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ATP-STIMULATED TRANSMITTER RELEASE AND CYCLIC AMP SYNTHESIS IN ISOLATED CHROMAFFIN GRANULES

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ATP stimulates chromaffin granules from the bovine adrenal medulla to release epinephrine and specific soluble proteins. ATP analogs substituted in the β-γ position with either nitrogen or carbon were also found to be effective at inducing release from isolated chromaffin granules. However, an ATP analog substituted at the α-β position with carbon was strongly inhibitory. Cyclic AMP was also found to be synthesized by isolated chromaffin granules under release conditions. ATP analogs were effective as substrates for adenylate cyclase in the same order as their efficiency for inducing release from vesicles. Hydrolysis at the β-γ linkage of ATP therefore is probably not necessary for release; however, hydrolysis at the α-β position may be important in the release process. Cyclic AMP may be produced and play a regulatory role in this event.

INTRODUCTION

The release of most hormones and neurotransmitters occurs by the process of exocytosis. Fusion of vesicle and cell membranes is believed to occur, and secretory products within the vesicle are released (1). The evidence is mainly from electron microscopy (2). However, there is little chemical information available on the regulation of this process.

Chromaffin granules (secretory vesicles) from the bovine adrenal medulla can be induced to release epinephrine, chromogranin A and dopamine-β-hydroxylase in response to the addition of ATP and other ions (3–5). This may be a useful model system for studying the molecular basis of the regulation of secretion from whole cells.

Previous workers have shown that ATP has other functions in the chromaffin granule system besides stimulation of release. For example, it also appears to mediate an uptake mechanism for catecholamines (6–8). A Mg^{++}-ATPase in the chromaffin granule membrane is thought by some investigators to couple ATP to uptake (8). The same Mg^{++}-ATPase has been implicated in release (4). Therefore, the relationship between ATP, uptake, and release is somewhat complicated.

We have approached this problem by studying ATP-stimulated release with various ATP analogs. These include: β-γ-imino-adenosine 5′-triphosphate (AMP-PNP); β-γ-methylene-adenosine 5′-triphosphate (AMP-CPP); and α-β-methylene-adenosine 5′-triphosphate (AMP-CPP). Our results show that uptake and release are distinct processes. Uptake requires an intact β-γ-oxygen in the ATP molecule while release requires an intact oxygen at the α-β position.

METHODS

Chromaffin granules were isolated from fresh bovine adrenal glands and purified by differential centrifugation in 0.3 M sucrose (8). Intact granules (0.1–1.0 mg protein) were incubated in a mixture containing 1 mM magnesium acetate, 90 mM choline chloride, 50 mM Na-HEPES buffer (pH 6.0), 100 mM sucrose, and variable amounts of ATP or ATP analogs. Tracer amounts of L-epinephrine (^{3}H) were added to study uptake. Following incubation at 37°C for 10 min, 2 ml of ice-cold 0.3 M sucrose were added. The whole mixture was then centrifuged for 30 min at 20,000 × g. The supernatant pellets were analyzed for epinephrine by the trihydroxyindole reaction (10). Protein was determined by the method of Lowry (10), using bovine serum albumin as a standard. ATPase activity was determined using release of ^{32}P from δ-^{32}P. ATP cyclic AMP was measured by a competitive protein-binding assay (cAMP kit from Amersham-Searle) using internal standards. Adenylate cyclase was also measured by the method of Salomon et al. (11). Nucleotide analogs were obtained from I.C.N. or Miles (Elkhart, Indiana).

RESULTS

Release of protein and epinephrine was found to be linear for up to 30 min at appropriate ATP concentrations. The initial rate of release was found to be a saturable function of ATP concentration with a $K_{½}$ of 0.22 mM. Under optimal conditions all of the transmitter and soluble protein could be released in approximately 10 min.

As summarized in Table I, AMP-PNP and AMP-PCP also catalyzed the release process. However, under conditions optimal for ATP-stimulated release, AMP-PNP and AMP-PCP were less effective. In a separate study, it was found that the $K_{½}$ for AMP-PNP and AMP-PCP were higher by an order of magnitude for the release process than was ATP. At 5 mM concentrations, AMP-PNP was much more active than AMP-PCP. These differences may be related to substantial differences in the pK_a of the different analogs (12).

AMP-PNP and AMP-PCP did not support epinephrine uptake by granules. In addition, they proved to be efficient inhibitors of granule ATPase activity. As noted earlier, uptake is believed to be dependent on the ATPase. Thus, these data suggest that hydrolysis at the β-γ of oxygen of ATP is required for uptake, but it is not necessarily required for ATP-stimulated release.

To test the necessity of an intact α-β oxygen in ATP for release, AMP-CPP was tested. However, as shown in Table I, the result was ambiguous. AMP-CPP did not support release. However, this analog also did not support uptake, and it substantially inhibited

TABLE I. Effect of ATP Analogs on Release, Uptake, and ATPase Activity

Adenosine nucleotide analog[1]	% release	% uptake	Influence on ATPase
ATP	100[2]	100[3]	substrate
AMP-PNP	30	0	inhibitor[4]
AMP-PCP	20	0	inhibitor[4]
AMPCPOP	0	0	inhibitor[4]

[1] 1 mM Mg^{++}-nucleotide.
[2] 160 nM epinephrine/mg protein/min.
[3] 20 nM ^{3}H-epinephrine/mg protein/min in presence of 10^{-5}M epinephrine.
[4] 1 mM ATP analog in the presence of 0.5 mM Mg-ATP.

ATPase activity. Therefore, the importance of the α-β oxygen for release could not be tested directly using ATP analogs.

An alternative approach to this question was to test for products of hydrolysis of the ATP α-β oxygen bond, such as cyclic AMP. Purified granules were examined for their capacity to synthesize cyclic AMP in the presence of ATP and ATP analogs. As shown in Fig. 1, isolated chromaffin granules were able to synthesize cyclic AMP in the presence of exogenous ATP. Chloride appeared to have a slight stimulatory effect. Endogenous synthesis of cAMP was small. This implied that the ATP inside the granule (estimated concentration ~ 0.12 M) is not available for the reaction. As summarized in Table II, granules

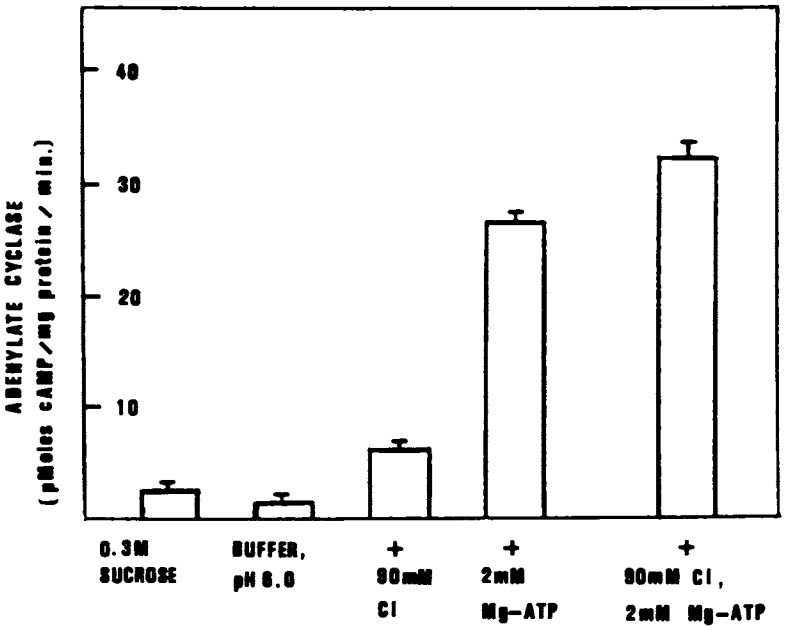

Fig. 1. Synthesis of cyclic AMP by releasing chromaffin granules. The conditions for the assay are as described in the text for release at pH 6.0. The reaction was terminated with 5% TCA. Cyclic AMP was then estimated by a competitive protein-binding assay using internal standards to quantitate yield. Enzyme activity was defined by the difference of cAMP at 0 time and after 10 min of incubation.

TABLE II. Influence of ATP Analogs on Synthesis of Cyclic AMP by Chromaffin Granules

Adenosine nucleotide analog[1]	% cAMP synthesized[2]	Adenylate cyclase activity[3]
ATP	100	20 pmole/mg/min
AMP-PNP	20	4 pmole/mg/min
AMP-PCP	2	ND[4]
AMPCPOP	0	ND[4]

[1] 2 mM nucleotide in presence of 1 mM Mg acetate.
[2] 100% = 30 pmoles/mg protein/min at 37°C and pH 6.0, assayed by the protein-binding method.
[3] Assayed using alpha-^{32}P-ATP and alpha ^{32}P-AMP-PNP. Nucleotide concentration was 1 mM. $MgCl_2$ concentration was 5 mM.
[4] ND, not detected.

were also able to use the ATP analogs as substrates; however, they differed in their efficiency as substrates for this reaction. The order of efficiency of ATP analogs in the synthesis of cyclic AMP appeared to be similar to their order of efficiency at inducing release from isolated granules.

DISCUSSION

The relationship between ATP utilization by granules for release and cyclic AMP synthesis is at this point highly circumstantial. However, it is clear from these studies with ATP analogs that only substrates for adenylate cyclase can induce release. This fact may be related to the observation that carbachol, an adrenal medulla secretagogue, induces a 20-fold increment in cyclic AMP levels in adrenal medulla tissue both in vitro and in vivo (13).

These data tend to speak against a β-γ-adenosine triphosphatase activity being involved in secretion from isolated granules. This is in direct contrast to theories of release which invoke either a calcium-activated or actomyosin-like ATPase activity (14).

On a purely biochemical level, these studies demonstrate that the chromaffin granules are metabolically active and not passive structures acted upon only by outside forces. However, it is not clear what exact relation these data have to the cellular process of release. We view the process of secretion as two half-reactions. One is the granule release system which is dependent on magnesium. The second is the coupling of granules and plasma membranes which may require calcium. The isolated chromaffin granule system may allow a dissection of the complex secretion process into single components.

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