The Role of Adenosine Triphosphate and Adenosine Triphosphatase in the Release of Catecholamines from the Adrenal Medulla

III. Similarities between the Effects of Adenosine Triphosphate on Chromaffin Granules and on Mitochondria

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SUMMARY

Further studies on the effect of ATP on chromaffin granules isolated from bovine adrenal medullae have been carried out. Granules isolated by density gradient centrifugation in an isotonic medium respond to ATP in the same way as do those isolated by Millipore filtration: release of catecholamines and fall in optical density. The effect of ATP was present when the KCl in the incubation medium was replaced by LiCl or choline chloride, and when the $MgCl_2$ was replaced by $CoCl_2$. ITP also caused the release of catecholamines but was less active than ATP. Bovine serum albumin caused a slight potentiation of the effects of ATP. The response to ATP was reduced as the pH was raised above 7.0. These effects are discussed in relation to the effects of ATP on mitochondria.

INTRODUCTION

It has been shown that ATP acts on chromaffin granules from the adrenal medulla, causing the release of soluble constituents (catecholamines, ATP, and protein) and that this process is accompanied by structural changes (1–4). During analyses of this action of ATP, certain similarities between its effects on chromaffin granules and on mitochondria were noted (1, 2). This paper reports further findings on the action of ATP on chromaffin granules and extends the comparison with mitochondrial contraction.

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METHODS

Preparation of chromaffin granules. Chromaffin granules were prepared from bovine adrenal medullae as previously described (1). This procedure involves Millipore filtration and differential centrifugation. In addition, some chromaffin granule preparations were obtained by density gradient centrifugation using a Ficoll–sucrose–D_2O gradient as follows. Adrenal medullae were homogenized in 5 volumes of 0.27 M sucrose and centrifuged for 10 min at $1200 \times g$. The supernatant fraction was then centrifuged for 10 min at $24{,}200 \times g$. The loose, upper layer of the sediment was washed away by swirling with 0.27 M sucrose, and the pellet was then suspended in 0.27 M sucrose and layered over a solution of Ficoll and sucrose in D_2O (final sp.

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gr. 1.205). An aliquot (2.0 ml) of the granule suspension was layered over 7.5 ml of the Ficoll–sucrose solution, which was composed of 0.27 M sucrose and 19.5% Ficoll, (w/v) dissolved in pure D_2O. The tube was centrifuged in the No. 50 angle rotor in a Beckman model L ultracentrifuge for 60 min at $110{,}000 \times g$. The tube was then inverted and drained, the inside of the tube was wiped dry, and the pellet was washed by swirling with 0.3 M sucrose. Finally, the pellet was resuspended in 0.3 M sucrose.

Incubation of granules for release of catecholamine, and light-scattering measurement. To test the effects of ATP on release of catecholamine and on optical density, granules were incubated as described previously (1, 2). The specific media will be described under RESULTS. Catecholamines were assayed by the method of Anton and Sayre (5).

Chemicals and reagents. ATP and ITP were obtained from Boehringer-Mannheim, New York; TES buffer [N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid], from Calbiochem; Ficoll, from Pharmacia; D_2O from New England Nuclear; and bovine serum albumin, from Sigma Chemical Company.

RESULTS

Effect of ATP on release of catecholamine and light scattering of chromaffin granules prepared by Ficoll–sucrose–D_2O density gradient. Previous reports on the effects of ATP on chromaffin granules have described results using granules obtained by Millipore filtration. Such granules have a small degree of mitochondrial contamination (1, 6). Purer granules can be obtained by density gradient centrifugation (7, 8), but the hypertonic sucrose employed has two disadvantages. First, isotonic sucrose inhibits the ATP-induced effects on chromaffin granules (1, 2). Second, if hypertonic sucrose is replaced by isotonic media, the chromaffin granules lyse (9). Because of these considerations, a Ficoll–sucrose–D_2O gradient was employed that has a density equivalent to about 1.6 M sucrose, but a tonicity comparable to 0.3 M sucrose. The degree of mitochondrial contamination, using monoamine oxidase as

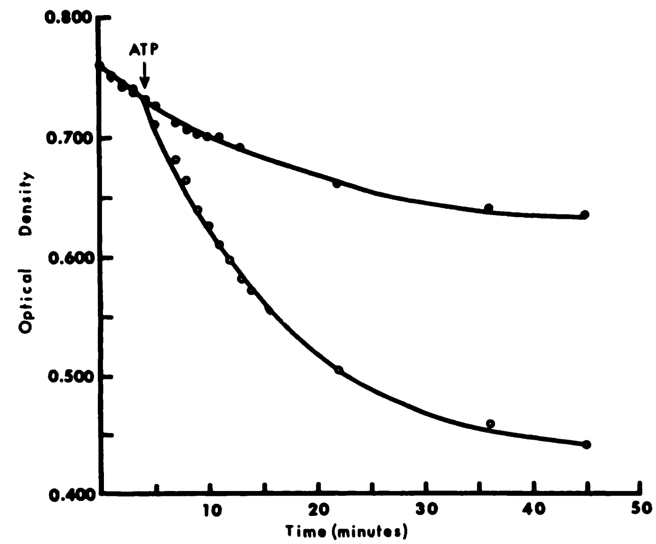

FIG. 1. ***Effect of ATP on optical density of chromaffin granules isolated by Ficoll–sucrose–D_2O density gradient centrifugation***

Two aliquots of chromaffin granules were incubated at room temperature in a medium containing KCl, 160 mM; NaCl, 5 mM; potassium phosphate buffer (pH 7.0), 10 mM; $MgCl_2$, 0.5 mM; and EDTA, 0.05 mM. Optical density at 540 mμ was followed for 45 min. At the arrow, 0.5 mM ATP was added to one aliquot (O).

a mitochondrial marker, was less in the granules prepared with the Ficoll–sucrose–D_2O gradient than in those prepared by Millipore filtration: the former contained 0.016 ± 0.003 and the latter 0.161 ± 0.004 unit/mg of protein.[1]

Granules prepared in this way could be diluted in the incubation media so that the sucrose concentration was negligible and there was no decrease in tonicity.

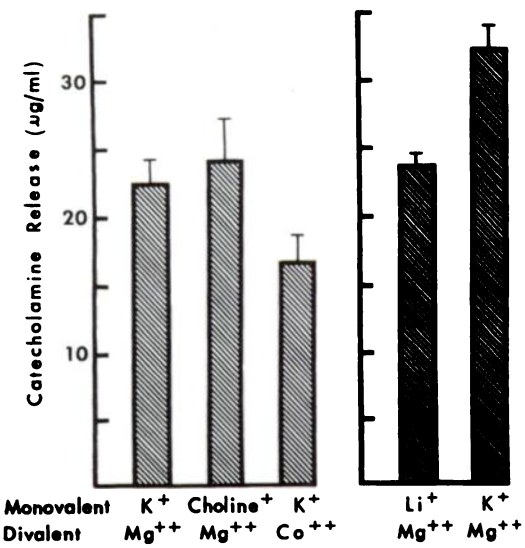

FIG. 2. *Effect of various cations on ATP-induced release of catecholamines from isolated chromaffin granules*

Chromaffin granules (isolated by Millipore filtration in this and all following figures) were incubated for 40 min at 30° in a medium containing potassium phosphate buffer (pH 7.0), 10 mM; ATP, 0.5 mM; and monovalent cation (165 mM) and/divalent cation (0.5 mM) as indicated. The results of two experiments done in triplicate are illustrated. Catecholamine release is expressed as micrograms released per milliliter above that of control tubes incubated without ATP.

When ATP was added to chromaffin granules prepared in this fashion, catecholamine release occurred and it was associated with a fall in optical density (Fig. 1). In an experiment in which these granules were incubated in triplicate for 10 min at 30° (after a 15-min incubation) in the medium described in Fig. 1, the mean ($\pm$ standard error) release of catecholamines was $0.5 \pm 0.4\%$ of the bound hormones; in the presence of ATP (0.5 mM) the release increased to $23.4 \pm 1.8\%$. These chromaffin granules thus behave in the same way as those isolated by the Millipore technique.

Effect of various monovalent and divalent cations on ATP-induced catecholamine release. Previous experiments indicated that the ATP-induced release of catecholamines proceeded equally well in a medium containing either Na^+ or K^+ as the predominant monovalent cation (1). In order to determine whether there were any specific requirements for the cations, experiments were carried out in which other chloride salts replaced the KCl usually employed. ATP produced its characteristic release of catecholamines when the predominant cation in the incubation medium was either choline or lithium (Fig. 2). Earlier examination of divalent ion requirements indicated that Mg^{++} or Mn^{++}

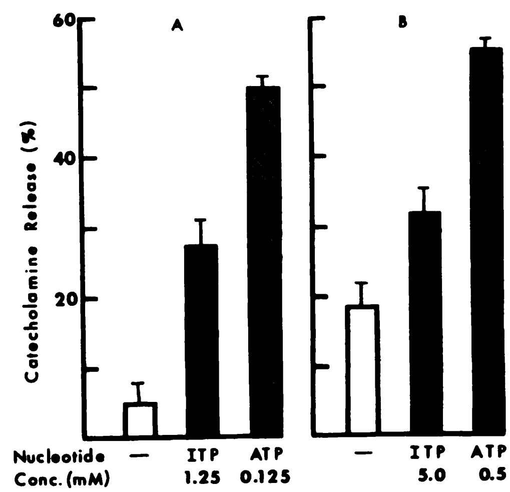

FIG. 3. *Effect of ATP and ITP on release of catecholamines from isolated chromaffin granules*

After a 10-min incubation, chromaffin granules were incubated at 30° for 30 min (A) or 10 min (B) in a medium of the following composition: KCl, 160 mM; NaCl, 5 mM; TES buffer (pH 7.0), 10 mM; and $MgCl_2$, 5.0 mM. ATP or ITP (at the concentrations indicated) was added at the end of the first incubation period. Release of catecholamines is expressed as a percentage of bound amines. Two preparations of chromaffin granules were used.

[1] J. Dworkind, manuscript in preparation.

could support the ATP-induced release of catecholamines, but that Ca^{++}, Ba^{++}, or Sr^{++} could not. To test another nonalkaline earth ion, cobaltous chloride was used. Figure 2 shows that cobalt also can support the effect of ATP on chromaffin granules although it is less effective than Mg^{++}.

Effect of ITP on catecholamine release. Earlier experiments showed that while ATP causes catecholamine release from chromaffin granules, this property is not shared by CTP and AMP (1). In the present study, ITP proved to be less than 10% as effective as ATP (Fig. 3).

Effect of bovine serum albumin on ATP-induced release of catecholamines. In two experiments, albumin (2 mg/ml) caused a small decrease in the spontaneous rate of release of catecholamine, and a small increase in the ATP-evoked release (Fig. 4). The net evoked release (ATP-treated minus control) was 1.5 and 2.0 times as much in the granules exposed to albumin as in those in the regular medium.

Effect of variation in pH levels on ATP-induced release of catecholamines. The effect of ATP on catecholamine release was examined at various pH values from 6.2 to 7.8. The spontaneous release of catecholamines did not vary much between these values although a slight tendency toward increased release was observed as the pH was raised (Fig. 5). However, the ATP-induced release of catecholamines showed a marked decrease as the pH was raised above 7.0 (Fig. 5). Since many workers have incubated chromaffin granules at pH 7.4, this may offer another reason why the effect of ATP was not observed by previous workers (see also ref. 1).

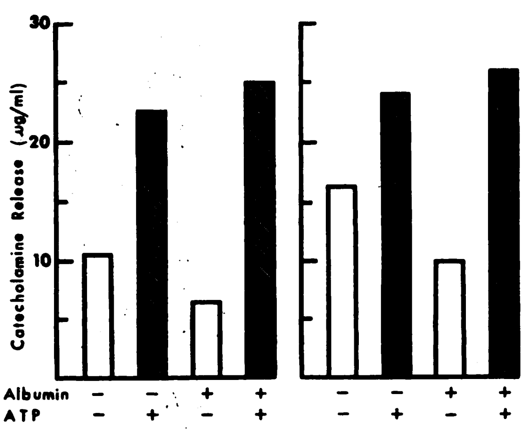

FIG. 4. *Effect of bovine serum albumin on ATP-induced release of catecholamines from isolated chromaffin granules*

Isolated chromaffin granules were incubated for 10 min at 30° in a medium containing KCl, 160 mM; NaCl, 5 mM; TES buffer (pH 7.0), 10 mM; $MgCl_2$, 0.5 mM; and EDTA, 0.05 mM. The concentrations of albumin and ATP, when present, were 2 mg/ml and 0.5 mM, respectively. Catecholamine release is expressed as micrograms of bound amine released per milliliter. Two separate experiments are illustrated.

DISCUSSION

The present experiments have extended previous observations on the effects of ATP on chromaffin granules. Granules prepared by a new method, density gradient centrifugation on an isotonic medium, respond to ATP in the same way as do granules obtained by Millipore filtration, even though the principles of separation by the two methods differ. Preparations obtained by isotonic density gradient centrifugation may prove useful for the study of subcellular particles that cannot withstand the shock of transfer from hypertonic to isotonic media.

The experiments describing the effects of the chemical environment on the ATP-induced release of catecholamines point out the similarities between these effects and those of ATP on mitochondria (see refs. 1 and 2). Studies on the effects of cations have now shown that a wide variety of monovalent cations—Na^+, K^+, Li^+, or choline—are sufficient to support the effect of ATP on chromaffin granules. This lack of specificity is also characteristic of the effects of ATP on mitochondria (10). Of the divalent cations tested so far, Mn^{++} was slightly superior to Mg^{++} (1), while cobalt was less effective. Ca^{++}, Ba^{++}, and Sr^{++} were reported earlier to be ineffective (1). On mitochondria, Mn^{++} has also been reported to be slightly better than Mg^{++} in supporting contraction induced by ATP (11).

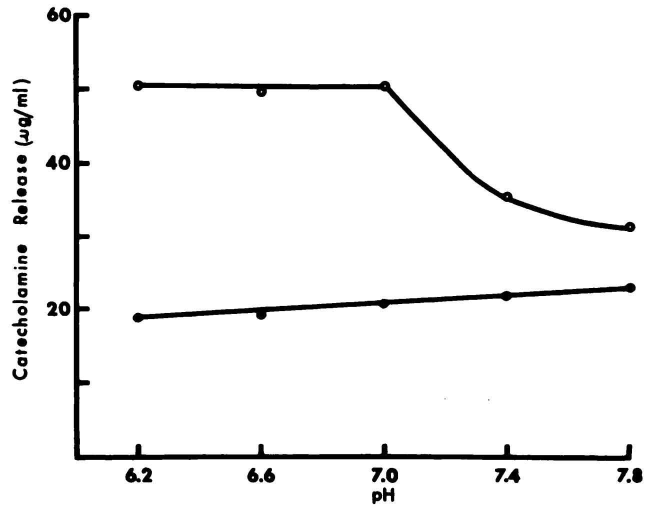

FIG. 5. *Effect of pH on spontaneous and ATP-induced release of catecholamines from isolated chromaffin granules*

Chromaffin granules were incubated for 30 min at 30° in medium containing KCl, 160 mM; $MgCl_2$, 0.5 mM; and potassium phosphate buffer (at various pH values), 10 mM. Catecholamine release in the absence (●) or presence (○) of ATP, 0.5 mM, is expressed as micrograms of bound amine per milliliter. The means of duplicate experiments are shown.

Inosine triphosphate has been reported to be effective by some (12), but ineffective by other workers (10), in producing an effect comparable to ATP on mitochondria. On chromaffin granules, ITP behaved like ATP, but it was less than 10% as active. ITP has been shown to act like ATP in supporting accumulation of catecholamines by chromaffin granules (13).

Serum albumin potentiates the action of ATP on mitochondria (10). The mechanism of action is not entirely understood but may be related to the binding of lipids. Albumin showed only a slight tendency to potentiate the action of ATP on chromaffin granules, and further experiments will be necessary to determine optimal concentrations and the mechanism of action. It should be noted that chromaffin granules on exposure to albumin do not lyse, as do zymogen granules prepared from the parotid gland (14) or from the pancreas.[2] Perhaps this difference reflects variations in the chemical composition of the membranes of chromaffin granules and those of zymogen granules. Thus, chromaffin granules are known to be rich in lysolecithin (15, 16).

The effects of pH on the ATP-induced release of catecholamines probably involve a complex set of interactions, since variations in pH levels could alter (a) rates of enzymatic reactions, (b) ionization of charged groups on the granule membrane, and hence its structural properties, or (c) stability of the intragranular catecholamine storage complex. It is of interest that the spontaneous leakage of catecholamines responded in the opposite way to increased pH from that of the ATP-induced release. This finding suggests that the heightened response to ATP from pH 7.0 down to 6.2 does not result from a greater lability of the granules in this pH range.

These studies have extended the list of similarities noted previously (1, 2) between ATP-induced effects on mitochondria and on chromaffin granules. Studies in progress in our laboratory show further similarities (ATP-induced volume changes and extractability of granule ATPase by hypertonic KCl). The results reported here strengthen our earlier suggestion that the effects of ATP on mitochondria and on

[2] Unpublished observations.

chromaffin granules may reflect a similar phenomenon, and that this is contractile in nature (1, 2).

Studies on the actions of ATP on secretory granules from other sources (17, 18) indicate that this phenomenon is not restricted to chromaffin granules, but rather may be a general mechanism for extrusion of secretory substances.

REFERENCES

1. A. M. Poisner and J. M. Trifaró, *Mol. Pharmacol.* **3**, 561 (1967).
2. J. M. Trifaró and A. M. Poisner, *Mol. Pharmacol.* **3**, 572 (1967).
3. A. M. Poisner and J. M. Trifaró, *Mol. Pharmacol.* **4**, 196 (1968).
4. M. Oka, T. Ohuchi, H. Yoshida and R. Imaizumi, *Jap. J. Pharmacol.* **17**, 199 (1967).
5. A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.* **138**, 360 (1962).
6. M. Oka, T. Ohuchi, H. Yoshida and R. Imaizumi, *Life Sci.* **5**, 427 (1966).
7. P. Banks, *Biochem. J.* **95**, 490 (1965).
8. A. D. Smith and H. Winkler, *Biochem. J.* **103**, 480 (1967).
9. N.-Å. Hillarp and B. Nilson, *Acta Physiol. Scand.* **31**, Suppl. 113, 79 (1954).
10. A. L. Lehninger, *Physiol. Rev.* **42**, 467 (1962).
11. A. L. Lehninger, *J. Biol. Chem.* **234**, 2465 (1959).
12. J. B. Chappell and S. V. Perry, *Nature* **173**, 1094 (1954).
13. A. Carlsson, N.-Å. Hillarp and B. Waldeck, *Med. Exp.* **6**, 47 (1962).
14. M. Schramm, B. Eisenkraft and E. Barkai, *Biochim. Biophys. Acta* **135**, 44 (1967).
15. H. Blaschko, H. Firemark, A. D. Smith and H. Winkler, *Biochem. J.* **104**, 545 (1967).
16. W. W. Douglas, A. M. Poisner and J. M. Trifaró, *Life Sci.* **5**, 809 (1966).
17. A. M. Poisner and W. W. Douglas, *Mol. Pharmacol.* **4**, 531 (1968).
18. A. M. Poisner, W. W. Douglas and R. Poisner, *Pharmacologist* **10**, 199 (1968).