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Depolarizing influences regulate preprotachykinin mRNA in sympathetic neurons

(superior cervical ganglion/neuronal plasticity/substance P)

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ABSTRACT **We have been studying mechanisms regulating neurotransmitter plasticity in sympathetic neurons. Neurons of the rat superior cervical ganglion (SCG) synthesize multiple putative transmitters, including the peptide substance P (SP). We have now examined steady-state levels of the mRNA encoding preprotachykinin (PPT), the SP precursor. A cloned cDNA probe was used to examine regulation mRNA levels in culture and *in vivo*. In RNA gel blot experiments, a single band (1.1 kilobases long) was observed in all cases in which an RNA was detected. A low level of PPT mRNA was detected by RNase protection assay in uncultured ganglia, suggesting that the low levels of SP previously observed in the normal ganglion *in vivo* are synthesized locally. When ganglia were maintained in culture, with consequent denervation, the steady-state level of PPT mRNA increased by 25-fold over the first 24 hr, and the high level was maintained for at least 7 days. RNase protection experiments indicated that the major message in the SCG is the β-PPT mRNA, encoding both SP and neurokinin A peptide regions. Accumulation of the PPT mRNA in cultured ganglia was sharply inhibited by the depolarizing agent veratridine, and this effect was blocked by tetrodotoxin. Therefore, one form of neuronal plasticity, change in neurotransmitter metabolism, is regulated at least in part by altering steady-state levels of specific mRNA. More generally, extracellular signals may contribute to neuronal plasticity through changes in gene expression.**

Multiple lines of evidence indicate that continuous functional change in the nervous system governs such varied processes as sensory perception, motor behavior, learning, and memory. A central problem in neuroscience concerns the mechanisms through which neural activity alters the functional state of neuron ensembles. Analysis at the level of single neurons has been particularly fruitful in identifying activity-dependent processes. For example, activity influences diverse properties, including neuronal morphology (1), the propensity to generate subsequent action potentials (2), synaptic ultrastructure (2), the mix of co-localized transmitters that are released (3), and the synthesis of neurotransmitters.

The regulation of transmitter metabolism has attracted wide interest, since these signals are prime agents of neural communication. Moreover, the realization that single neurons use multiple transmitters has vastly expanded our appreciation of the potential for mutability at the neuronal level. Recent studies indicate that activity may differentially alter concentrations of individual co-localized transmitters.

Sympathetic neurons of the rat superior cervical ganglion (SCG) have served as useful models, since they are accessible for *in vivo* manipulation, conveniently studied in culture, and since they synthesize multiple putative transmitters. Recent work from our laboratory has indicated that the putative transmitters substance P (SP) and somatostatin, as well as the classical transmitter norepinephrine, are synthesized by the SCG (4, 5). Moreover, impulse activity differentially regulates transmitter levels in sympathetic neurons. Interruption of impulse activity *in vivo*, surgically or pharmacologically, increases ganglion SP severalfold (6, 7). In contrast, transsynaptic stimulation has long been known to biochemically induce tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), E.C. 1.14.16.20], the rate-limiting enzyme in catecholamine biosynthesis, with augmented norepinephrine synthesis (8).

In initial studies, we have begun to explore regulatory mechanisms in cultured ganglia. Upon explanation and deafferentation, ganglia exhibit a 20- to 50-fold increase in SP over 4 days (5, 6), while tyrosine hydroxylase is unchanged. Depolarizing agents prevent the dramatic increase in SP, mimicking the effects of impulse activity *in vivo* (5, 6). The regulatory profile of somatostatin parallels that of SP. Conversely, depolarizing stimuli increase tyrosine hydroxylase catalytic activity and enzyme molecule number (8, 9).

The molecular mechanisms mediating activity-dependent regulation of transmitter levels have yet to be characterized. Does depolarization increase synthesis of SP precursor peptide, conversion of precursor to the SP undecapeptide, or even stabilization of SP itself? To approach these questions, we have examined mRNA encoding the SP precursor preprotachykinin (PPT). Using a cDNA clone for rat β-PPT mRNA (10), we found that depolarization exerts striking regulation of PPT mRNA. Our observations raise the possibility that impulse activity regulates transmitter gene expression.

METHODS

Culture of Ganglia. Ganglia were removed from newborn Sprague–Dawley rats within 24 hr of birth and cultured on collagen-coated (11) 30-mm plastic culture dishes in FF-12 medium, consisting of Ham's F-12 medium (12) supplemented with transferrin, insulin, progesterone, putrescine, and selenium (13). Nerve growth factor was added to a final concentration of 100 ng/ml in all cultures. Cultures were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2/95% air.

RNA Purification. RNA was prepared by the method of Chirgwin *et al.* (14), quantitated spectrophotometrically, and examined by ethidium bromide staining of analytical agarose gels before use in blotting experiments. Selection for poly(A)-containing transcripts was performed using oligo(dT)-cellulose (type III, P-L Biochemicals) and the procedure of Aviv and Leder (15).

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Abbreviations: SP, substance P; PPT, preprotachykinin; SCG, superior cervical ganglion.

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RNA Blot Hybridization. Formaldehyde-denatured RNA was electrophoresed through 1.2% agarose/2.2 M formaldehyde gels and transferred to nitrocellulose by capillary blotting (16). DNA probes were hybridized with RNA on filters in 50% formamide/5× SSC (17)/10% dextran sulfate at 42°C for 20 hr, and washed at 50°C in 0.2× SET (17)/0.02% sodium pyrophosphate/0.1% $NaDodSO_4$.

RNase Protection Experiments. Solution hybridization and RNase digestion were performed as described (18) with the difference that RNase T2 (Bethesda Research Laboratories) at 30 units/ml was used in 50 mM NaOAc, pH 4.5/2 mM EDTA. Digestion products were electrophoresed through 5% acrylamide/50% urea gels (17), which were dried and autoradiographed.

RESULTS

PPT mRNA in Cultured Ganglia. Explantation and consequent denervation increases SP in SCG neurons 20- to 50-fold within 4 days in culture (5, 6). Although previous studies have suggested that SP elevation is due to enhanced net synthesis of peptide, conclusions were largely inferential (5). Since increased protein synthesis is often accompanied by an increased steady-state level of message, the PPT cDNA clone (10) was used as a probe to detect mRNA in blot hybridization experiments. Ganglia were maintained in culture for various periods, ranging from 3 hr to 7 days, and PPT mRNA levels were measured. A single band [1.1 kilobases (kb) long] was present at all times that message was detectable (Fig. 1); this message was comparable in size to that previously reported for PPT mRNAs in bovine (19) and rat (10) striatum.

PPT mRNA exhibited a dramatic increase in culture, reaching a maximum plateau level after 48 hr that was maintained through 7 days. Using the present approach, message was not detectable in uncultured ganglia, but increased to detectable levels after only 3 hr in culture. In contrast, SP peptide did not increase until after 6 hr under similar conditions (6), indicating that the increase in mRNA precedes the increase in SP itself.

To examine uncultured (*in vivo*) ganglia more precisely, and to quantitate increases in message, a more sensitive RNase protection assay was used. Radiolabeled RNA complementary to PPT mRNA was synthesized and incubated with RNA samples, allowing complementary sequences to anneal. Subsequent digestion with a RNase specific for single-stranded RNA left the probe annealed to endogenous message (now double-stranded) intact. Analysis of digestion products allowed quantitation of mRNA complementary to the probe.

To construct the probe, the 1.0-kb PPT cDNA fragment from pJK28-1 (10) was subcloned into pSP65 (18), resulting in the plasmid pPT-2 shown in Fig. 2. The plasmid was cut with *Pvu* II endonuclease and transcribed with SP6 polymerase (18), yielding a 1.2-kb RNA, complementary to 0.9 kb of PPT

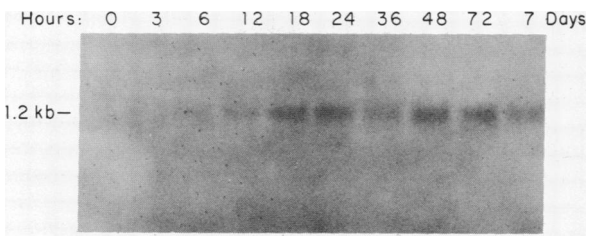

FIG. 1. PPT mRNA in cultured SCGs. Autoradiograph of gel blot of RNA extracted from cultured ganglia. RNA was purified from uncultured ganglia (0) and ganglia cultured for 3, 6, 9, 12, 18, 24, 48, and 72 hr, and 7 days. Eight micrograms of total cellular RNA from each sample was electrophoresed, blotted, and hybridized with the pJK28-1 cDNA probe.

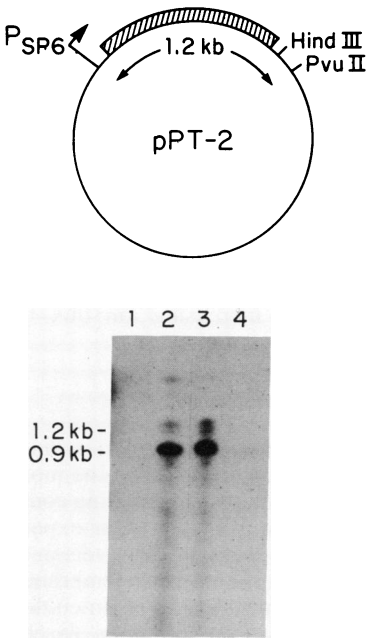

FIG. 2. RNase protection assay of PPT mRNA. (*Upper*) The RNA probe used in this experiment was made by cutting pPT-2 with *Pvu* II and transcribing the resulting template with SP6 polymerase and [^{32}P]UTP. (*Lower*) Autoradiograph of protected RNAs. Uniformly labeled RNA probe (5×10^5 cpm) was hybridized with sample RNAs and digested with RNase T2, and the resulting mixture was electrophoresed through 5% acrylamide/50% urea gel and autoradiographed. RNA samples were 5 μg of liver total RNA (lane 1), 0.20 μg of poly(A)$^+$ RNA from 24-hr cultured SCG plus 4.8 μg of liver total RNA (lane 2), 5 μg of poly(A)$^+$ RNA from uncultured ganglia (lane 3), and 5 μg of total RNA from uncultured ganglia (lane 4).

mRNA and 0.3 kb of vector sequences. To detect message in ganglia, the probe was hybridized to high C_0t (100 mol·s·liter^{-1}) with SCG RNA and digested with RNase T2. Poly(A)-selected RNA (0.20 μg) from ganglia cultured for 24 hr protected the probe to the expected length of 0.9 kb (Fig. 2, lane 2). In contrast, hybridization with 5 μg total cellular RNA from control liver failed to protect, as anticipated (lane 1).

Comparison of Expression *in Vivo* and in Culture. Uncultured neonatal ganglia are known to contain low levels of SP (5, 6). However, PPT mRNA was not detectable in these ganglia in the RNA blot experiment (Fig. 1). With the more sensitive nuclease protection experiment, 5 μg of total RNA from uncultured ganglia did yield a detectable signal (Fig. 2, lane 4), indicating that specific message is, indeed, expressed *in vivo*. Moreover, 5 μg of poly(A)$^+$ containing RNA from uncultured ganglia protected much more of the probe from digestion (lane 3), demonstrating that the PPT mRNA in SCG is polyadenylylated. Approximately equal amounts of probe were protected by 0.2 μg of poly(A)$^+$-containing RNA from 24-hr cultured SCG, and 5.0 μg from uncultured SCG, indicating that the steady-state level of PPT mRNA increased by ≈25-fold during the first 24 hr in culture. Furthermore, the presence of detectable amounts of PPT mRNA in uncultured ganglia suggests that the low levels of SP in the SCG prior to explantation or decentralization are due to synthesis within the ganglion itself, rather than presence of the peptide in terminals of extraganglionic origin. Therefore, sympathetic neurons respond to explantation with a striking increase in PPT-specific mRNA accumulation accompanying a large increase in the neuropeptide SP.

The Effect of Veratridine on PPT mRNA. The alkaloid depolarizing agent veratridine, which holds voltage-sensitive sodium channels open (20), is known to prevent SP elevation

after explantation, and this effect is blocked by the sodium channel antagonist tetrodotoxin (21). To determine whether veratridine prevents the increase in peptide by inhibiting the increase in PPT mRNA, ganglia were cultured with the alkaloid or toxin. SCGs were grown for 24 hr in basal medium or in medium supplemented with 50 μM veratridine, 0.1 μM tetrodotoxin, or both. RNA was isolated, electrophoresed, and hybridized as described above. The dramatic increase in PPT mRNA observed in control cultured ganglia was prevented by veratridine (Fig. 3). Tetrodotoxin blocked the veratridine effect, suggesting that veratridine prevented mRNA elevation by increasing ion flux through sodium channels.

DISCUSSION

By studying the effects of depolarization on transmitter mRNA levels, we hope to approach the more general problem of molecular mechanisms mediating communication and plasticity in the nervous system. Our experiments indicate that PPT mRNA is present at low levels in the SCG *in vivo* and that depolarization and transmembrane Na^+ influx influence steady-state message levels in culture.

Initial experiments were designed to characterize PPT mRNA in cultured ganglia. The RNA gel blot and RNase protection studies indicated that polyadenylylated PPT mRNA increased progressively with time in culture. Message exhibited a detectable increase after only 3 hr in culture and by 48 hr attained plateau levels that were maintained through 1 week. By comparison, SP peptide does not increase until 6 hr and achieves plateau levels after 4 days (5, 6). These temporal relationships suggest that explantation, with consequent denervation, elicits an increase in PPT mRNA that mediates the increase in the peptide itself.

Results obtained with the uncultured SCG *in vivo* support these contentions. Previous work had indicated that low, but detectable, levels of SP are present in the rat SCG *in vivo* (5, 6). We were surprised, then, that PPT mRNA was undetectable by RNA gel blot. However, the more sensitive RNase protection assay did reveal PPT mRNA *in vivo*, indicating that SP is, indeed, synthesized within the ganglion and not simply transported from external sources. Furthermore, the low levels of message detected *in vivo* suggest that physiologic impulse activity may normally suppress PPT mRNA.

Previous work has indicated that the increase of SP peptide in culture is prevented by veratridine depolarization in a tetrodotoxin-sensitive manner (5, 6). While these studies

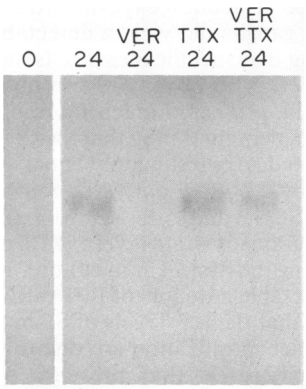

FIG. 3. PPT mRNA in culture with veratridine. Autoradiograph of gel blot of RNA extracted from uncultured ganglia (lane 0), or ganglia cultured for 24 hr with no drug (lane 24), 50 μm veratridine (lane VER 24), 0.1 μM tetrodotoxin (lane TTX 24), or both veratridine and tetrodotoxin (lane VER TTX 24). Ten micrograms of total RNA was in each sample, except for the VER TTX lane, in which 5 μg was used.

suggest that depolarization and attendant transmembrane Na^+ influx suppress SP, the molecular site(s) of action were undetermined. The present experiments begin to address this issue. Veratridine completely blocked the 20-fold increase in PPT mRNA, and tetrodotoxin prevented the alkaloid's effect. Consequently, depolarization and Na^+ influx appear to suppress steady-state levels of mRNA encoding a specific putative transmitter. Calcium influx through voltage-sensitive calcium channels mediates some of the effects of neuronal depolarization. Data recently obtained (A.R. and I.B.B., unpublished observations) indicates that transmembrane calcium flux, in the absence of veratridine, can prevent the 25-fold increase in PPT mRNA in culture. We have not yet determined whether depolarization decreased transcription itself or decreased stability of message. Nevertheless, our studies raise the intriguing possibility that depolarization of the neuronal membrane regulates the readout of specific genes, thereby mediating molecular plasticity.

The regulation of specific mRNA levels by membrane depolarization may occur in a number of systems. For example, blockade of the spontaneous contractile activity of cultured chicken myotubes with tetrodotoxin elicits a 17-fold increase in the mRNA encoding the α subunit of the acetylcholine receptor (22). Steady-state levels of preproenkephalin mRNA in cultured adrenal medulla increase to 74 times the *in vivo* level, and this increase is prevented by the depolarizing agents veratridine and 50 mM KCl (23). Consequently, activity-dependent repression of entirely different species of mRNA involved in signal transmission may occur in diverse systems and may underlie plasticity in different areas of the nervous system.

Although we have not yet definitively identified the structure of the PPT mRNA, our experiments provide useful hints. The multiple PPT mRNAs observed in rat and bovine striatum arise from a single gene (10). The gene has seven exons, and gives rise to multiple mRNAs through alternate splicing of primary transcripts. β-PPT mRNA contains sequences from all seven exons and encodes a precursor protein including the two tachykinin peptides, SP and neurokinin A (substance K). α-PPT mRNA does not encode neurokinin, since exon 6 is lost during splicing, while γ-PPT mRNA is missing exon 4 (10). The RNA blot technique presently used does not provide sufficient resolution to distinguish between these messages, which differ in length by <100 nucleotides. However, the experiment depicted in Fig. 2 provides preliminary insights. The synthetic probe used in that study should have been protected as a single 900-nucleotide fragment by β-PPT mRNA or as two shorter fragments by either α-PPT or γ-PPT mRNA due to digestion of the unprotected exons. Only the 900-nucleotide band was detected, suggesting that β-PPT mRNA was present exclusively or predominantly. Furthermore, ongoing work indicates that immunoreactive neurokinin, in addition to SP, is present in the SCG and also increases with time in culture (D. M. Katz and I.B.B., unpublished data).

Together, these data suggest that β-PPT mRNA is expressed in rat SCG and that steady-state levels are regulated by external depolarizing signals. More generally, extracellular information appears to play a critical role in neurotransmitter plasticity by potentially regulating gene expression.

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