The Effect of Uncouplers on Catecholamine Incorporation by Vesicles of Chromaffin Granules

By C. LINDSAY BASHFORD, ROBERT P. CASEY, GEORGE K. RADDA and GILLIAN A. RITCHIE
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

(Received 6 February 1975)

It is shown that uncouplers inhibit the incorporation of catecholamines by vesicles of chromaffin granules in parallel with their stimulatory effect on the membrane-bound adenosine triphosphatase.

The catecholamine-storage vesicles of the adrenal medulla (the chromaffin granules) are characterized by their high content of catecholamine (0.5M), nucleotide (0.125M-ATP) and acidic protein (120mg/ml) (Kirshner & Kirshner, 1971). Kirshner (1962) and Carlsson *et al.* (1963) showed that the granules contain a catecholamine pump that requires ATP. Taugner (1971) demonstrated that this pump was localized in the membrane that surrounds the chromaffin granules, and Phillips (1974a) has observed ATP-dependent catecholamine incorporation by chromaffin-granule 'ghosts'. Von Euler & Lishajko (1969) showed that the flux of nor-adrenaline across the membrane of the storage vesicles from splenic nerve is sensitive to a number of metabolic inhibitors and particularly to mitochondrial uncouplers. In the present paper we report that the uncoupler sensitivity of the ATPase* activity of bovine adrenomedullary granules (Bashford *et al.*, 1975) is reflected by changes in the uptake of catecholamines by both intact granules and their 'ghosts' prepared by osmotic lysis.

Chromaffin granules were prepared from bovine adrenal medulla as described by Kirshner (1962) except that the iso-osmotic sucrose (0.3M) was buffered with 10mM-Hepes buffer, pH adjusted to 7.4 with 2M-KOH. The final pellet of such a preparation is contaminated with lysosomes and with mitochondria (Smith & Winkler, 1967), but further purification by density-gradient centrifugation gives rise to preparations of greater fragility and impaired catecholamine-transport activity (Phillips, 1974a). Chromaffin-granule membranes were prepared by lysing the granules by dilution with 10mM-Hepes buffer, pH adjusted to 7.4 with 2M-KOH, and centrifuging the suspension at 48000g for 10min at 4°C in the SS-34 rotor of a Sorvall RC 2B centrifuge. Under these conditions the granule membranes pellet as a loosely packed reddish layer on top of a denser brown layer of mitochondrial membranes, from which they can be separated by gentle swirling with iso-osmotic buffered sucrose as above. The granule

membranes were subjected to further osmotic shock by dilution with 10mM-Hepes buffer, pH 7.4, and were centrifuged as before. After three cycles of this procedure the chromaffin-granule membranes were resuspended in 10mM-Hepes buffer, pH 7.4, and were used within 6h of their preparation. Transport experiments were performed at 37°C in 0.3M-sucrose (buffered as above) (total volume of incubation 0.5ml), by using 100 μ M-DL-[7-¹⁴C]-adrenaline (specific radioactivity 8.25 mCi/mmol), 5mM-ATP, 2.5mM-MgCl₂ and other reagents at the concentration specified. Reactions were started by the addition of the membranes (final concentration approx. 500 μ g of protein/ml) and were terminated by the addition of 1 ml of ice-cold buffered sucrose containing 1.5 μ M-reserpine. The membrane vesicles were collected by centrifugation at 48000g for 10min at 4°C as before. The pellets were washed with buffered sucrose containing reserpine and finally taken up in 1 ml of water. Radioactivity was determined by liquid-scintillation counting in toluene scintillation fluid containing 2,5-diphenyloxazole (5g/litre), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/litre) and 25% (v/v) Triton X-100.

DL-[7-¹⁴C]Adrenaline DL-bitartrate (33 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K., and Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

The incorporation of adrenaline by chromaffin-granule-membrane vesicles is shown in Fig. 1. The uptake requires the presence of ATP and MgCl₂ and reaches a maximum after 20min. This pattern of incorporation is essentially that observed by Phillips (1974b), though he observed maximal incorporation after incubation for 1h. The earlier appearance of maximal adrenaline incorporation in our experiments is probably due to the more rigorous conditions of our membrane-preparation procedure, which may make the vesicles more leaky. The incorporation of adrenaline in the presence of a final concentration of 1 μ M-N-(3-t-butyl-5-chlorosalicyl)-2-chloro-4-nitroanilide (S-13), a potent mitochondrial uncoupler, is also shown in Fig. 1. In this

* Abbreviations: ATPase, adenosine triphosphatase; Hepes, 2-(N-2-hydroxyethyl)piperazin-N'-yl)ethanesulphonic acid.

case the incorporation does not differ significantly from the control experiments in the absence of ATP and MgCl_2 . We can conclude therefore that mitochondrial uncouplers can inhibit the ATP-dependent catecholamine incorporation by chromaffin-granule-membrane vesicles.

Fig. 1. Incorporation of adrenaline by chromaffin-granule vesicles

Incorporation of DL-[7- ^{14}C]adrenaline was determined as described in the text for different times of incubation. The points represent the mean of four different experiments and the bars the standard deviation for each determination. The incorporation was measured in the presence (closed symbols) and absence (open symbols) of $1\text{ }\mu\text{M}$ -*N*-3-*t*-butyl-5-chlorosalicyl)-2-chloro-4-nitroanilide (S-13) and in the presence (□, ■) and absence (○, ●) of 5 mM -ATP and 2.5 mM - MgCl_2 .

Previous experiments have indicated that the ATPase activity of the chromaffin granule is sensitive to a number of perturbations (Bashford *et al.*, 1975). Table 1 summarizes the effects of similar perturbations on the ATP-dependent incorporation of catecholamine in the system described above. At equivalent concentrations the salicylanilide uncoupler (S-13) is the most potent inhibitor of adrenaline incorporation and it was also the most effective stimulator of ATPase activity. Another procedure that perturbs membrane permeability, incubation with NH_4CNS , also inhibits the ability of the membrane vesicles to incorporate adrenaline despite their increased ATPase activity. We have made similar observations with preparations of intact chromaffin granules and these are included in Table 1. Because of the high endogenous concentrations of catecholamine and the leakiness of the membrane it is difficult to interpret these observations quantitatively. As we do not know the specific radioactivity of the adrenaline present in the assay owing to the dilution by the endogenous catecholamine, the results with intact granules are expressed in terms of the c.p.m. incorporated/10 min per μg of protein. The results with intact granules are qualitatively similar to those with membrane vesicles, and the incorporation of exogenous DL-[7- ^{14}C]adrenaline in the presence of ATP and MgCl_2 is inhibited by mitochondrial uncouplers.

We have shown that the ATPase activity of chromaffin-granule membranes is associated with changes in the fluorescence of the extrinsic probe 8-anilidonaphthalene-1-sulphonic acid (Bashford *et al.*, 1975). These changes are reversed by uncouplers

Table 1. Effect of uncouplers on adrenaline incorporation by chromaffin granules and their membrane vesicles

Incorporation of DL-[7- ^{14}C]adrenaline was determined as described in the text. The control contained 0.5 ml of a mixture of buffered sucrose, 5 mM -ATP, 2.5 mM - MgCl_2 , $100\text{ }\mu\text{M}$ -DL-[7- ^{14}C]adrenaline and $250\text{ }\mu\text{g}$ of protein. The incorporation of adrenaline in the absence of ATP and MgCl_2 was determined as the ATP blank. Other experiments included the reagents at the concentrations specified. Incorporation by intact granules is expressed as c.p.m. found in the pellet after a 10 min incubation in the assay medium. Abbreviations: compound S-13, *N*-3-*t*-butyl-5-chlorosalicyl)-2-chloro-4-nitroanilide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Addition	Adrenaline incorporation by chromaffin-granule-membrane vesicles		Adrenaline incorporation by chromaffin granules	
	(pmol/10 min per μg of protein)	(percentage of control)	(c.p.m./10 min per μg of protein)	(percentage of control)
Control	13.2	100	1248	100
$1\text{ }\mu\text{M}$ -Compound S-13	1.0	7.6	101	8.1
$1\text{ }\mu\text{M}$ -Bis-hexafluoroacetone	1.7	13	297	23.8
$1\text{ }\mu\text{M}$ -FCCP	2.3	17.5		
1 mM - NH_4CNS	10.1	76.5		
5 mM - NH_4CNS	2.9	22		
ATP blank	1.2	9		
ATP blank + $1\text{ }\mu\text{M}$ -compound S-13	1.1	8.3		

and other membrane perturbations, with a concomitant enhancement of ATPase activity. The observations described in the present paper extend these findings and indicate the ATP-dependent incorporation of catecholamine is also sensitive to uncouplers and the other changes in membrane permeability. The observation that on addition of uncouplers the ATPase activity of chromaffin-granule membranes is enhanced and adrenaline incorporation is suppressed has important implications for the mechanism of catecholamine incorporation by the vesicles. Since uncouplers are believed to equilibrate protons across or within a membrane the ATPase activity of the chromaffin granule is likely to be associated with the movement of protons and a concomitant development of an electromechanical potential. Catecholamine transport could then be driven by such a potential and could occur by an exchange or co-transport mechanism. In view of the very unfavourable stoichiometry of the process, 250 mol of ATP being hydrolysed for every mol of catechol-

amine incorporated (Phillips, 1974b), it is possible that a more complex mechanism must be sought.

We thank the Science Research Council for financial support. G. A. R. is a recipient of an S.R.C. training award and C. L. B. and R. P. C. are recipients of M.R.C. training awards. C. L. B. thanks Hertford College for a Senior Scholarship.

- Bashford, C. L., Radda, G. K. & Ritchie, G. A. (1975) *FEBS Lett.* **50**, 21–24
Carlsson, A., Hillarp, N.-Å. & Waldeck, B. (1963) *Acta Physiol. Scand.* **59**, Suppl. **215**, 1–38
Kirshner, N. (1962) *J. Biol. Chem.* **237**, 2311–2317
Kirshner, N. & Kirshner, A. G. (1971) *Phil. Trans. Roy. Soc. London Ser. B* **261**, 279–289
Phillips, J. H. (1974a) *Biochem. J.* **144**, 311–318
Phillips, J. H. (1974b) *Biochem. J.* **144**, 319–325
Smith, A. D. & Winkler, H. (1967) *Biochem. J.* **103**, 480–482
Taugner, G. (1971) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **270**, 392–406
Von Euler, U. S. & Lishajko, F. (1969) *Acta Physiol. Scand.* **77**, 298–307