Biochemical Pharmacology, Vol 23, pp 2190–2192 Pergamon Press, 1974 Printed in Great Britain

Drug-resistant effect of adenine nucleotides and magnesium on catecholamine efflux from isolated adrenal medullary storage vesicles

(*Received* 14 *September* 1973; *accepted* 7 *December* 1973)

ADENINE NUCLEOTIDES and divalent cations play vital roles in the uptake, storage and release of the catecholamines of adrenal medullary vesicles. The incorporation of amines into isolated vesicles is stimulated by ATP and magnesium and inhibited by reserpine.[1,2] The intravesicular storage complex probably consists of four molecules of catecholamines combined with one molecule of ATP,[3–7] and ATP and magnesium have also been implicated in the mechanism of catecholamine secretion.[8–11] A question which has attracted some attention is whether ATP and magnesium exert a direct effect upon the rate at which amines leak outward across the vesicle membrane. In 1967, Taugner and Hasselbach[12] reported that ATP and magnesium accelerated the influx of amines into the vesicles but had no effect upon amine efflux; they also found that *N*-ethylmaleimide, which inhibits the ATPase utilized for catecholamine uptake, accelerated efflux. In contrast, Lishajko[13] found that ATP and magnesium both accelerated amine influx and reduced efflux and that reserpine prevented most but not all of the dual effect. Subsequent studies from our laboratory[14,15] showed that uptake inhibitors, such as reserpine or *N*-ethylmaleimide, themselves produced no change in efflux but could prevent completely the effect of ATP and magnesium, suggesting that the apparent reduction in efflux might actually represent re-uptake of amines into the vesicles. Unfortunately, the experiments from the various laboratories involved different species as well as different amine and vesicle concentrations, all of which could have affected the results and interpretations. Specifically, in most studies in which efflux has been measured, concentrations of storage vesicles have been sufficiently high so that the extravesicular amine concentration (from breakage of vesicles during preparation) has generally exceeded 10^{-4} M. Since the K_m of the ATP and magnesium-stimulated amine uptake system is estimated to be 4×10^{-5} M,[16] the addition of ATP and magnesium during efflux always produces re-uptake during these conditions. In the current studies, we have examined amine efflux at low concentrations to determine whether ATP and magnesium exert an effect exclusive of uptake. Isotonic sucrose medium has been utilized to avoid the ATP-Mg^{2+}-stimulated release which occurs in chloride-containing ionic media,[9] a phenomenon which involves all-or-none release of the entire soluble contents of the storage vesicle;[17] in contrast, the efflux observed in isotonic sucrose is a completely different process, representing leakage of catecholamines (but not of soluble proteins) across an intact vesicle membrane.[14]

Male albino Wistar rats (Hilltop), weighing 200–300 g, were sacrificed by decapitation, and the adrenals were homogenized in isotonic sucrose buffered at pH 7·4 with 25 mM *tris*. Iproniazid (10^{-5} M) was added to inhibit monoamine oxidase For each experiment, consisting of four to six efflux curves, glands from 15 to 25 rats were pooled in a total volume of 10 ml. After centrifugation at 800 *g* to remove debris, the vesicle-containing supernatant was added to 1·5 ml of 1 mM epinephrine, 1·5 ml of 50 mM ATP and magnesium, 1 5 ml of ^{14}C-epinephrine (10 μCi/ml) and sucrose-*tris* to a final volume of 15 ml. After incubation at 30° for 30 min, the mixture was centrifuged at 26,000 *g*, and the vesicular pellet was washed and recentrifuged twice with fresh sucrose-*tris* Under these conditions, labeling occurs solely into storage vesicles.[18] The final pellet was resuspended in sucrose-*tris*, and 0·5-ml aliquots, containing a total of approximately 3 μg catecholamines, were added to an equal volume of sucrose-*tris* and used for efflux

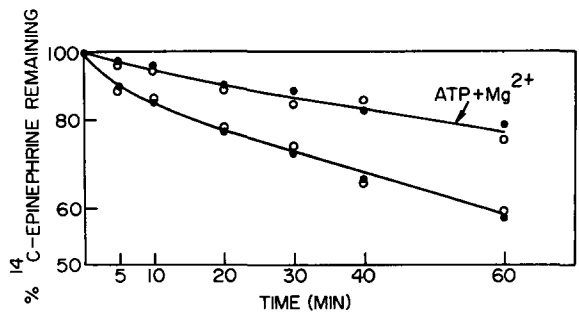

FIG. 1. Efflux of ^{14}C-epinephrine in isotonic sucrose at 30°, in the presence (○) and absence (●) of 0 01 mM reserpine, with and without ATP and magnesium. The ordinate is logarithmic.

determinations at 30 . In some experiments, other agents were present in the efflux mixture: adenine nucleotides (5 mM), magnesium chloride (5 mM), reserpine (0·01 mM), harmine (0·1 mM), epinephrine (0·1 mM) and EDTA (2 mM). In experiments utilizing *N*-ethylmaleimide (0·1 mM), the addition of ATP and magnesium was delayed for 7·5 min to allow the *N*-ethylmaleimide to react with sulfhydryl groups in the vesicle membrane.[12] Each efflux curve consisted of at least seven time points from 0 to 60 min; the detailed methodology has been described previously.[14,15,18] The effluxes of ^{14}C-epinephrine and endogenous catecholamines gave similar results, except that the efflux of endogenous amines could not be measured in experiments where 0·1 mM epinephrine was present. In most experiments, the extravesicular amine concentration averaged about 3×10^{-6} M, and unless epinephrine was added, never exceeded 8×10^{-6} M, thus ensuring that re-uptake would be minimal. The efflux rate varied from preparation to preparation, thus requiring the determination of control curves with and without ATP and magnesium for each set of experiments. Each experiment was performed twice, and the results were in good agreement Data are reported from individual curves.

A typical experiment appears in Fig 1. ATP and magnesium substantially reduced the rate of ^{14}C-epinephrine efflux (half-life of 181 min for late phase vs 100 min without ATP and magnesium). Reserpine in concentrations which produce nearly complete blockade of uptake[1,2,14] had little or no effect on efflux

TABLE 1. EFFECTS OF NUCLEOTIDES, MAGNESIUM AND DRUGS ON THE EFFLUX OF ^{14}C-EPINEPHRINE FROM ADRENAL MEDULLARY VESICLES

	Nucleotide (5 mM)	Magnesium (5 mM)	Other	Half-life of late efflux component (min)
A		−		96
		−	Harmine (0·1 mM)	100
	ATP	+		185
	ATP	+	Harmine	185
B		−		87
		−	Epinephrine (0·1 mM)	95
	ATP	+		228
	ATP	+	Epinephrine	224
C		−		80
		−	EDTA (2 mM)	76
	ATP	+		156
	ATP	−	EDTA	108
		+		81
D		−		55
	ATP	+		110
	ADP	+		75
	AMP	+		57
		−	NEM (0 1 mM)	62
	ATP	+	NEM	122

with or without ATP and magnesium, suggesting that re-uptake was not responsible for the reduced efflux in the presence of ATP and magnesium. Harmine, which also blocks uptake,[16] demonstrated the same lack of effect on efflux as reserpine (Table 1, A). Since these observations were substantially different from those reported previously for other species,[12-15] we repeated the experiments with bovine adrenal medullary vesicles in concentrations equivalent to those in the present studies with rat vesicles and obtained results identical to those reported here. Thus, the differing results[12-15] do not reflect species differences but rather the fact that the higher vesicle concentrations used in earlier studies produced a correspondingly high extravesicular amine concentration; consequently, the addition of ATP and magnesium had previously resulted in re-uptake and the complete or partial masking of the currently observed, reserpine-resistant, direct effect of ATP and magnesium on efflux.

To demonstrate further that the reduced efflux in the presence of ATP and magnesium was independent of the uptake phenomenon, the labeled vesicles were exposed to high (0·1 mM) concentrations of unlabeled epinephrine during efflux of ^{14}C-epinephrine. Since the extravesicular specific activity is, therefore, very

low, very little re-uptake of labeled epinephrine should take place. If re-uptake is responsible for the effect of ATP and magnesium, then the apparent reduction in efflux of ^{14}C-epinephrine should disappear. As shown in Table 1 (B), the unlabeled epinephrine did not alter the actions of ATP and magnesium, confirming the hypothesis that the effect is direct and unrelated to uptake.

Both ATP and magnesium are required in the stimulated uptake system, and ADP is totally ineffective in stimulation of amine uptake.[1] In contrast, a partial reduction in amine efflux was observed with ATP alone (with EDTA added to chelate endogenous magnesium) as well as with ADP and magnesium (Table 1, C and D); AMP and magnesium did not reduce efflux, nor did magnesium alone. These data suggested that the membrane ATPase, which requires magnesium[19] and utilizes ATP, might not be involved in the reduction of efflux. To test this hypothesis, the actions of *N*-ethylmaleimide (NEM) were studied. Although this agent blocks amine uptake and inhibits the vesicle membrane ATPase by about 50 per cent,[19,20] it did not alter efflux either in the presence or absence of ATP and magnesium (Table 1, D). The apparent lack of dependence on the ATPase again differentiates the effect of ATP and magnesium on efflux from that on uptake.

It is interesting to note that the data reported here parallel to a great extent observations that adenine nucleotides, divalent cations and reserpine decrease the binding of catecholamines to purified adrenal storage vesicle membranes.[21] The similarities include: partial activity of ATP without magnesium, lack of effect of magnesium alone, partial activity of ADP, lack of effect of AMP, failure of uptake inhibitors to alter the actions of ATP and magnesium, and lack of dependence on the ATPase. These data suggest that the efflux of catecholamines from the vesicles is not a simple diffusion process, but rather may require the interaction of the amine with a specific component of the vesicle membrane, and that the reduction in amine efflux produced by the addition of ATP and magnesium may result from interference with this interaction.

In conclusion, these studies demonstrate that ATP and magnesium reduce amine efflux by a mechanism which is independent of amine uptake into the vesicles and is not blocked by drugs which inhibit uptake. This effect may play a role in the stability of catecholamine storage.

Acknowledgements—Supported by USPHS DA-00465-01, the Council for Tobacco Research—USA, and by grants-in-aid from the American Heart Association and the North Carolina Heart Association.

Department of Physiology and Pharmacology,
Duke University Medical Center,
Durham, N.C. 27710, U.S.A.

THEODORE A. SLOTKIN*
HANNAH O. GREEN

REFERENCES

1. N. KIRSHNER, *J. biol. Chem.* **237,** 2311 (1962).
2. A. CARLSSON, N.-Å. HILLARP and B. WALDECK, *Medna. exp.* **6,** 47 (1962).
3. N.-Å. HILLARP, *Acta physiol. scand.* **42,** 321 (1958).
4. N.-Å. HILLARP, *Acta physiol. scand.* **47,** 271 (1959).
5. N.-Å. HILLARP, *Acta physiol. scand.* **50,** 8 (1960).
6. K. H. BERNEIS, A. PLETSCHER and M. DAPRADA, *Nature, Lond.* **224,** 281 (1969).
7. K. H. BERNEIS, A. PLETSCHER and M. DAPRADA, *Br. J. Pharmac. Chemother.* **39,** 382 (1970).
8. M. OKA, T. OHUCHI, H. YOSHIDA and R. IMAIZUMI, *Life Sci.* **5,** 433 (1966).
9. A. M. POISNER and J. M. TRIFARÓ, *Molec. Pharmac.* **3,** 561 (1967).
10. R. M. FERRIS, O. H. VIVEROS and N. KIRSHNER, *Biochem. Pharmac.* **19,** 505 (1970).
11. F. LISHAJKO, *Acta physiol. scand.* (Suppl.), 362 (1971).
12. G. TAUGNER and W. HASSELBACH, *Biochem. J.* **102,** 22 (1967).
13. F. LISHAJKO, *Acta physiol. scand.* **76,** 159 (1969).
14. T. A. SLOTKIN, R. M. FERRIS and N. KIRSHNER, *Molec. Pharmac.* **7,** 308 (1971)
15. T. A. SLOTKIN and N. KIRSHNER, *Molec. Pharmac.* **7,** 581 (1971).
16. H. O. GREEN and T. A. SLOTKIN, *Molec. Pharmac.*, **9,** 748 (1973).
17. R. M FERRIS, O. H. VIVEROS and N. KIRSHNER, *Biochem. Pharmac.* **19,** 505 (1970).
18. T A. SLOTKIN and N. KIRSHNER, *Biochem. Pharmac.* **22,** 205 (1973).
19. N. KIRSHNER, in *Pharmacology of Cholinergic and Adrenergic Transmission* (Eds. G. B. KOELLE, W. W. DOUGLAS and A. CARLSSON), p. 225. Czechoslovak Medical Press, Prague (1965).
20. J. M. TRIFARÓ and J. DWORKIND, *Molec. Pharmac.* **7,** 52 (1971).
21. T. A. SLOTKIN and N. KIRSHNER, *Biochem. Pharmac.* **22,** 2492 (1973).

* Recipient of Faculty Development Award in Pharmacology from the Pharmaceutical Manufacturers Association Foundation.