PHARMACOLOGICAL REVIEWS
Vol. 18, No. 1, Part I

G. MEDULLARY PARTICLES

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The medullary hormones epinephrine (E) and norepinephrine (NE) are not evenly distributed throughout the medullary cells, but are held in specific particles, the chromaffin granules. These granules store 70 to 90% of the hormones, as shown by Hillarp *et al.* (16) as well as by Blaschko and Welch (3). Electron microscopic studies of adrenal medullary tissue showed densely osmiophilic granules nearly 0.2 μ in diameter. They are limited by a membrane and easy distinguishable on a morphological basis from other cell organelles (21, 31). From homogenates of adrenal medulla the storage granules can be isolated by differential centrifugation and separated from other cell particles such as mitochondria and microsomes by density-gradient centrifugation. Granules separated by this technique show a double layered membrane surrounding the filaceous ground-structure. The comparison of granules isolated from human medullary tissue or pheochromocytoma with granules obtained from the suprarenal medulla of the chicken shows no differences in structure and size (1, 20).

The granules contain besides catecholamines large amounts of ATP and also proteins, lipids, and RNA (12, 15, 23). The molar ratio of catecholamines to ATP as determined in different animal species is approximately 4:1 (10, 29). For example, in pure medullary granules from cattle, obtained by density-gradient centrifugation, this molar ratio is, according to Hillarp (11), 4.5:1; this value is somewhat higher than the value for equivalent amounts of base and acid, since 1 molecule of ATP has 4 negative charges. If it is assumed that the small amounts of intragranular ADP, AMP, and inorganic phosphate present in these granules are products split from ATP, the ratio amines to ATP would be 3.9:1. The positive charges of the catecholamines are in this case balanced by the sum of the negative charges of the adenine nucleotides. This quantitative relationship of catecholamines and ATP in medullary granules shows a remarkable constancy under different conditions which lead to amine depletion, as reported by several authors (6, 13, 17, 27, 29). On the other hand if the above mentioned ratio amine to ATP of 4.5:1 is valid, then about 10% of the amines cannot be bound to ATP. This is in accordance with our observations that after osmotic lysis of medullary granules of cattle the whole amount of ATP is released but only 90% of the catecholamines (25).

The observation that the granules can be stored at 0°C in isotonic sucrose for several days without losing their catecholamine and ATP content implies a storage mechanism that is not metabolically dependent (13, 29). The above-mentioned findings and investigations *in vitro* on catecholamine-nucleotide complexes by nuclear magnetic resonance spectroscopy (33) led to the hypothesis that the catecholamines form together with ATP, and perhaps with intragranular protein as a third component, a nondiffusible storage-complex within the granules (11). In accordance with this assumption are results of Kirshner (18), as well as of Carlsson *et al.* (7), who found that at 0°C the granules did not exchange their E with radioactive E, and further the observation that the incorporation of P^{32} into the ATP of the medullary granules is a very slow process *in vivo* in contrast to that of mitochondria (26).

If the catecholamine and ATP content of the granules from normal suprarenal tissue and from pheochromocytoma is compared, the molar ratio of amine to ATP is found to be different (28). The ratio in granules isolated from normal suprarenal medulla was 5.3, whereas those of 3 pheochromocytomas were 11.6, 35.4 and 10.3; in other words, there is a relative deficiency of ATP. Similar results have been obtained recently (32). In these granules, therefore, most of the catecholamines cannot be stored together with ATP as a complex. Possibly they are bound directly to lipid or lipoprotein material in the granular structure, as suggested by Euler (9). This observation could perhaps explain the high rate of the release of E and NE from such tumors.

Whereas the storage mechanism is probably independent of metabolic processes, the uptake of catecholamines into the granules appears to be strongly dependent on such processes, as the rate of amine uptake increases with the temperature and can be accelerated 5 to 6 times by the addition of ATP and magnesium (7, 18).

Since according to Kirshner (18, 19) the interior surface of the granular membrane shows dopamine-β-hydroxylase activity, the biosynthesis of NE from dopamine may be assumed to take place inside the granules. Therefore the mechanism of uptake of exogenous catecholamines is of importance for the formation of NE. It provides the granules with dopamine, as this amine is taken up to about the same extent as NE and E. The uptake can be inhibited by small doses of reserpine but is unaffected by ouabain, which is also known to block transport mechanisms (7, 18).

Recently Kirshner (19) suggested that the membrane of the chromaffin granules is impermeable to catecholamines and that therefore both the uptake of amines into the granules and their release from the granules is mediated by an active transport. The author postulated that 1 molecule of intragranular ATP could be utilized for the transport of 3 to 4 molecules of amines, but this view is not compatible with earlier observations of Carlsson and Hillarp (5, 14), who demonstrated the permeability of the granular membrane to catecholamines. Furthermore the hypothesis does not account for two of our own observations. 1) In incubation experiments with isolated medullary granules, calcium ions release catecholamines and ATP to the same percentage extent at 37°C as at 0°C (30). According to Kirshner's view, calcium ions should have activated the outward transport at 0°C less than at 37°C. 2) Tyramine releases from the intragranular pool into the suspension medium exclusively catecholamines and no ATP. Therefore no energy for the transport would be available (29).

On the other hand if the membrane is permeable to catecholamines, these amines should be stored in some bound form and should not exist as free ions, since at 0°C practically no diffusion of amines takes place. The facts that during incubation of granules at 37°C a spontaneous release of catecholamines together with ATP takes place and that this release is dependent on temperature suggest that the release is initiated by an enzymatic decomposition of the storage complex and that thereafter the amines are released by diffusion.

Together with Dr. Philippu we have performed the following experiments in order to prove the possible role of other components for the storage of the ATP-catecholamine complex in bovine medullary granules (25). In incubation experiments at 37°C we were able to show (23) that the spontaneous release of amines and ATP is accompanied by a loss of RNA in the same percentage. At 0° no decrease of the 3 substances is observed. In order to prove whether a causal relationship exists between the decreases of RNA and amines, we incubated granules with increasing amounts of a purified RNase preparation. The addition of this enzyme produces an increased and dose-dependent release of catecholamines, ATP, and RNA to the same degree, but does not change the protein content. The depolymerizing effect of the added RNase on the RNA is obviously connected with the release of amines and ATP. Similarly the spontaneous release of catecholamines and ATP at 37°C could be due to a primary depolymerization of the RNA by an intragranular RNase. We therefore investigated pure medullary granules obtained from a discontinuous density gradient and found that this granular fraction contains besides catecholamines, ATP, and RNA sufficient RNase activity to catalyze the spontaneous release of RNA, catecholamines, and ATP at 37°C (24).

In previous investigations (22, 30) we had shown that calcium in concentrations of 2.5×10^{-3} M, the physiological concentration of the blood plasma, is capable of releasing proportional amounts of catecholamines and ATP from isolated medullary granules. This result is of importance in connection with the

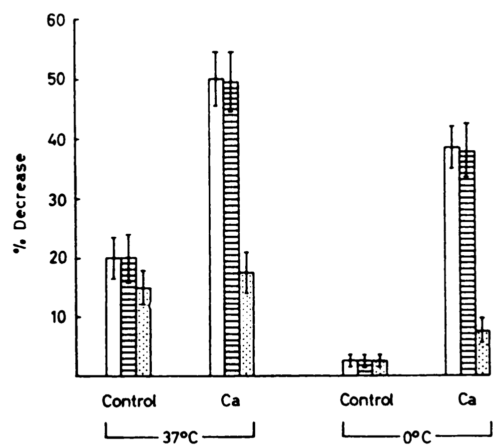

FIG. 1. Release of catecholamines, ATP, and RNA by calcium (5×10^{-3} M). ☐ Catecholamines, ⊟ ATP, ⚄ RNA. The values represent the means of 6 experiments ± standard deviations.

TABLE 1

ATP-, calcium-, and magnesium content of chromaffin granules isolated from 100 mg medulla

ATP	Ca	Mg	ATP/CA + Mg
	μmoles		
0.417 ± 0.045	0.230 ± 0.022	0.114 ± 0.014	1.210 ± 0.155

The values represent the means of 37 experiments ± standard deviations.

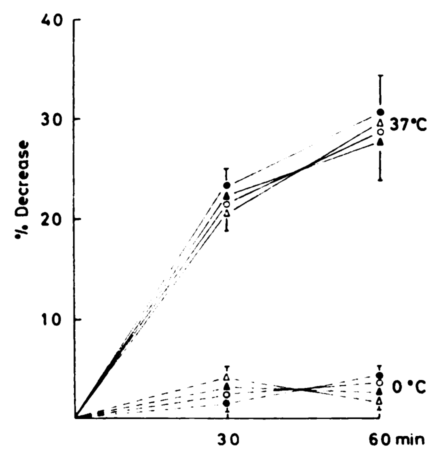

FIG. 2. Spontaneous release of catecholamines, ATP, calcium, and magnesium. ● Catecholamines, △ ATP, ▲ calcium, O magnesium. The values represent the means of 6 experiments ± standard deviations.

observation of Douglas and Rubin (8) that calcium liberates catecholamines also from the intact medullary cell. This action of calcium could perhaps be mediated by an activation of the intragranular RNase (fig. 1). At both 37° and 0°C, however, calcium releases amines and ATP without affecting the RNA content of the granules. Therefore calcium cannot act by an activation of this enzyme at least under our experimental conditions. It could rather act directly on the storage complex by displacement of another bivalent ion.

To prove this possibility we determined at first the magnesium, calcium, and ATP content of chromaffin granules (table 1). Granules isolated from 100 mg medulla contain calcium as well as magnesium. The molar ratio ATP to calcium and magnesium is 1.21; this means that one mole of bivalent ions, calcium plus magnesium, corresponds to 1 mole ATP (25). Our values of calcium and magnesium are somewhat higher than those reported recently by Borowitz *et al.* (4). Further experiments show (fig. 2) that the spontaneous release of ATP and amines at 37° is accompanied by a release of calcium and magnesium in the same percentage and that at 0°C neither amine and ATP nor calcium and magnesium are liberated.

TABLE 2

Effect of releasing agents on the amine, ATP, Ca^{++}, and Mg^{++} content of isolated medullary granules

	% Release			
	Amine	ATP	Ca^{++}	Mg^{++}
RNase	14 ± 0.8	14 ± 1.1	15 ± 0.9	22 ± 3.5
Reserpine	44 ± 2.7	43 ± 2.0	37 ± 1.2	40 ± 2.8
Prenylamine	25 ± 1.9	29 ± 1.5	25 ± 0.8	29 ± 2.3
Tyramine	22 ± 0.9	0	2 ± 0.1	4 ± 0.1

Incubation: 60 min, 37°C. The values represent the means of 4 experiments ± standard deviations. RNase, 240 μg/ml; reserpine, 2×10^{-4} M; prenylamine, 6×10^{-5} M; tyramine, 10^{-2} M.

In order to get more information about the release of these bivalent ions, incubation experiments were performed with catecholamine- and ATP-releasing agents. The agents used were purified RNase, reserpine, prenylamine [Segontin, N-(3′-phenylpropyl-(2′))-1,1-diphenylpropyl-(3)-amine)], and tyramine. Table 2 shows the results. RNase, reserpine, and prenylamine release, along with catecholamines and ATP, calcium and magnesium in the same percentage, whereas the protein content remains practically unchanged. Tyramine, on the other hand, releases exclusively catecholamines and has no effect on the ATP, calcium, magnesium, or protein content. This is in agreement with our earlier findings that tyramine displaces the less basic hormones E and NE from their binding sites to ATP and is itself taken up in equivalent amounts (29).

From these investigations it can be concluded that calcium and magnesium are released only if simultaneously ATP is liberated, as for example in the presence of RNase, prenylamine, and reserpine. Tyramine, on the contrary, releases catecholamines but no ATP and therefore no calcium and magnesium.

Our results indicate that bivalent ions as well as RNA or a ribonucleoprotein may participate in the formation of the storage complex, but they do not permit conclusions about the structure of the hypothetical complex. The observation of Blaschko and Helle (2) that soluble protein from bovine adrenal medullary granules can interact with E, ATP, and magnesium favors the existence of a similar complex.

SUMMARY

The reviewed results show that the medullary granules are surrounded by a membrane which contains on its interior surface dopamine-β-hydroxylase. An active transport mechanism which is activated by ATP and magnesium and inhibited by reserpine catlyzes the uptake of catecholamines into the granules. Whether or not there exists also an active mechanism for the release of catecholamines is not yet settled. The results discussed favor the assumption that the granule membrane permits the release of catecholamines by diffusion. Our own experiments are in agreement with the hypothesis that the main part of the

catecholamines is stored as a complex with ATP, bivalent ions, and a ribonucleoprotein or RNA.

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