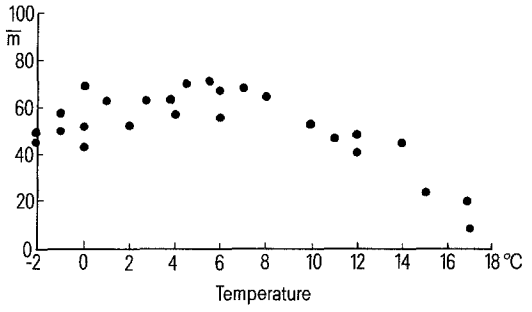

Figure 1. Effect of temperature on quantal release of transmitter in extraocular muscle of *Pagothenia borchgrevinki*. m̄ is average quantal content recorded at 10 Hz for 200 endplate potentials. Each point represents one determination of m. All points are from a single muscle fiber. Recording conditions and analysis of epps are described in the text.

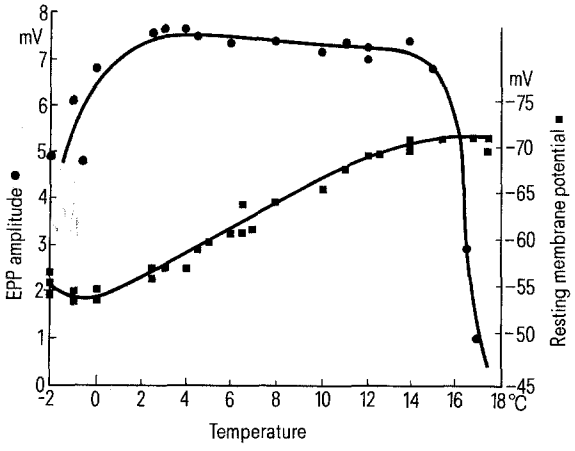

Figure 2. Effect of temperature on endplate potential amplitude and resting potential in extraocular muscle of *Pagothenia borchgrevinki*. Each circle represents average amplitude of 200 endplate potentials and each square one determination of resting membrane potential of the muscle fiber, the same as that in figure 1. Recording conditions are described in the text.

transmitter release failed at 16–17 °C (fig. 2). The progressive decrease in quantal content above 9 °C parallels a similar decrease in isotonic contraction for *P. borchgrevinki* extraocular muscle[7] and is probably the main factor responsible for high temperature failure of neuromuscular transmission in antarctic fishes.

The fact that transmitter release occurs at all at −2 °C indicates that *P. borchgrevinki* has made considerable adaptation to low temperature life. However, the fact that release is more effective at +5 °C than at the fish's ambient temperature of −1.9 °C (mean of 10 determinations of quantal content made between −3 °C and −1 °C on 6 muscle fibers each from a different fish was significantly different from mean of eight determinations made between +4 °C and +6 °C on the same muscle fibers: $0.005 < p < 0.01$, Student's t-test) shows that the adaptation is incomplete. Perhaps lack of competition in the antarctic environment has meant that there is no selective pressure for further fine-tuning of the cold adaptation, or perhaps the biochemical and biophysical constraints of membrane structure have not permitted adaptation to proceed further.

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Cholera toxin B-subunit incorporation into synaptic vesicles of the neuromuscular junction of the rat*

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Summary. The B-subunit of cholera toxin, a nontoxic macromolecule which binds specifically to GM1 ganglioside, was conjugated to colloidal gold and injected into skeletal muscle of the rat. It was taken up rapidly in vesicles in the terminal axons at the neuromuscular junctions. Injection of albumin-colloidal gold conjugates resulted in an insignificant uptake. The results indicate that uptake of extracellular macromolecules into the terminal axon of the neuromuscular junction may be greatly enhanced by binding to gangliosides at the presynaptic membrane, and that it may occur without association with vesicular recycling related to transmitter release.

Key words. Synaptic vesicles; endocytosis; cholera toxin; ganglioside; neuromuscular junction; rat.

Uptake of endo- and exogenous macromolecules occurs at the terminal axon of the neuromuscular junction[1] and has been considered an unspecific phenomenon related to recycling of synaptic vesicles during transmitter release[2]. Some of the incorporated macromolecules undergo retrograde axonal transport to the central nervous system[3]. Efficient transport of radio-

actively labeled cholera toxin to the spinal cord has been demonstrated after i.m. injection[4]. This property of cholera toxin is shared by some other macromolecules such as tetanus toxin, but not by albumin[5]. Retrograde transport of cholera toxin may be blocked by preincubation with GM1 ganglioside[4], a specific receptor for cholera toxin[6]. Gangliosides represent a large proportion of the components of neuronal membranes[7]; GM1 ganglioside is abundant in the peripheral nervous system[8]. Specific binding sites for macromolecules on the nerve terminal membrane are thought to be a prerequisite for efficient retrograde axonal transport[4]. The purpose of the present investigation was to find out whether there is a more significant uptake into the terminal axon of the B-subunit of cholera toxin, which binds specifically to GM1 ganglioside[9], than of albumin. The B-subunit of cholera toxin, which consists of five identical polypeptides (mol.wt 11,500 each)[9], was used after labeling it with colloidal gold in order to detect the molecules ultrastructually at high resolution. Its uptake was contrasted with that of albumin (mol.wt 69.000) conjugated with colloidal gold.

Methods. Colloidal gold was prepared by a method described previously[10]. It entails the mixing of a solution containing 1.5 ml 1% $HAuCl_4$ and 5.4 ml 0.1 M K_2CO_3 in 120 ml H_2O with a solution containing white phosphorus in diethyl ether. The mixture is heated at the boiling point for 5 min during which the colloidal gold is formed. Colloidal gold particles made in this way are about 5 nm in diameter. Stable colloidal gold-protein conjugates were prepared by mixing either a solution of the B-subunit of cholera toxin (manufactured by Institut Merieux, France, and kindly provided by Prof. J. Holmgren, Institute of Medical Microbiology, Göteborg, Sweden) or of rat albumin (Sigma) with the colloidal gold at a final concentration of 0.02 mg protein/ml. The colloidal gold-protein conjugates were filtered through Millipore filters (0.2 µm pore size) and centrifuged twice at 80,000× g for 30 min to get rid of unbound protein. The affinity of the conjugate of colloidal gold and the B-subunit of cholera toxin for GM 1 ganglioside was confirmed by a method taking advantage of the affinity of gangliosides for polystyrene. The method used was a modification of that described by Holmgren et al.[11]. Male Sprague-Dawley rats weighing 150–200 g were anesthetized by chloral hydrate. The palmaris longus muscle was exposed by an incision through the skin and 10 µl of one of the colloidal gold-protein conjugates injected into the belly of the muscle. One group of rats was injected with colloidal gold conjugated with the β-subunit of cholera toxin and another with colloidal gold-albumin conjugate. Each group consisted of four rats. 5 min after the injections, the rats were perfused with 2.5% glutaraldehyde in sodium phosphate buffer. Slices of the muscles were fixed in 2% OsO_4 and embedded in resin. Semi-thin sections were cut to identify the innervation regions and ultrathin sections of these regions were cut for electromicroscopy and examined without contrasting them. 20 randomly chosen neuromuscular junctions of each group of rats were photographed.

Results. Both types of colloidal gold-protein conjugates diffused into the synaptic clefts where they appeared at a concentration similar to that in the surrounding extracellular compartment (figs 1 and 2). Very few colloidal gold-albumin particles were found in synaptic vesicles in the terminal axon. In contrast many particles of colloidal gold conjugated with the B-subunit of cholera toxin were present in synaptic vesicles of the terminal axon. Sometimes several particles were seen in one vesicle. In addition, particles were sometimes observed in coated vesicles, and occasionally in elongated membrane-bound structures of smaller diameter than the synaptic vesicles, possibly cisternae. The relation between the number of gold particles in the terminal axon and the number in the primary synaptic cleft and between the Schwann cell and terminal axon was determined for each neuromuscular junction. It was found that 71 (±4)% (mean ± SEM) of the conjugate of colloidal gold and the B-subunit of cholera toxin were present in the terminal axon. The corresponding figure for colloidal gold albumin was 12 (±3)%. The difference is statistically significant ($p < 0.01$).

Discussion. The results indicate that the B-subunit of cholera toxin, when administered i.m., is taken up rapidly into the terminal axon while albumin is not. This result may explain why cholera toxin is efficiently transported to the spinal cord after intramuscular administration[4], whereas albumin is not. It may

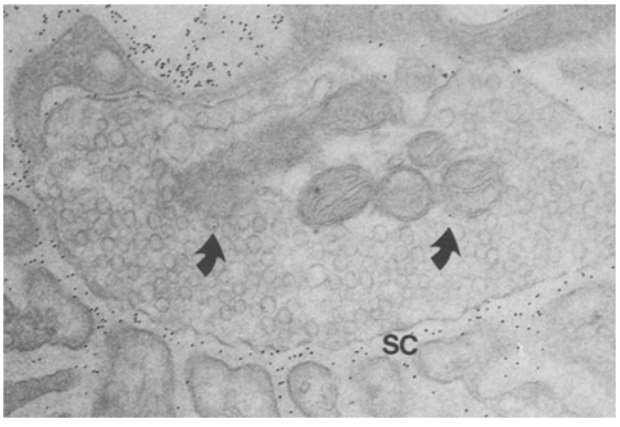

Figure 1. Section of the terminal axon 5 min after i.m. injection of albumin conjugated with colloidal gold. Many colloidal gold particles lie in the extracellular compartment, including the synaptic cleft (sc). Few particles (arrows) are seen in synaptic vesicles of the axon. ×40,000.

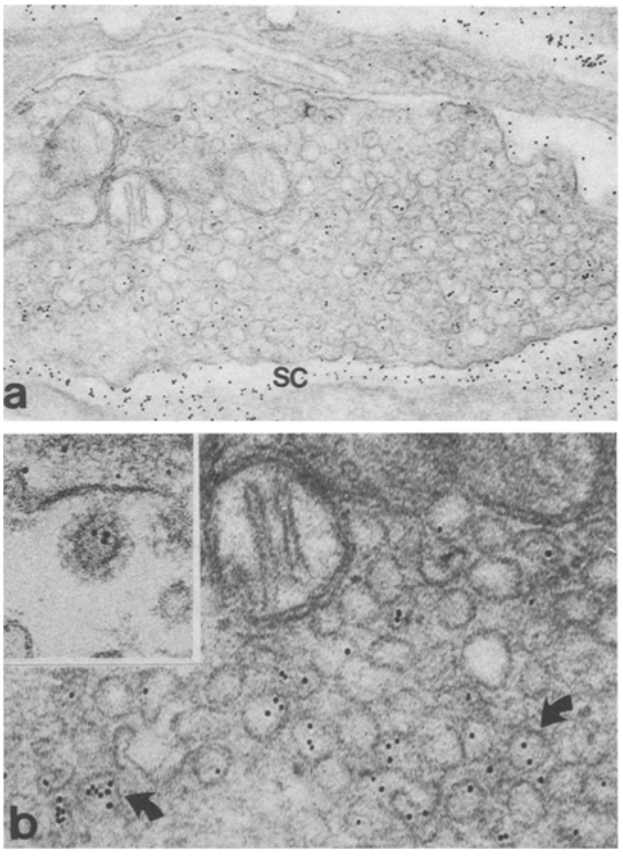

Figure 2. *a* Section of the terminal axon 5 min after i.m. injection of the B-subunit of cholera toxin conjugated with colloidal gold. The extracellular compartment including the synaptic clefts (sc) shows a similar high concentration of colloidal gold particles to that in figure 1. Numerous particles are also seen in the terminal axon. ×40,000.
b The particles are generally located within synaptic vesicles (arrows) but sometimes in coated vesicles (inset). ×80,000.

be postulated that the presence of GM1 ganglioside on the surface of the presynaptic membrane is involved in the rapid uptake of the B-subunit of cholera toxin, since it binds specifically to GM1 ganglioside[6]. It has been shown that tetanus toxin, conjugated to colloidal gold, is selectively taken up in nerve endings in the iris of the rat[12]. Like cholera toxin, tetanus toxin binds specifically to gangliosides[11]. Thus, the binding of macromolecules to gangliosides at the surface of the presynaptic membrane may greatly enhance endocytosis. This may be an important function of gangliosides which occur in high concentrations at the synaptic regions[13].

According to the recycling model of synaptic vesicles of Heuser et al.[2, 14, 15] which is based on studies on frog neuromuscular junction, synaptic vesicles coalesce with the axolemma during transmittor release. Membrane is then retrieved by coated vesicles which coalesce to form cisternae from which new synaptic vesicles are formed. During this recycling, exogenous macromolecules may be endocytosed[2, 14]. It has been shown in the neuromuscular junction of the frog that efficient uptake of horseradish peroxidase into synaptic vesicles is a process dependent on stimulation of the nerve for a considerable period[16]. It has not been established whether the uptake of macromolecules which is related to retrograde axonal transport is dependent on stimulation of the nerve, although synaptic activity may to some extent influence the amount of material retrogradely transported[17]. The finding in the present study of numerous colloidal gold particles within the terminal axon as early as 5 min after administration into an inactive muscle may indicate occurrence of endocytosis which is independent of recycling of vesicles during transmitter release. This is further supported by the fact that macromolecules are also taken up in sensory nerve terminals[18, 19]. Since the endocytosed particles appeared in both synaptic and coated vesicles the type of vesicle involved in the endocytosis could not be determined in this study.

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Hypotensive activity of histidine-containing analogues of C-terminal hexapeptide of Substance P

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Summary. Four new hexapeptide analogues of C-terminal Substance P fragment with increased solubility in aqueous solutions are described. The peptides contain histidine in positions 6, 8, 9 and 10, respectively. The effect of the structural changes on the hypotensive activity and antigenic properties of analogues was compared. It was found that substitution of amino acid residues in various positions in the C-terminal hexapeptide of Substance P resulted in different effects on the hypotensive and antigenic properties, respectively. Only the [His^6] SP_{6-11} analogue had an unchanged antigenic structure when compared with the C-terminal region of Substance P, but it showed an almost total loss of hypotensive activity. The [His^9] SP_{6-11} analogue retained 50% of the hypotensive activity of the C-terminal hexapeptide but showed a markedly reduced expression of the antigenic epitope localized in this region of Substance P.

Key words. SP_{6-11} analogues; hypotensive activity; antigenic properties.

Substance P, discovered in horse brain by von Euler and Gaddum[1], is an undecapeptide with sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH_2. The C-terminal hexapeptide and its pyroglutamyl-analogue [pGlu^6] SP_{6-11} show the full activity of the native Substance P in most biological tests[2] and are a very useful model to study the structure-function relationships of this neuropeptide. Previous studies showed that the substitution of the C-terminal MetNH_2 and Phe^7 by other amino acids leads to a complete loss of biological activity[3].

Owing to the poor solubility of the C-terminal hexapeptide in aqueous medium the C-terminal hexapeptide of Substance P is not very convenient to use. Therefore we attempted to synthetize analogues of the C-terminal hexapeptide with improved solubility. For this purpose, analogues containing histidine in positions known from previous studies not to be involved in the biological function of SP_{6-11} were prepared and their biological activity tested.

Material and methods. Peptides. Four hexapeptides were synthesized by a classical solution method[4]. The purity of the peptides was checked routinely by thin-layer chromathography (TLC) on Merck silica gel plates. In addition, melting points and specific rotations were taken for all peptides and compared with those from the literature when available (table 1). Final products of synthesis, i.e. all hexapeptides, were characterized by amino acid