *in situ* hybridization.

For immunohistochemistry (15) slices of the ganglia were immersed in 10% formalin/0.3% picric acid (16) for 2 hr. After rinsing in phosphate buffer containing 10% sucrose, the tissue was cut at 14 μm in a cryostat (Dittes, Heidelberg). The sections were incubated with TyrOHase antiserum (1:400) (17) or NPY antiserum (1:400) (Peninsula Laboratories), rinsed, incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (Boehringer Mannheim Scandinavia, Stockholm) rinsed, mounted, and examined in a Nikon Microphot FX fluorescence microscope equipped with proper filter combinations. For control purposes the sections were incubated with normal rabbit serum (control for TyrOHase antiserum) or NPY antiserum preabsorbed with NPY peptide (1 μM; Peninsula Laboratories). After incubation with control sera, no cell bodies or processes were stained.

For *in situ* hybridization (11) the slices were immersed in ice-cold saline and frozen within 1 hr, and sections were cut at 14 μm in a cryostat (as above) and were treated as described by Schalling *et al.* (18): they were air-dried at room temperature for 20 min, immersed in 10% formalin in phosphate-buffered saline (PBS; 0.74% NaCl, 0.01 M Na_2HPO_4/NaH_2PO_4) at room temperature for 30 min, rinsed in PBS, transferred through ethanol, and incubated for 5 min in chloroform. The slides were then put in humidified boxes and hybridized with synthetic oligonucleotide probes complementary to bases 1–48, 812–859, and 171–218 of human RNA sequences for TyrOHase (8), DBHase (9), and NPY (10), respectively. Probes were synthesized on Applied Biosys-

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Abbreviations: DBHase, dopamine β-hydroxylase; LI, like immunoreactivity; NPY, neuropeptide tyrosine; TyrOHase, tyrosine hydroxylase.

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tems DNA synthesizer model 381A and purified using an oligonucleotide purification cartridge (Applied Biosystems). They were labeled at the 3′ end to a specific activity of 0.4–1.2 × 1.0^9 cpm/μg using terminal deoxynucleotidyltransferase (International Biotechnologies) and dATP[^{35}S] (New England Nuclear) in a cobalt-containing buffer. The labeled probes were purified on an NENsorb column (New England Nuclear). Hybridization was carried out at 42°C for 18 hr with 1 × 10^6 cpm of the probe in a solution containing 50% formamide, 4× SSC (SSC = 0.15 M NaCl/15 mM sodium citrate), 1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 1% sarcosyl, 0.02 M $NaPO_4$ (pH 7.0), 10% dextran sulfate, 500 μg of yeast tRNA per ml, 250 μg of salmon sperm DNA per ml, and 60 mM dithiothreitol. The sections were rinsed in 1× SSC at 55°C for 75 min with five changes of SSC, transferred through distilled water, air-dried, covered with Hyperfilm-β_{max} autoradiography film (Amersham), and later dipped in NTB 2 nuclear track emulsion (Kodak). After development [LX24 (Kodak) for film and D19 (Kodak) for emulsion] and fixation [Unifix (Kodak) for film and G333 (Agfa) for emulsion], the autoradiograms were used for quantitative analysis and the emulsion-dipped slides were analyzed in a Leitz light microscope using a dark-field condenser. The slides were stained with cresyl violet to obtain a rough estimation of the proportion of labeled ganglion cells.

Quantitation of autoradiograms was carried out with standards prepared by addition of dATP[^{35}S] in varying concentrations to brain paste (18). The brain paste was frozen and sectioned in a cryostat and coexposed with analyzed ganglia. Measurements were performed on an IBAS 2000 image analysis system (Zeiss/Kontron) as described (18).

RESULTS

By using immunohistochemistry a majority of the cells on the stimulated and unstimulated side showed positive immunoreactivity for TyrOHase and NPY (Fig. 1 *a–d*). There was, however, a clear difference in that TyrOHase-like immunoreactivity (LI) was seen in almost all ganglion cells, whereas NPY-LI was found only in a subpopulation of neurons. No apparent difference in TyrOHase-LI or NPY-LI was seen between the two sides (Fig. 1 *a–d*). With *in situ* hybridization no activity or only weak activity was seen on the film autoradiographs from the unstimulated side with the exposure time used (Fig. 2 *a–c*), but in the emulsion-dipped sections a few cells were fairly strongly labeled (Fig. 2 *d* and *f*). In addition, 20–30% of the principal ganglion cells had a slightly stronger activity than background. On the stimulated side, intense labeling was observed on films over the stimulated ganglia after hybridization with each of the three probes (Fig. 2 *a–c*). In the emulsion-dipped sections numerous principal ganglion cell bodies with a very high grain density could be observed, again with all three probes (Fig. 2 *a–c*, *e*, and *g*). Thus, about 75% of all principal ganglion cells were strongly labeled for TyrOHase mRNA and DBH mRNA. With regard to NPY about half of all neurons showed strong labeling. The increase was most marked in sections hybridized with the DBHase probe, as revealed with image analysis (Fig. 3). Changes in all three patients examined were of the

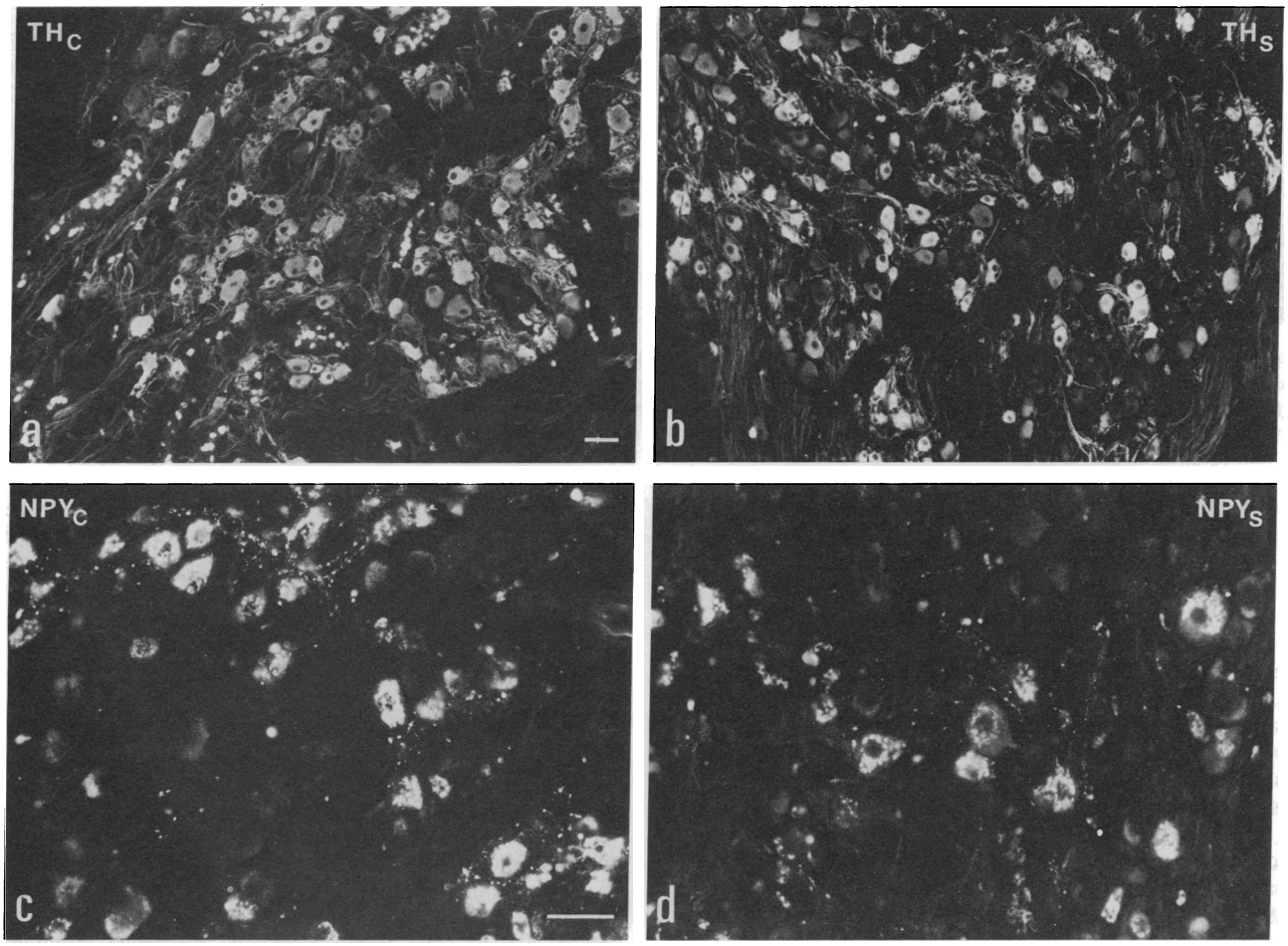

FIG. 1. Immunofluorescence micrographs of control (C) (*a* and *c*) and stimulated (S) (*b* and *d*) ganglia after incubation with antiserum to TyrOHase (TH) (*a* and *b*) and NPY (*c* and *d*). Almost all cell bodies are immunoreactive to TyrOHase, whereas only a subpopulation contains NPY-LI. No obvious difference can be seen between the control and stimulated sides. [Bars = 50 μm (*a* = *b*, *c* = *d*)].

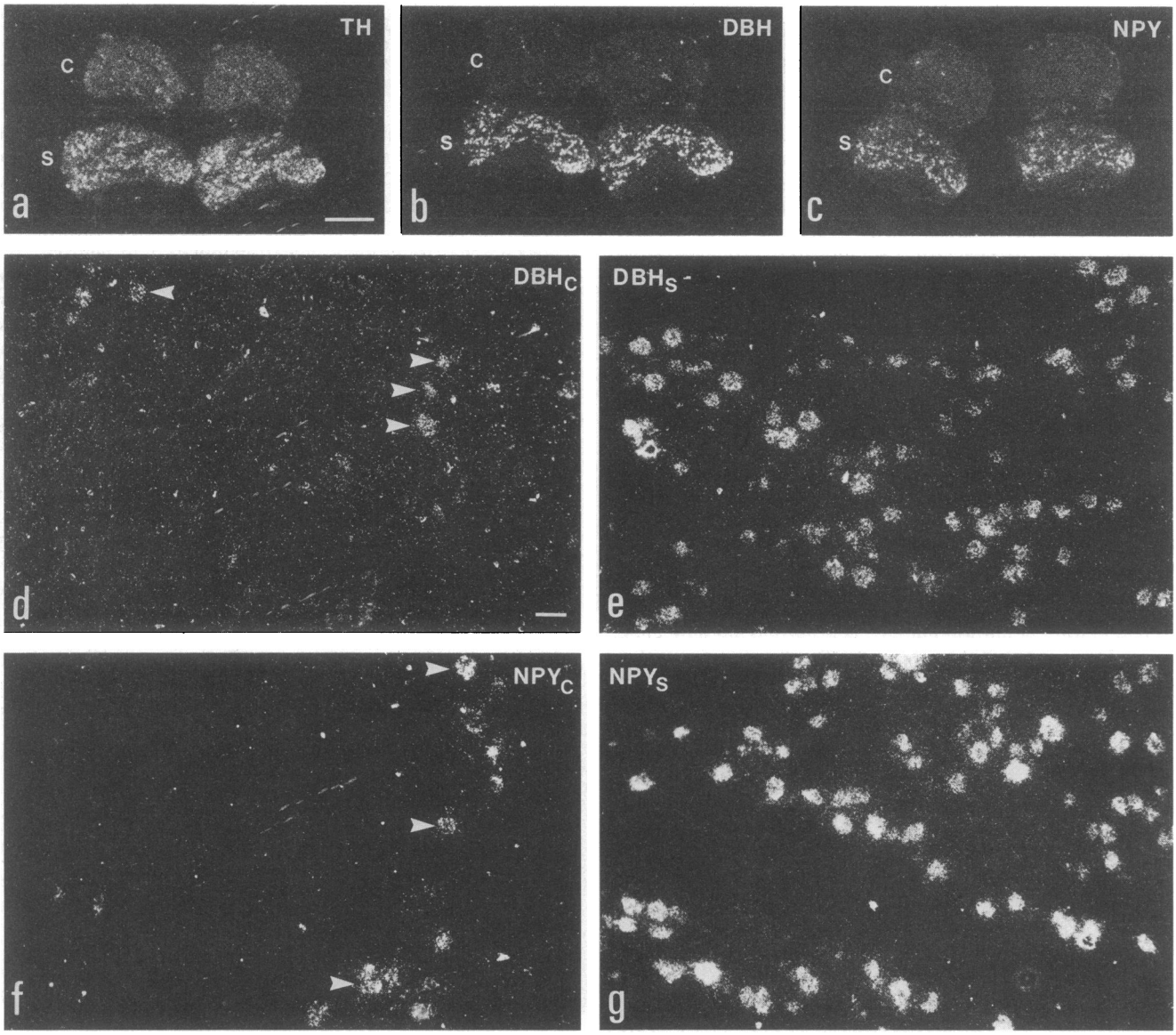

FIG. 2. (*a–g*) Film (*a–c*) and emulsion (*d–g*) autoradiographs of control (C) (*a–d* and *f*) and stimulated (S) (*a–c*, *e*, and *g*) ganglia after hybridization with oligonucleotide probes complementary to TyrOHase mRNA (TH) (*a*), DBHase mRNA (DBH) (*b*, *d*, and *e*), and NPY mRNA (*c*, *f*, and *g*). (*a–c*) Note marked difference in grain density over control (C; upper parts) and stimulated (S; lower parts) ganglia. (*d–g*) Only a few cells are labeled in control sections (*d* and *f*), whereas numerous strongly labeled cells can be seen in stimulated ganglia (*e* and *g*). [Bar = 1 mm (*a–c*) or 50 μm (*d–g*)].

same magnitude. Thus, DBHase mRNA showed a 5- to 6-fold increase, NPY mRNA showed a 3- to 5-fold increase, and TyrOHase mRNA showed a 2- to 3-fold increase.

DISCUSSION

The present results demonstrate that in patients with essential palmar hyperhidrosis electrical stimulation of preganglionic fibers to a sympathetic ganglion in <20 min induces a marked increase in mRNA for three markers of sympathetic neurons, the enzymes TyrOHase and DBHase and the coexisting neuropeptide NPY. Even including the interval (≈1 hr) on ice before freezing, this increase appears rapid, since studies on catecholamine cells in the adrenal gland and noradrenergic neurons in the locus coeruleus (19) have shown that treatment with the catecholamine-depleting drug reserpine causes changes in TyrOHase and NPY mRNAs no earlier than 3 hr after injection with a peak at 1–2 days after this stimulus (12, 13). In contrast, we were not able to observe any changes in the translation products—i.e., in TyrOHase- or NPY-LI—with immunohistochemistry under our experimental conditions. An interesting question is whether or not an increase in TyrOHase-LI and NPY-LI could have been seen if ganglia could have been analyzed at a much later time point after electrical stimulation—i.e., when the increase in TyrOHase and NPY mRNA could be expected to reflect an increase in translation.

The increase was most marked for DBHase mRNA followed by NPY mRNA, whereas the increase in TyrOHase mRNA was moderate. It may be speculated that at least one reason for the strong effect on DBHase mRNA and NPY mRNA is the fact that both DBHase (20) and the peptide (21), in contrast to TyrOHase, are released during the exocytotic process and thus have to be rapidly replaced.

The cellular analysis of emulsion-dipped sections revealed that in control ganglia single, fairly strongly NPY and DBHase labeled cells were observed. Electrical stimulation caused labeling of the majority of cells with TyrOHase mRNA and DBHase mRNA probes, whereas NPY mRNA labeled somewhat fewer cells. This may suggest that a major

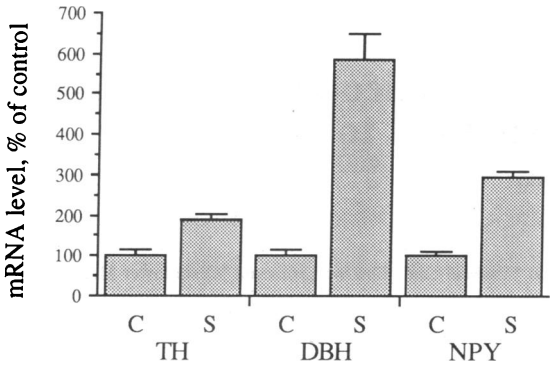

FIG. 3. Diagram illustrating changes in TyrOHase (TH), DBHase (DBH), and NPY mRNA in one of three patients examined. Marked increases were present in all three markers, although DBHase and NPY mRNA increases were more pronounced. C, control; S, stimulated. Changes were similar in all three patients.

portion of the overall increase in mRNA levels is the recruitment of cells with low mRNA levels for these enzymes and peptide.

Our results open up an interesting aspect in that the present methodology may be used to map projections in the peripheral and the central nervous system. Thus, comparatively brief electrical stimulation of a cell group or pathway may transsynaptically activate gene expression of transmitter enzymes or peptides in next order neurons or effector cells. This is somewhat similar to the recent demonstration that electrical stimulation of peripheral fibers can rapidly induce expression of c-fos-encoded protein in neurons in the dorsal horn and that their laminar distribution is related to the nature of the sensory stimulation (22). However, the present approach could in addition disclose what type of transmitter/peptide is present in target neurons.

In conclusion, the present study shows that regulation of enzyme and peptide synthesis in human sympathetic ganglia is a very rapid process and that synthesis of transmitter and coexisting peptide is regulated in parallel. To what extent the disease state (hyperhidrosis) may influence this regulation is at present not known. Furthermore, *in situ* hybridization is shown to be a powerful method for analysis of gene expression in human tissues under normal and experimental conditions. The molecular mechanism(s) underlying this rapid increase in gene expression in the sympathetic nervous system may be of relevance for understanding of stress and of diseases of the cardiovascular system in humans.

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