The Effect of Salts on Catecholamine Fluxes and Adenosine Triphosphatase Activity in Storage Vesicles from the Adrenal Medulla

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1. Influx and efflux of catecholamine and adenosine triphosphatase activity in storage vesicles from the adrenal medulla were studied with DL-[¹⁴C]adrenaline in different media. 2. The lowest values for flux and adenosine triphosphatase activity were observed in sucrose media in which an ATP-dependent influx of catecholamine compensated for an efflux of the same magnitude. Efflux in the presence or absence of ATP was similar. 3. In media containing sodium succinate or glutarate adenosine triphosphatase activity was higher and the ATP-dependent influx of catecholamine was about twice that observed in iso-osmotic sucrose medium. In the presence of ATP influx and efflux of catecholamine were balanced; in its absence there was a net release of catecholamine, since efflux was more than twice the influx. Efflux in the presence or absence of ATP was similar. 4. In media containing sodium or potassium chloride and in the presence of ATP influx and adenosine triphosphatase activity were further enhanced, but in the absence of ATP there was no further increase in influx, since catecholamine was released with or without ATP at the same rate. Efflux was therefore twice as high in the presence of ATP as in its absence. 5. Sodium nitrate suppressed the ATP-dependent influx nearly completely, but caused a greatly enhanced efflux, which was twice as high in the presence of ATP as in its absence. 6. The extinction of vesicular suspensions remained unchanged in the presence of ATP under conditions where the catecholamine efflux was balanced by the influx. Under conditions where the efflux was not compensated by influx, the extinction of the suspensions decreased in the presence of ATP more than in its absence.

Poisner & Trifaró (1967, 1968, 1969) and Trifaró & Poisner (1967) reported that the release of catecholamines from the adrenal medullary vesicles is enhanced by magnesium-ATP provided that the assay medium contained potassium chloride or sodium chloride or both. This finding led the authors to propose an ATP-dependent secretion mechanism for catecholamine release (Poisner & Trifaró, 1967). The authors suggest that the release is brought about by an ATP-consuming contraction of a contractile protein present in the vesicular membrane. This concept would provide a molecular basis for the proposed exocytosis mechanism for amine release (Banks & Helle, 1965; Blaschko, Comline, Schneider, Silver & Smith, 1967) and also for results of Viveros, Aqueros, Connet & Kirshner (1969), who found that the vesicular membrane remains within the cell after depletion.

A similar concept has been proposed by Hillarp (1958), who in contrast to Poisner & Trifaró (1967) assumes that the intravesicular and not the extravesicular ATP is utilized in this process. Poisner & Trifaró (1968) assume that the cytoplasmic concentration of ATP is very low to prevent catechol-

amine release inside the cell by the ATP present there, an assumption that would seem to conflict with its function as the energy donor for the accumulation of catecholamines by the medullary vesicles (Taugner & Hasselbach, 1966a,b; Kirshner, Kirshner & Kamin, 1966). The results on which the latter conclusion is based were obtained in assay media containing iso-osmotic sucrose instead of potassium chloride and sodium chloride. Since it seems unlikely that the replacement of sucrose by these salts would disclose a completely new mechanism, vesicular preparations obtained by centrifugation in iso-osmotic sucrose media were studied in parallel in iso-osmotic sucrose and different iso-osmotic salt solutions.

A preliminary report of some of these results has been published (Taugner, 1969).

MATERIALS AND METHODS

The preparation of the vesicles containing catecholamines from the bovine adrenal medulla was carried out in unbuffered 0.3M-sucrose at 2–4°C by the method described by Taugner & Hasselbach (1966a). The adrenal

medullas of six to eight animals were passed through a nylon sieve and homogenized; nuclei and unbroken particles were removed by centrifugation at 900g for 6 min and the supernatant was centrifuged at 12000g for 15 min. The storage vesicles of higher density were separated from the upper, loosely packed layer of vesicles with a lower catecholamine/protein ratio. The upper layer was discarded by swirling with ice-cold 0.3M-sucrose. This procedure was repeated twice after centrifugation at 9000 and 7000g respectively. The catecholamine/protein ratio had a final value of about 2.84 (2.29–3.16) $\mu\text{mol/mg}$ of protein (the mol.wt. of catecholamine being taken as 180). A sample was taken from each preparation for determination of the protein content by the Kjeldahl method. The catecholamine content was measured colorimetrically by the method of von Euler & Hamberg (1949). The isolated vesicles were incubated with constant stirring at 31°C, always on the day of preparation. The samples contained 0.5–1.2 mg of vesicular protein/ml in the assay.

Measurement of catecholamine fluxes. The influx across the vesicular membrane was measured by using DL-[^{14}C]adrenaline in different incubation mixtures as mentioned in the Results section. The specific radioactivity of the added [^{14}C]adrenaline was 9 or 37 mCi/mmol; the specific radioactivity in the media, however, was lower owing to the catecholamine quantities released from the vesicles. All samples contained 5 mM-MgCl₂ and, in the experiments containing ATP, the concentration of added ATP was 5 mM. The incubation media were buffered with sodium glycerophosphate (0.04M final concentration) to pH 7.4. The incubation procedure, determination of the ^{14}C radioactivity and calculation of the initial velocity of the catecholamine influx was described by Hasselbach & Taugner (1970). The average of three successive values (t_5 , t_{10} , t_{15}) of the first 15 min of the experiment were taken as influx rate. The supernatant was used to assay the rate of catecholamine release, which was estimated from the increasing catecholamine content in the medium as mean rate, the average of three successive values from the first 15 min of the experiment. 'Release' is defined as the analytically measured increase of the catecholamine concentration in the medium, in contrast with the 'efflux', which represents the total catecholamine outward flux across the vesicular membrane, i.e. the sum of release + influx. Since the amount of protein released during the incubation from the vesicles was too small to determine by the Kjeldahl method it was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) after precipitation of the protein in the supernatant with 10% (w/v) trichloroacetic acid and then dissolving the protein pellet in NaOH.

Assay of adenosine triphosphatase activity. The adenosine triphosphatase activity was always measured in the same sample as the catecholamine fluxes. The reaction was terminated after appropriate time-intervals by precipitating samples with 10% (w/v) trichloroacetic acid. Inorganic phosphate was measured by the method of Rockstein & Herron (1951). The protein was determined by the Kjeldahl method in a portion of the vesicular suspension.

Measurement of the extinction. The extinction of the vesicular suspension was measured in a Zeiss spectrophotometer at 560 nm. Experiments were started by the addi-

tion of 0.1–0.2 mg of the vesicular protein/ml of the medium containing 5 mM-ATP + 5 mM-MgCl₂, buffered with sodium glycerophosphate to pH 7.4. In control experiments carried out at the same time ATP was omitted. After the initial mixing measurements were made at room temperature on the unstirred mixture.

Chemicals. ATP (disodium salt) was from P-L Biochemical Inc., Milwaukee, Wis., U.S.A.; DL-[^{14}C]adrenaline (specific radioactivity 9 mCi/mmol, and a later charge 37 mCi/mmol) was from Farbwerke Hoechst, Frankfurt a.M., Germany; Omnifluor (98% 2,5-diphenyloxazole + 2% *p*-bis-(*o*-methylstyryl)-benzene) was from NEN Chemicals G.m.b.H., Frankfurt a.M., Germany.

RESULTS

Catecholamine fluxes and extinction in media containing sucrose. The results of the control experiments in iso-osmotic sucrose were in full agreement with those described in previous papers (Taugner & Hasselbach, 1966a,b, 1968; Hasselbach & Taugner, 1970). The release of catecholamine that takes place in the absence of ATP and which is the result of a low catecholamine efflux and an even lower catecholamine influx stops completely when ATP is present, because the activated catecholamine influx now largely compensates for the unchanged catecholamine efflux. In the described experiment the ATP-dependent influx even exceeded the catecholamine efflux, so that a small net catecholamine uptake was observed (Table 1). The elevation of the sucrose concentration in the medium from 0.3 to 1.0M caused both in the absence and presence of ATP an equal decrease of the catecholamine efflux. Likewise the catecholamine influx rates were diminished by 40–50%. As a result, in the absence of ATP, the release is decreased relative to that at 0.3M-sucrose whereas in the presence of ATP a net catecholamine loss occurs (Fig. 1; Table 1).

At the beginning of the experiment the concentration of free catecholamine in the medium was on average 70 nmol/mg of protein, which corresponds to approximately 3% of the total catecholamine present in the assay mixture. In comparison with this initial catecholamine concentration, the concentration of the water-soluble protein was relatively high. Approximately 15–20% of the total protein was usually found in the supernatant (Table 2). In the presence of ATP the soluble protein remained unchanged during the course of the experiment but increased in the absence of ATP. This low protein release did not give rise to a detectable change in the extinction of the vesicular suspension (Fig. 1).

Catecholamine fluxes and changes in extinction in media of iso-osmotic solutions of sodium glutarate or succinate. When the assay media were supplemented with the sodium salts of glutarate or

Table 1. Catecholamine fluxes and adenosine triphosphatase activity of vesicular preparations in the presence and absence of ATP

The experiments were started by the addition of DL-[14C]adrenaline and the vesicular suspension to the various media as described in the Materials and Methods section. All media contained 0.04 M-sodium glycerophosphate at pH 7.4. Fluxes were calculated as described in the text. Results are shown as means $\pm$ s.e.m. of the number of experiments given under *n*. * Indicates that in some experiments net uptake takes place.

Medium	n	Catecholamine flux (nmol of catecholamine/min per mg of protein)						Adenosine tri-phosphatase activity (nmol of P _i /mg of protein per min)	n
		With ATP (5 mM)			Without ATP				
		Influx	Release	Efflux	Influx	Release	Efflux		
Sucrose (0.3 M)	13	3.42 ± 0.55	-0.31* ± 0.39	3.11	0.83 ± 0.15	2.74 ± 0.41	3.57	16.7 ± 0.7	10
Sucrose (1.0 M)	4	1.89 ± 0.72	0.75 ± 0.18	2.64	0.38 ± 0.13	1.89 ± 0.54	2.27	13.05 ± 1.3	3
Sodium glutarate (0.1 M)	4	7.08 ± 0.65	-0.62* ± 0.22	6.46	1.37 ± 0.55	5.57 ± 0.64	6.94	21.5 ± 1.7	3
Sodium succinate (0.1 M)	4	7.29 ± 0.81	0.13* ± 0.25	7.42	1.45 ± 0.28	5.06 ± 0.37	6.51	22.1 ± 1.9	3
0.16 M-KCl + 0.005 M-NaCl	9	8.86 ± 1.10	6.02 ± 1.27	14.88	1.40 ± 0.24	6.15 ± 0.87	7.55	26.8 ± 1.4	7
Choline chloride (0.16 M)	4	8.04 ± 0.39	3.62 ± 0.77	11.66	1.87 ± 0.04	5.44 ± 0.96	7.31	26.3 ± 2.5	3
NaNO ₃ (0.16 M)	3	2.59 ± 0.42	20.18 ± 1.09	22.77	1.16 ± 0.38	11.56 ± 1.59	12.72	—	—

Fig. 1. Time-courses of the release of catecholamines (a) and of the extinction (b) of vesicular suspensions incubated in 0.3 M-sucrose (○, ●) and 1.0 M-sucrose (△, ▲) respectively in the presence (○, △) or absence (●, ▲) of ATP. The experiments were started by the addition of the vesicular suspension to the medium at 31°C and were terminated by cooling of samples, after the time-intervals indicated, in an ice-bath for 5 min and subsequent centrifugation. The catecholamine released was determined from the supernatant as described in the Materials and Methods section. The light-scattering experiment was started by the addition of the vesicular suspension. Absorption is expressed as per cent of the value at zero time.

succinate instead of sucrose, the catecholamine influx and the catecholamine efflux increased irrespective of the presence of ATP. In the absence of ATP, efflux and influx were increased twofold compared with the experiments performed in 0.3 M-sucrose. This led to an increased release of catecholamine (Table 1).

In the presence of ATP, both influx and efflux were stimulated twofold in comparison with values obtained in iso-osmotic sucrose medium but there

Table 2. Liberation of catecholamine and protein from vesicles in the presence and absence of ATP

The catecholamine and protein in the supernatant are expressed as percentages of the total amounts present in the reaction mixture. All media contained 0.04 M-sodium glycerophosphate at pH 7.4. Results are shown as means $\pm$ s.e.m. of the number of experiments (*n*).

Medium	<i>n</i>	Catecholamine release (% of total catecholamine)				Protein release (% of total protein)			
		With ATP (5 mM)		Without ATP		With ATP (5 mM)		Without ATP	
		0 min	15 min	0 min	15 min	0 min	15 min	0 min	15 min
Sucrose (0.3 M)	13	3.16 $\pm$ 0.33	3.09 $\pm$ 0.48	3.46 $\pm$ 0.44	5.89 $\pm$ 0.71	20.3 $\pm$ 5.2 (<i>n</i> 4)	20.8 $\pm$ 6.3	15.0 $\pm$ 4.3	18.9 $\pm$ 4.5
Sucrose (1.0 M)	14	5.05 $\pm$ 0.61	5.25 $\pm$ 0.52	4.53 $\pm$ 0.49	5.77 $\pm$ 0.58	—	—	—	—
Sodium glutarate (0.1 M)	4	5.17 $\pm$ 0.84	4.90 $\pm$ 0.65	6.46 $\pm$ 0.94	9.44 $\pm$ 1.05	—	—	—	—
Sodium succinate (0.1 M)	4	5.11 $\pm$ 0.79	4.88 $\pm$ 0.63	6.42 $\pm$ 0.62	8.99 $\pm$ 0.83	7.5 $\pm$ 2.4 (<i>n</i> 4)	7.9 $\pm$ 1.9	6.5 $\pm$ 1.7	7.6 $\pm$ 2.2
0.16 M-KCl + 0.005 M-NaCl	9	6.67 $\pm$ 0.74	10.98 $\pm$ 1.08	6.57 $\pm$ 1.07	10.74 $\pm$ 0.99	7.8 $\pm$ 2.1 (<i>n</i> 4)	14.0 $\pm$ 3.5	7.8 $\pm$ 1.4	10.4 $\pm$ 2.4
Choline chloride (0.16 M)	4	6.91 $\pm$ 0.59	8.99 $\pm$ 0.74	7.83 $\pm$ 0.55	10.58 $\pm$ 0.86	—	—	—	—
NaNO ₃ (0.16 M)	3	9.96 $\pm$ 1.08	20.63 $\pm$ 1.97	11.51 $\pm$ 0.98	18.00 $\pm$ 1.63	—	—	—	—

Fig. 2. Time-course of the release of catecholamine (a) and the extinction (b) of vesicular suspensions incubated in 0.1 M-sodium glutarate (○, ●) and 0.1 M-sodium succinate (△, ▲) respectively in the presence (○, △) or absence (●, ▲) of ATP. Experimental conditions are given in Fig. 1.

was no net loss of catecholamine (Fig. 2). The same results are obtained when potassium glutarate and succinate were used in these media instead of the sodium salts.

The initial catecholamine concentration in these media was nearly twice the concentration in 0.3 M-sucrose, whereas less non-sedimentable protein was found (Table 2). Its quantity remained unchanged during the incubation period and the extinction values did not decline (Fig. 2).

Catecholamine fluxes and extinction in iso-osmotic solutions of sodium and potassium chloride and choline chloride. In the standard iso-osmotic medium of Poisner & Trifaró (1967) (160 mM-KCl + 5 mM-NaCl) or iso-osmotic choline chloride in the absence of ATP practically the same enhancement of influx was observed as with the glutarate and succinate salts. In the absence of ATP the efflux in the KCl + NaCl medium was 20% higher than

Fig. 3. Time-course of the release of catecholamine (a) and of the extinction (b) of vesicular suspensions incubated in 0.16M-KCl+0.005M-NaCl (○, ●) and 0.16M-choline chloride (△, ▲) respectively in the presence (○, △) or absence (●, ▲) of ATP. Experimental conditions are given in Fig. 1.

in succinate or glutarate, resulting in a net loss of 5–6nmol of catecholamine/min per mg of protein. In the presence of ATP, however, the efflux rate increased to values four- to five-fold those in sucrose or twice as high as in the presence of the glutarate or succinate (Table 1). In contrast, the influx did not exceed essentially the rates obtained in the other salt media. It follows that in the presence of the chlorides of Na^+ and K^+ catecholamines were released in the presence of ATP as fast as in its absence (Fig. 3). In the medium containing choline chloride, which was studied to discriminate between the effects of cations, the efflux and the release enhancement were somewhat lower than in the presence of KCl or NaCl or both. That the observed enhancement of the catecholamine effluxes is sensitive to anions is clearly demonstrated by the effect of sodium nitrate. In the absence of ATP sodium nitrate induced an efflux fourfold the control value in 0.3M-sucrose and a still higher increase

Fig. 4. Relation between catecholamine fluxes and adenosine triphosphatase activity of catecholamine vesicles suspended in the various media: ●, catecholamine influx; ▲, catecholamine efflux. The experiments were carried out as described in the Materials and Methods section. The catecholamine flux rates as well as the ATP-splitting rates were obtained as averages of three successive values of the first 15 min of the experiments.

occurred in ATP-containing media. Simultaneously the influx was decreased, resulting in a much higher loss of catecholamine (Table 1).

Loss of catecholamines, as determined in the chloride media, was accompanied by the liberation of soluble protein. Although the catecholamine losses in the presence and in the absence of ATP were equal more protein was released when ATP was present (Table 2). The enhanced protein release in the presence of ATP led to a change in extinction of the suspension (Fig. 3). It decreased in the medium containing KCl+NaCl and ATP by 10% during 15min and only by 5% in the absence of ATP. If vesicles were used 1–2 days after preparation, or if the tissue was homogenized more vigorously than usual, in the presence of ATP the extinction diminished by 20–25% within 15min.

Consumption of ATP in relation to the catecholamine fluxes. The results presented in the last column of Table 1 and in Fig. 4 show that the hydrolysis of the added ATP by the adenosine triphosphatase of the vesicles varied with the catecholamine fluxes. In the sucrose and organic salt solutions the catecholamine influx and efflux changed in parallel with the adenosine triphosphatase activity. However, in the KCl+NaCl and choline chloride-containing solutions both the influx and the efflux were not proportional to the adenosine

triphosphatase activity. The catecholamine influx increased only slightly, whereas the efflux was strongly enhanced. From the increase of the linear part of the influx curve a transport ratio (nmol of catecholamine/nmol of ATP hydrolysed) of 0.7 was calculated, which is in good agreement with earlier findings (Hasselbach & Taugner, 1970). No correlation was found between adenosine triphosphatase activity and the rate of catecholamine release.

Control experiments with adrenal medullary mitochondria. To exclude the possibility that the changes in the adenosine triphosphatase activity of the vesicular preparations were disturbed by adenosine triphosphatase present in mitochondrial impurities, the adenosine triphosphatase activity of mitochondrial preparations was examined under the conditions described above. Mitochondria suspended in sucrose or glutarate medium hydrolysed 60 nmol of ATP/min per mg of protein. In the KCl+NaCl medium 63 nmol of P_i /min per mg of protein was liberated. The small difference of 3 nmol of P_i /mg of protein may be due to vesicular contaminations of the mitochondrial preparations.

DISCUSSION

The present results support the view that catecholamine storage in vesicles from the adrenal medulla is an active, ATP-dependent, process, whereas release in the experimental system studied is the result of an uncompensated efflux (Taugner & Hasselbach, 1966a, 1968; Hasselbach & Taugner, 1970) that can be facilitated by various salts. All the electrolyte media examined caused, even in the absence of ATP, a distinct increase in the efflux rate compared with that obtained in sucrose media. The fact that catecholamine release in sucrose media is much slower than in the salt media has been interpreted as an inhibitory effect of sucrose on the ATP-evoked catecholamine release (Poisner & Trifaró, 1967, 1969). However, the same slow release takes place in the absence of ATP. It is possible that the efflux of catecholamine is decreased by an osmotic shrinkage of the membrane proteins (Diamond, 1966). The surprising observation that in the iso-osmotic sucrose medium more soluble protein (about 20% of the total vesicular protein) was present in the supernatant than in the salt solutions may perhaps be related to the proposed membrane shrinkage. Obviously the membrane does not shrink in the presence of glutarate or succinate, since no protein release could be observed. Since multivalent anions predominate in the cytoplasm it can be assumed that the high flux rates obtained in the bivalent organic salt solutions correspond more closely to the flux rates under physiological conditions than those observed in sucrose or chloride media. Whereas the presence

of chloride ions in the medium did not cause any marked change in catecholamine fluxes in the absence of ATP, in its presence they stimulated the efflux so that it was no longer compensated by the ATP-dependent influx; therefore under these conditions a release of catecholamine was observed. It may be fortuitous that the release rate was not different from that observed in the absence of ATP. However, in contrast with the findings of Poisner & Trifaró (1967), the addition of ATP to a chloride medium did not accelerate catecholamine release from fresh vesicle preparations. To account for the efflux enhancement by chloride ions in ATP-containing solutions a specific interaction with the membrane during ATP-stimulated catecholamine transport might be assumed. This assumption is supported by the properties of the vesicular adenosine triphosphatase.

In sucrose and in the presence of the bivalent anions catecholamine influx and efflux and the adenosine triphosphatase activity changed in parallel. That it is the influx and not the efflux which is coupled with the adenosine triphosphatase activity is supported by the finding that ATP protects influx and adenosine triphosphatase activity against poisoning by *N*-ethylmaleimide whereas no such protection of efflux by ATP against *N*-ethylmaleimide is observed (Taugner & Hasselbach, 1966b, 1968).

On the other hand, in the chloride-containing media there was no correlation between adenosine triphosphatase activity and the catecholamine fluxes: efflux was increased proportionally more than influx. The prevailing catecholamine efflux thus causes the catecholamine release. That a direct correlation between the catecholamine efflux and the adenosine triphosphatase activity does exist in the sense that ATP splitting is required for the catecholamine release seems very unlikely on the basis of the analysis performed with transport inhibitors mentioned above. If according to Poisner & Trifaró (1967, 1969) the release of catecholamines is brought about by an ATP-consuming contraction of the membrane proteins, a correlation between release and ATP splitting should exist. However, such a correlation was not detected. Therefore it might be assumed that chloride increases specifically the permeability of the membrane in the presence of ATP and simultaneously stimulates the adenosine triphosphatase activity, although the latter can be used only partially for the catecholamine uptake. The high efflux is then not compensated by the influx, the rate of which is restricted to 8–9 nmol/min per mg of protein, and catecholamine starts to leak out. The decompensation of the catecholamine transport system becomes especially accentuated in the sodium nitrate-containing medium because, in

addition to the enhanced efflux, the influx is inhibited.

The fact that a release of protein occurs in parallel with the loss of catecholamine, however, requires some modifying assumptions. If the catecholamine efflux is accompanied by a protein efflux under all conditions, the protein concentration in the incubation medium might be expected to rise, since it is difficult to conceive of a protein influx coupled to the catecholamine influx. Since in the succinate and glutarate media with ATP present relatively high flux rates were observed without any protein loss, one has to assume that catecholamine can leave the vesicles independently of the water-soluble vesicular protein. The efflux threshold at which protein loss starts is clearly ATP-dependent, because in the absence of ATP efflux was always accompanied by a loss of protein whereas in the presence of ATP, and in media containing sucrose, glutarate or succinate, no protein loss occurred at the same efflux rate (Table 2). Under these conditions ATP appears to inhibit the release of protein. In the presence of the chloride ion, on the other hand, it would appear that ATP no longer inhibits the release of protein and the accompanying loss of catecholamine, providing an additional or alternative explanation for the enhanced release of catecholamine in chloride media containing ATP.

It is noteworthy that ATP was less effective in this respect when aged or less carefully prepared vesicles were used.

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