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SHORT COMMUNICATION

Stimulation by Acetylcholine of Sulfated Mucopolysaccharide Release from the Perfused Cat Adrenal Gland

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SUMMARY

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Sulfated complex carbohydrates in the adrenal medulla were labeled with $^{35}SO_4^=$ after the administration of insulin to deplete catecholamine stores. Twenty-four hours later the left adrenal gland was perfused *in situ* with Locke's solution. Addition of acetylcholine to the perfusion solution resulted in a 12–20-fold increase in the release of sulfate-labeled non-dialyzable material, together with the usual large increase in catecholamine secretion. Digestion of the sulfate-labeled macromolecular material with bacterial chondroitinases demonstrated that 65–80% of the radioactivity was in the form of chondroitin 4- and 6-sulfate. The remaining radioactivity was present in an *N*-sulfated mucopolysaccharide which was tentatively identified as heparan sulfate. The results of these experiments are consistent with our previous finding of sulfated mucopolysaccharides, consisting mostly of chondroitin 4- and 6-sulfate, in purified chromaffin granules from bovine adrenal medulla, and are discussed in terms of the possible role of anionic complex carbohydrates in the storage and release of biogenic amines.

Several reports have suggested that biogenic amines are stored in tissues in the form of complexes containing sulfated mucopolysaccharides. Experimental evidence has been presented in support of such a storage mechanism for mast cell granules and rat thrombocytes, in which histamine and serotonin are considered to be linked by electrostatic bonds to a mucopolysaccharide-protein complex (1–3). Other studies have suggested that sulfated mucopolysaccharides may be involved in the binding and storage of catecholamines in chromaffin granules of adrenal medulla (4) and in adrenergic vesicle fractions from sympathetically innervated tissues, peripheral sympathetic nerves, and brain (5).

We have previously demonstrated the presence of chondroitin 4- and 6-sulfate and of glycoproteins with a high sialic acid content in purified chromaffin granules

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from bovine adrenal medulla (6). However, the number of anionic sulfate and carboxyl groups present in these complex carbohydrates was much less than required to account for the binding of the large quantities of catecholamines found in the isolated granule fractions. In an attempt to obtain more information concerning the possible role of mucopolysaccharides and glycoproteins in the storage and release of catecholamines, we have investigated the effects of acetylcholine stimulation on the release of sulfated complex carbohydrates from the perfused adrenal gland.

To produce hypoglycemia, and thereby deplete catecholamine stores in the adrenal medulla, male and female cats (1.6–2 kg) were given subcutaneous injections of crystalline zinc insulin (0.3 unit/kg) dissolved in 0.5 ml of dimethylsulfoxide. This was followed after 30 min by $Na_2{}^{35}SO_4$ (20 mCi/kg intraperitoneally), and 24 hr were then allowed for the labeling of sulfated complex carbohydrates in the adrenal medulla.

Cats were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally), and the left adrenal gland, acutely denervated, was perfused *in situ* by the aortic route and the effluent was collected from the adrenal vein according to the procedure of Douglas and Rubin (7). Perfusions were carried out with normal Locke's solution, equilibrated at room temperature with 95% oxygen and 5% carbon dioxide, and having a pH of 7.0. The solution contained NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2 mM; $MgCl_2$, 0.5 mM; $NaHCO_3$, 12 mM; and glucose, 10 mM. The flow rate was maintained at an average of 1.2 ml/min by regulating the perfusion pressure, and after four control periods of 10 min each perfusion was begun with Locke's solution containing acetylcholine (55 μM), resulting in a decrease in flow rate of approximately 20%. The effluent was collected during alternating control periods of 5–10 min and during acetylcholine stimulation as shown in Fig. 1. The data are expressed as macromolecular (i.e., nondialyzable) sulfate radioactivity per milliliter of effluent. Total catecholamines in the effluent were determined by the method of Anton and Sayre (8). Chondroitinase digestions and analyses of sulfated mucopolysaccharides were performed as described previously (9, 10).

Addition of acetylcholine at a concentration of 55 μM to Locke's solution perfusing the cat adrenal gland resulted in a 12–20-fold stimulation of the release of macromolecular sulfate radioactivity. The results of experiments on eight animals, in which acetylcholine stimulation was alternated with control periods, are summarized in Fig. 1. In three of these experiments the acetylcholine concentration was increased 10-fold to 550 μM after the first period of stimulation, but produced only a 14% greater increase in the release of sulfate-labeled material as compared to that obtained with 55 μM acetylcholine. These experiments were therefore not treated separately in the calculation of average values for Fig. 1.

In four cats which had been treated with considerably larger doses of insulin (1–2 units/kg) before the administration of labeled sulfate, the stimulation produced by acetylcholine was only 75% of that observed in animals treated with 0.3 unit/kg. These experiments are not included in the data summarized in Fig. 1. In the absence of prior treatment with insulin only a relatively small stimulation (2–4-fold) was observed in the release of macromolecular sulfate radioactivity after the addition of acetylcholine to the perfusion solution.

An average of 16% of the sulfate radioactivity released into the perfusion solution during the control periods was in the form of nondialyzable macromolecular material, while 54% of the sulfate radioactivity in the samples collected during acetylcholine stimulation was nondialyzable. There was no significant difference in the percentage of sulfate radioactivity which was nondialyzable in the early or later control samples, or in the effluents collected during successive acetylcholine stimulations. The dialyzable sulfate radioactivity could be quantitatively recovered as a single retarded peak which was eluted with 0.1 M NaCl from a 1 × 50 cm column of Sephadex G-25 at an effluent volume corresponding to that of inorganic sulfate.

Treatment with bacterial chondroitinase ABC of material pooled from either control

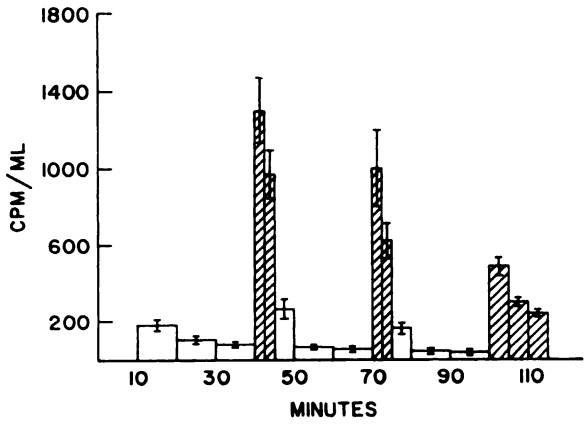

FIG. 1. *Mucopolysaccharide sulfate radioactivity released from perfused cat adrenal gland in the presence and absence of added acetylcholine*

The effluents collected during acetylcholine stimulation are indicated by the hatched bars. The figure shows the mean sulfated mucopolysaccharide radioactivity in the perfusate from eight experiments (means ± standard errors).

or acetylcholine stimulation effluents converted 65–80% of the nondialyzable radioactivity to low molecular weight disaccharides, which could be separated from the undigested material by gel filtration on Sephadex G-25 eluted with 0.2 M NaCl. Treatment with chondroitinase AC gave the same quantitative yield of disaccharides as did chondroitinase ABC, indicating the absence of significant amounts of labeled dermatan sulfate in the perfusion effluent.

The single retarded peak of disaccharides was desalted on Sephadex G-10 and chromatographed on Whatman No. 3MM paper using butanol–acetic acid–1 N ammonia (2:3:1, v/v). Examination of the strips with a radiochromatogram scanner revealed that approximately 60% of the radioactivity migrated as 3-*O*-*β*-Δ 4, 5-glucuronosyl-*N*-acetyl-D-galactosamine—4-*O*-sulfate (derived from chondroitin 4-sulfate), while the remainder was in the form of the corresponding disaccharide from chondroitin 6-sulfate. Fully 75% of the radioactivity in the chondroitinase-undigestible material could be removed under conditions sufficient for the hydrolysis of sulfoamino bonds (0.04 N HCl, 90 min, 100°), and all the sulfate in this fraction was removed by hydrolysis in 1 N HCl for 4 hr at 100°. These results indicate that labeled heparan sulfate (or heparin), containing *N*-sulfated hexosamine residues, was also released into the perfusate together with chondroitin 4- and 6-sulfate.

Concomitant with the increased release of sulfated mucopolysaccharides produced by acetylcholine was the occurrence of a 100-fold or greater increase in catecholamine secretion (Fig. 2).

These studies demonstrate that chondroitin 4- and 6-sulfate, and a chondroitinase-undigestible, *N*-sulfated mucopolysaccharide which is probably heparan sulfate, are released from the perfused adrenal gland in small amounts under basal conditions. Acetylcholine stimulation produced the expected large increase in catecholamine secretion, together with a 12–20-fold increase in the release of sulfated mucopolysaccharides.

We have previously reported that mucopolysaccharides are present in purified chromaffin granules from bovine adrenal medulla at a concentration of 1.5% by weight of the lipid-free protein residue (6). These were identified as 61% chondroitin 4-sulfate, 35% chondroitin 6-sulfate, and 4% of a glucosamine-containing sulfated mucopolysaccharide. The amounts of chondroitin sulfate radioactivity in the perfusate which were present as chondroitin 4- and 6-sulfate were essentially the same as the relative amounts of these two mucopoly-

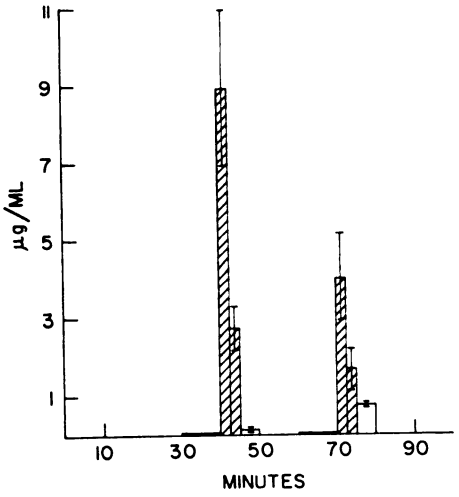

FIG. 2. *Catecholamine release from perfused cat adrenal gland in the presence (hatched bars) and absence of added acetylcholine (55 μM)*
Time intervals correspond to those in Fig. 1.

saccharides found in isolated chromaffin granules, while the heparan sulfate radioactivity was relatively greater than the small contribution (4%) of glucosamine-containing mucopolysaccharides to the total material present in bovine chromaffin granules. Although this may have been due partly to species differences, the distribution of radioactivity is consistent with the available data on sulfate turnover in the mucopolysaccharides of other types of nervous tissue such as brain, where heparan sulfate has a rapid turnover rate, while the chondroitin sulfates turn over at a slower rate which is approximately the same for both isomers (9).

The role of mucopolysaccharides and glycoproteins in chromaffin granules is presently unclear, although we have previously concluded that the amounts present there are not in themselves sufficient to account for the simple ionic binding of the large quantities of stored catecholamines.

Other constituents of the chromaffin granules, such as chromogranin A, dopamine β-hydroxylase, and adenine nucleotides and their metabolites, are also secreted together with catecholamines following stimulation of adrenal medullary cells. While the similar release of mucopolysaccharides may merely reflect their presence in chromaffin granules, it is possible that they interact with other cell organelles, such as lysosomes, in the process of exocytosis. Schneider (11) has reported that carbachol stimulates the release of several lysosomal enzymes from the isolated perfused bovine adrenal gland, and it has been shown that in pituitary and other cells unused secretory granules are disposed of by direct fusion with previously existing lysosomes by a process which is specific for secretion granules. Because of the distinctive features of this granule disposal mechanism, deDuve (12) has coined the term "crinophagy" to distinguish the process from autophagy. Crinophagy appears to be a general function of lysosomes in pituitary cells (13), and although relevant findings in other systems are not as complete, there is suggestive evidence that the process also occurs in neurosecretory neurons (14, 15), α cells of the pancreatic islets (16), and adrenal medulla (17). Although the possible relationship between the secretion of mucopolysaccharides and lysosomal enzymes bears further investigation, it appears unlikely that the released mucopolysaccharides are themselves of lysosomal origin, since the reported increase in enzyme secretion upon carbachol stimulation was only 50–300%, as compared to the 12–20-fold increase in release of sulfated mucopolysaccharides.

It was also observed that when prior treatment with insulin (to deplete catecholamine stores and allow resynthesis of chromaffin granules in the presence of labeled sulfate) was omitted, acetylcholine produced a much smaller than usual increase in the secretion of sulfate-labeled mucopolysaccharides. These results indicate that labeled mucopolysaccharides present in newly synthesized granules are preferentially released in response to stimulation of the adrenal medulla, in agreement with other evidence demonstrating the preferential release of newly synthesized biogenic amines.

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