The Effects of Univalent Anions 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 were studied in storage vesicles of bovine adrenal medulla. 2. In the absence of ATP the influx of catecholamine was slow and was not influenced by various anions, whereas the efflux increased in the sequence of anions given by the lyotropic series. 3. In the presence of ATP the efflux was enhanced compared with that in the absence of ATP; the anion-dependent sequence, however, in which the efflux increased was the same as in the absence of ATP. 4. The ATP-dependent catecholamine influx and the adenosine triphosphatase activity are correlated. The sequence in which anions affect adenosine triphosphatase activity and catecholamine influx, however, is completely different from the lyotropic anion series. 5. No correlation was found between adenosine triphosphatase activity and the efflux of catecholamine.

Under various conditions the ATP-dependent fluxes of catecholamine across the vesicular membrane have been found to be correlated to the adenosine triphosphatase activity (Taugner, 1969, 1971). In media containing sucrose as well as in iso-osmotic solutions of the bivalent anions glutarate or succinate, influx and efflux of catecholamine remained balanced. In bivalent salt solutions flux rates were much higher than in the non-electrolyte-containing media; also the vesicular adenosine triphosphatase activity was markedly enhanced. In iso-osmotic solutions of chloride salts the adenosine triphosphatase activity was further stimulated; the catecholamine efflux exceeded the influx, resulting in a release of catecholamine in the presence of ATP similar to that obtained in its absence.

The release of catecholamine caused by ATP in solutions containing KCl and NaCl was interpreted in terms of an ATP-dependent secretion mechanism for catecholamines (Poisner & Trifaró, 1967; Trifaró & Poisner, 1967). The release is thought to be brought about by an ATP-consuming contraction of a contractile membrane component of the storage vesicles (Poisner & Trifaró, 1968).

The results presented in the present paper show that adenosine triphosphatase activity and catecholamine influx are changed proportionally by iso-osmotic solutions of halogen salts. The catecholamine efflux, however, changes independently from the adenosine triphosphatase activity. While the influx and the adenosine triphosphatase activity increase in the order $F^- < I^- < Cl^- < Br^-$ the efflux follows physico-chemical properties of the anions as known for the lyotropic series of Hofmeister (1891). Other anions of this series were also examined.

Materials and Methods

Catecholamine storage vesicles from bovine adrenal medulla were isolated in unbuffered 0.3M-sucrose at 2–4°C as described by Taugner & Hasselbach (1966). From each preparation a portion was taken to determine the content of protein by the Kjeldahl method and the content of catecholamine by the colorimetric method of von Euler & Hamberg (1949). The ratio of catecholamine/protein of the final preparation was 2.96 (2.46–3.19) $\mu\text{mol/mg}$ (the molecular weight of catecholamine being taken as 180).

Incubation media

Freshly isolated vesicles were incubated at 31°C with constant stirring. The incubation media consisted of 0.16M solutions of potassium halogen salts, potassium thiocyanate, sodium acetate or sodium trichloroacetate. All assays contained 5mM-MgCl₂ and were buffered to pH 7.4 with 50mM-sodium glycerophosphate. In the assays containing ATP, the ATP concentration was 5mM. The concentration of vesicular protein was 0.6–1.1mg/ml of the assay. Tracer amounts of ($\pm$)-[¹⁴C]adrenaline (specific radioactivity 37mCi/mmol) were added to the experiments for measuring the catecholamine fluxes.

Measurement of catecholamine fluxes

The experiments were started by addition of 1ml of the vesicular preparation to the incubation mixture, giving a final volume of 10ml. At chosen time-intervals samples (2ml) were taken, cooled for 5min

in an ice bath and centrifuged at 20000*g* for 15 min. The catecholamine content in the supernatant was determined by the method of von Euler & Hamberg (1949). The ^{14}C radioactivity of the sediment was extracted with 1 ml of ice-cold trichloroacetic acid (10%, w/v). The extract was transferred to 15 ml of a scintillation mixture of toluene/Triton X-100 (11:4, v/v) containing Omnifluor (4 g/litre). The radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer.

The initial velocity of the catecholamine influx was calculated from the formula $v_0 = a_0/t_1 \ln a_1/a_0$ where t_1 is the time (min) after the start of the experiment, a_0 is the radioactivity (c.p.m.) in the medium at zero time, a_1 is the radioactivity (c.p.m.) in the medium at t_1 and v_0 represents the rate of decrease of the radioactivity (c.p.m.) in the medium. The catecholamine influx was obtained by multiplying v_0 by the specific radioactivity of adrenaline present in the medium. The average of three successive values obtained after 5, 10 and 15 min (at t_5 , t_{10} and t_{15}) was taken as the rate of influx. The release of catecholamine from the vesicles was measured as the increase of catecholamine in the supernatant. The average of three successive values of the first 15 min of the experiment was taken as the release rate. The efflux represents the total outward flux of catecholamine across the vesicular membrane and, hence, is the sum of the release rate and the influx rate measured by the radioactivity procedure.

Determination of the adenosine triphosphatase activity

The medium for measurement of the adenosine triphosphatase activity of the vesicular preparation

was the same as described for the flux experiments except, that $(\pm)\text{-}[^{14}\text{C}]\text{adrenaline}$ was omitted. Measurement of enzyme activity was always carried out with the same vesicular preparation in parallel with the flux experiments. The reactions were terminated after appropriate time-intervals by the addition of 1 ml of 10% (w/v) trichloroacetic acid to 3 ml of the assay. P_i was measured by the method of Rockstein & Herron (1951). The rate of hydrolysis of ATP was determined from the average of three successive measurements (at t_5 , t_{10} , t_{15}) in the first 15 min of the experiment.

Chemicals

ATP (disodium salt) was obtained from Pharma Waldhof A.G., 68 Mannheim, Germany; $(\pm)\text{-}[\text{carbinol-}^{14}\text{C}]\text{adrenaline bitartrate}$ (specific radioactivity 37 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; Omnifluor [98% 2,5-diphenyloxazole + 2% *p*-bis(*o*-methylstyryl)benzene] was obtained from Merck, Darmstadt, Germany.

Results

Catecholamine fluxes and adenosine triphosphatase activity in media containing halogen ions

The results are summarized in Table 1. In the absence of ATP the catecholamine influx was equally slow, independent of the anion present in the media. In contrast, the anions exerted distinctly different effects on the catecholamine efflux. It increased in the following sequence: $\text{F}^- < \text{Cl}^- < \text{Br}^- < \text{I}^-$. The ratio of influx/efflux obtained varied only from 0.1–0.2.

Table 1. *Influence of iso-osmotic halogen salt solutions on catecholamine fluxes and adenosine triphosphatase activity*

The experiments were carried out at 31°C with constant stirring in either the absence or the presence of 5 mM-ATP (final concentration). In all experiments the Mg^{2+} final concentration was 5 mM. The reaction mixture (vol. 10 ml) was buffered with 50 mM-sodium glycerophosphate to pH 7.4. The concentration of protein was 0.6–1.1 mg/ml of mixture. Further experimental details are given in the legend of Fig. 1. The results are given as means $\pm$ S.E.M. of five experiments.

Catecholamine fluxes (nmol/min per mg of protein)					Adenosine triphosphatase activity (nmol of P _i /min per mg of protein)	Transport ratio
Medium	Influx	Release	Efflux	Ratio of influx/efflux		
ATP absent						
KF	1.44 ± 0.11	4.84 ± 0.45	6.28 ± 0.86	0.23		
KCl	1.32 ± 0.04	6.47 ± 0.30	7.79 ± 0.59	0.17		
KBr	1.17 ± 0.04	9.32 ± 0.75	10.49 ± 1.37	0.11		
KI	1.22 ± 0.06	11.84 ± 0.80	13.06 ± 1.47	0.09		
ATP present						
KF	4.04 ± 0.42	3.02 ± 0.23	7.05 ± 0.83	0.57	14.57 ± 1.58	0.28
KCl	9.32 ± 0.51	5.96 ± 0.67	15.28 ± 1.56	0.61	28.78 ± 1.08	0.32
KBr	10.19 ± 0.49	18.67 ± 2.70	28.86 ± 2.47	0.35	30.14 ± 1.15	0.34
KI	5.06 ± 0.19	29.50 ± 1.56	34.55 ± 3.13	0.15	18.83 ± 0.65	0.27

In the presence of ATP the sequence of the halogen ions in which the catecholamine efflux increased was the same as in the absence of ATP. However, with the exception of the media containing F^- ions, in which the catecholamine efflux was not affected by ATP, ATP enhanced the catecholamine efflux by a factor of 2 with Cl^- to 2.7 with Br^- and I^- . The catecholamine influx occurring in the presence of ATP in the halogen ion-containing media can be divided into two groups. In the media containing Cl^- or Br^- , the ATP-dependent catecholamine influx was about 7–9 times faster than in the absence of ATP. In the media containing F^- and I^- ATP accelerated the influx only three- to four-fold.

In all halogen ion-containing media the efflux exceeded the influx, resulting in catecholamine release. This is expressed by the ratio of influx/efflux,

which is lowest in the media containing I^- and highest in the media containing Cl^- . The results presented in Table 1 and Fig. 1 show that the hydrolysis of added ATP by the catecholamine vesicles suspended in media containing halogen salts is linearly correlated to the influx. The transport ratio, catecholamine influx/ATP hydrolysed, obtained in this media is about 0.3. No correlation, however, exists between the adenosine triphosphatase activity and the catecholamine efflux.

Catecholamine fluxes and adenosine triphosphatase activity in media containing sodium acetate, sodium trichloroacetate and potassium thiocyanate

The fluxes of catecholamine and the adenosine triphosphatase activity observed in the three media

Fig. 1. Rates of catecholamine influx and efflux corresponding to rates of ATP hydrolysis obtained in media of iso-osmotic solutions of potassium halogen salts and sodium acetate salts

The experiments were carried out at 31°C in the presence of 5 mM-ATP and 5 mM- Mg^{2+} , buffered to pH 7.4 with 50 mM-sodium glycerophosphate (final concentrations) in a volume of 10 ml. The experiments were started with the addition of the vesicular suspension to the mixture giving a final protein concentration of 0.6–1.1 mg/ml. The flux experiments were terminated by cooling 2 ml samples taken after various time-intervals and subsequently centrifuging them at 20000g. The experiments measuring hydrolysis of ATP were terminated by precipitation of 3 ml samples with 1 ml of 10% (w/v) trichloroacetic acid. The rates of influx (●) and efflux (○) (nmol of catecholamine/min per mg of protein) and ATP hydrolysis (nmol of P_i /min per mg of protein) were calculated by taking three successive values during the first 15 min of the experiments. Values are means of five experiments. The S.E.M. values are given in Tables 1 and 2.

Table 2. *Influence of iso-osmotic solutions of sodium acetate, sodium trichloroacetate and potassium thiocyanate on catecholamine fluxes and adenosine triphosphatase activity*

The experimental conditions are described in the legends of Table 1 and Fig. 1. The results are given as means $\pm$ S.E.M. of four experiments.

Medium	Catecholamine fluxes (nmol/min per mg of protein)				Adenosine triphosphatase activity (nmol of P _i /min per mg of protein)	Transport ratio
	Influx	Release	Efflux	Ratio of influx/efflux		
ATP absent						
KSCN	2.29 ± 0.15	44.40 ± 6.97	46.69 ± 6.14	0.05		
CH ₃ CO ₂ Na	1.96 ± 0.15	12.20 ± 2.59	14.17 ± 2.13	0.14		
CCl ₃ CO ₂ Na	2.73 ± 0.20	45.15 ± 6.07	47.88 ± 5.46	0.06		
ATP present						
KSCN	3.96 ± 0.15	60.83 ± 2.45	64.79 ± 3.58	0.06	—	—
CH ₃ CO ₂ Na	15.12 ± 0.72	5.52 ± 0.58	20.64 ± 2.62	0.73	21.83 ± 0.53	0.69
CCl ₃ CO ₂ Na	4.56 ± 0.22	41.67 ± 3.09	46.23 ± 4.64	0.10	9.23 ± 0.32	0.49

are presented in Table 2. The catecholamine influxes in the absence of ATP are about twice as fast as those obtained in the halogen ion-containing media. The corresponding catecholamine efflux observed in the acetate medium is as fast as in the presence of iodide and is strongly enhanced if trichloroacetate or thiocyanate are present. The ratio of influx/efflux is therefore very low.

ATP increases the efflux in the media containing acetate and thiocyanate, but not in media containing trichloroacetate, which in that respect resembles F⁻. In the media containing acetate the catecholamine influx is stimulated eightfold by ATP, whereas the efflux is only enhanced by a factor of 1.5. Therefore the ratio of influx/efflux is relatively high, but even in this case the efflux exceeds the influx, resulting in catecholamine release. In the media containing trichloroacetate and thiocyanate ATP stimulated the catecholamine influx only slightly. The influx/efflux ratios obtained in both solutions therefore are as low in the presence of ATP as in its absence.

The adenosine triphosphatase activity of the vesicles suspended in sodium acetate or sodium trichloroacetate changed with the catecholamine influx. The transport ratio, catecholamine influx/ATP hydrolysed, however, was considerably higher than in the halogen ion-containing media. The values obtained in the media containing acetate or trichloroacetate were shifted distinctly to the left (Fig. 1) indicating that the influx is brought about by a much lower ATP consumption than in the halogen salt-containing solutions. No correlations were found between efflux and adenosine triphosphatase activity.

Discussion

The present results clearly demonstrate that the influx and not the release of catecholamine depends

on the energy-consuming reaction of ATP hydrolysis. Indeed, catecholamine release occurs both in the absence and in the presence of ATP in all the media containing univalent anions.

In the absence of ATP the catecholamine release varies by a factor of 10 depending on the anion employed:

This order corresponds to the lyotropic anion series. Since the catecholamine influx in the absence of ATP is slow and remains unaffected by the anions in the medium, only the efflux of catecholamine is changed by the latter resulting in a net release of catecholamine.

The presence of ATP modifies this pattern of catecholamine release in a complex manner: release occurring in the presence of ATP is diminished with CH₃CO₂⁻ as the anion, remains equal with F⁻, Cl⁻ or CCl₃CO₂⁻, or is strongly enhanced with Br⁻, I⁻ or SCN⁻. Since the release is the resultant of catecholamine efflux opposed by the catecholamine influx, considerations based only on the release pattern may lead to wrong conclusions about the mechanism (Taugner & Hasselbach, 1966; Taugner, 1971). Both influx and efflux vary under the influence of the anions in the medium independently from one another. The catecholamine efflux is increased to a certain degree by ATP, but the influence of the anions remains the same as observed in the absence of ATP. In contrast the catecholamine influx changes with the adenosine triphosphatase activity, which is influenced by the anions present in the medium in an order other than that of the lyotropic series. That it is the adenosine triphosphatase activity which is primarily affected by the anions and that the influx directly depends on the adenosine triphosphatase activity is

shown by the correlation found between enzyme activity and catecholamine influx and, further, by the result that in the absence of ATP the catecholamine influx is not influenced by the different media.

These results are difficult to reconcile with the concept that the catecholamine influx is brought about by an ATP-stimulated binding of catecholamine to the storage complex, whose stability depends on the anions. In this case the effect of the anions in the absence of ATP can only be explained by an increase in the rate constants for dissolution; in the presence of ATP the effect of the anions can only be explained if the rate constants for both complex formation and dissolution increased. Even so to account for the results, the pattern in which the rate constants for complex formation were affected by anions would have to be completely different from that for the rate constants for complex dissolution. Such a mechanism is very unlikely.

The efflux is not correlated with the hydrolysis of ATP, although ATP induces enhancement of the catecholamine efflux. However, the anion sequence in which the efflux increases is similar to that in the absence of ATP. This indicates, that the efflux is directly evoked by the anion in the system. This view is supported by the observation of Lishajko (1969), who has shown that the release of catecholamine was proportional to the concentration of Cl^- ions present in the medium and that ADP can replace ATP in its enhancing effect on the catecholamine release.

Several reasons for the different effects of the anions on the catecholamine efflux are conceivable: (1) if the anion acts on the permeability of the vesicular membrane, both catecholamine pools of the adrenal medullary vesicles must be affected. The existence of two different, but interdependent compartments containing catecholamine within the vesicles has been shown (Taugner & Hasselbach, 1966). About 20–25% of the vesicular catecholamine is rapidly exchangeable, whereas the remaining 75–80% of the catecholamine stored within the vesicles is only slowly exchangeable. The latter probably represents the storage complex. Outflow experiments from vesicles loaded with [^{14}C]adrenaline have demonstrated that the catecholamine release occurs first from the smaller, highly radioactively labelled compartment; and subsequently the larger, weakly labelled pool is involved (Taugner & Hasselbach, 1966). Increased permeability of the vesicular membrane, caused by the anions, therefore might rapidly exhaust the smaller pool, leading to disintegration of the complex. Slotkin *et al.* (1971) confirmed the existence of two

catecholamine pools with different rates and capacities for catecholamine uptake. (2) The different effects of the anions could also be brought about by a decrease in the stability of the storage complex, thus enhancing the concentration of the 'free' intravesicular catecholamine and giving rise to a faster efflux. If all anions decrease the stability of the complex equally, the differences could be caused by different velocities of penetration of the anions across the vesicular membrane. A direct graded effect of the anions on the stability of the storage complex also seems possible. In the latter case, however, the membrane should not retard the diffusion of the anions. These considerations are valid for the efflux in the absence as well as in the presence of ATP.

The ATP-induced enhancement of the anion-effected catecholamine efflux, which, as discussed above, is not correlated with either ATP-splitting or the influx, is probably caused by a change in the membrane structure depending on the anion present. The fact that ATP is not incorporated into the core of the vesicles (Kirshner, 1962; Trifaró & Dworkind, 1971) but is bound to membrane structures (G. Taugner, unpublished work) supports this assumption.

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