Adenosine Triphosphate-Evoked Catecholamine Release in Chromaffin Granules

OSMOTIC LYSIS AS A CONSEQUENCE OF PROTON TRANSLOCATION

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Chromaffin granules suspended in Cl−-containing media release catecholamine and protein when ATP is added. This phenomenon is inhibited in hyperosmotic media and in the presence of uncouplers of oxidative phosphorylation. Release requires a permeant anion in the medium, but is independent of the cation. The release process appears to be driven by an inwardly directed proton-translocating adenosine triphosphatase. The resulting proton–anion influx causes osmotic lysis of the chromaffin granules.

Chromaffin granules, the catecholamine-storage vesicles of the adrenal medulla, may respond to ATP in vitro by either accumulating or releasing catecholamine. Energy-dependent catecholamine uptake requires ATP hydrolysis (Hasselbach & Taugner, 1970; Phillips, 1974) and seems to involve a proton-translocating ATPase* (Bashford et al., 1975b, 1976). However, in Cl−-containing media, ATP apparently induces the opposite phenomenon: chromaffin granules release their contents (Oka et al., 1965; Poisner & Trifaro, 1967; Trifaro & Poisner, 1967; Lishajko, 1969; Izumi et al., 1975) via a process that also requires ATPase activity (Poisner & Trifaro, 1967; Trifaro & Poisner, 1967). These apparently antagonistic effects have caused considerable confusion about the role of ATP in vivo.

The release phenomenon has been interpreted as a response in vitro of the secretory apparatus of the chromaffin granule, and a model for exocytotic secretion has been proposed on this basis (Poisner & Trifaro, 1967). However, catecholamine release can be explained as a simple consequence of osmotic lysis driven by the proton pump reported by Bashford et al. (1975a,b). Proton translocation, resulting from ATP hydrolysis, generates a membrane potential. In medium containing a permeant anion (such as Cl−), there is a consequent anion influx. This raises the osmolarity of the granule interior, resulting in osmotic lysis and release of granular contents. The experiments reported here show that this is the case. There is no reason to believe that this ATP-induced lysis is a phenomenon of importance in vivo.

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

Experimental

Chromaffin granules were isolated from bovine adrenal medullae as described by Bashford et al. (1975b). They were suspended in 0.3 m sucrose/10 mm Hepes, pH 7.0, at a concentration of 25–40 mg of protein/ml and kept on ice until required. All experiments were performed within 6–10 h of the cattle being slaughtered.

Measurement of extinction changes

Chromaffin granules (6 μl or 150 μg of protein) were diluted into 1 ml of medium in a cuvette as described in the Results section. The change in $E_{440}$ was monitored at 37°C by using a Unicam SP.1800 recording spectrophotometer fitted with a thermostatically controlled cell block. The initial $E_{440}$ was always between 0.4 and 0.7. The experimental results are expressed as a percentage of the initial extinction. Granules diluted into 10 mm Hepes, pH 7.0 (representing 100% lysis), were used as the blank.

Measurement of protein and adrenaline release

Experiments were initiated by adding chromaffin granules (0.1 ml or 3–4 mg of protein) to 1 ml of medium as described in the Results section. After incubation for 10 min at 37°C, the granules were cooled to 4°C and pelleted by centrifugation for 10 min at 27000 g and 4°C in the SS-34 rotor of a Sorvall RC2-B centrifuge. Supernatants were immediately removed, frozen, and stored at –20°C until assayed. Samples (5 μl) were assayed for adrenaline by the method of Anton & Sayre (1962), except that chromatography on alumina was found to be unnecessary.
Further samples (0.7 ml) were assayed for protein by using biuret reagent calibrated with bovine serum albumin. Before assay, protein was precipitated with 5% (w/v) trichloroacetic acid and re-dispersed in 3% (w/v) NaOH/2% (w/v) deoxycholate. Complete (100%) release was determined by lysing 0.1 ml of granules in 1 ml of 10 mM Hepes, pH 7.0, removing membranes by centrifugation and assaying released adrenaline and protein.

Materials

Bovine serum albumin, ATP (disodium salt) and adrenaline were from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. The uncoupler N-(3-t-butyl-4-chlorosalicylyl)-2-chloro-4-nitroanilide (compound S-13) was kindly provided by Dr. Britton Chance, University of Pennsylvania, Philadelphia, PA, U.S.A.

Results

The release of chromaffin-granule contents was monitored by three methods: release of protein, release of adrenaline and decrease in E540. Trifaro & Poisner (1967) observed that ATP-induced release of catecholamine was accompanied by a decrease in the E540 of the chromaffin-granule suspension. These changes were later shown to correlate with morphological changes observed by light-scattering and electron microscopy (Morris et al., 1974). The extinction measurements permit us to follow changes in both the rate and extent of the release process. The adrenaline and protein assays represent the extent of release after a fixed time, 10 min. However, since the rate of release is fairly constant over the first 10 min, these assays also give a good measure of the rate of release.

In almost all experiments reported here, some adrenaline and protein release and decrease in extinction occur in the absence of added ATP. This can be attributed to endogenous ATP, which is present in chromaffin granules in high concentrations (Smith, 1968). Since granules lyse spontaneously (Morris et al., 1974) some ATP will be released, and this ATP will in turn induce further release.

In the samples containing exogenous ATP, the adrenaline assayed is the excess of that released, either by lysis or efflux, over that actively re-incorporated by the granules. In most cases, more adrenaline is released than can be re-incorporated and a net adrenaline release is seen. However, in conditions that markedly inhibit the release phenomenon, ATP-driven catecholamine uptake dominates, causing the 'negative' release observed.

Effect of external osmotic pressure on ATP-induced release

Chromaffin granules were incubated in media (10 mM Hepes/KCl/sucrose, pH 7) of varying osmolarity. Osmolarity was varied either by increasing the KCl concentration or by increasing sucrose concentration, while maintaining KCl concentration at a constant 150 mM. ATP-induced release was followed by changes in E540 (Fig. 1). The rate at which E540 decreases after the addition of ATP clearly diminishes as the external osmotic pressure is increased by using either KCl or sucrose. The release of protein and adrenaline in the presence or absence of ATP was also measured as a function of external osmotic pressure (Figs. 2 and 3). These data are clearly consistent with the changes in extinction, and demonstrate that the ATP-dependent change in chromaffin-granule structure, which results in release of the granular contents, is inhibited by increasing the osmotic pressure of the external medium. At sufficiently high osmolarities, release is not sufficient to overcome ATP-induced uptake of catecholamine, resulting in negative adrenaline release.

Effect of ionic composition of the medium on ATP-induced release

Lishajko (1969) showed that ATP-induced catecholamine release is dependent on the presence of Cl−, and demonstrations of catecholamine release (Oka et al., 1965; Poisner & Trifaro, 1967; Izumi et al.,
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Chromaffin granules (0.1 ml, 3.6 mg of protein) were added to 1 ml of medium containing 10 mM-Hepes/150 mM-KCl, pH 7.0, and either sucrose (○) or additional KCl (●) to make up the indicated osmolarity. At each osmolarity, the difference between samples with and without ATP was calculated. Four such pairs were assayed at each osmolarity and the mean and s.d. of the differences are shown. ATP was added as 20 μl of 50 mM-ATP/25 mM-MgSO4/5 mM-Hepes, pH 7.0; 100% adrenaline release = 3.7 ± 0.5 μmol.

1975) have commonly involved the use of Cl−-containing media. To investigate the dependence of release on the external anion, we incubated chromaffin granules in iso-osmotic (310 mosM) media containing 10 mM-Hepes buffer, pH 7.1, and sucrose or various K+ salts in the presence and absence of ATP. Table 1 shows the anion-dependence of protein and adrenaline release. Extinction changes consistent with these data are shown in Fig. 4. Background release, which is attributable to endogenous ATP, shows the same order of anion-dependence.

Medium containing choline chloride is as effective in supporting release as KCl medium (Fig. 4 and Table 1). This medium contained no cations other than choline (150 mM) and Tris (10 mM), except for approx. 5 mM-Na+ added as ATP (sodium salt) and those ions present in the chromaffin-granule preparation. Consequently, it is unlikely that release requires a permeant cation.

Effect of mitochondrial uncouplers on release

Two uncouplers of oxidative phosphorylation, dinitrophenol and compound S-13, inhibit ATP-induced adrenaline and protein release (Table 2) and abolish the ATP-dependent change in extinction (Fig. 5). At the concentrations used, these uncouplers also stimulate chromaffin-granule ATPase activity, inhibit adrenaline uptake, and abolish the ATP-induced enhancement of 8-anilinonaphthalene-1-sulphonate fluorescence in chromaffin granules (Bashford et al., 1975a,b; R. P. Casey, D. Njus, G. K. Radda & P. A. Sehr, unpublished work).

Discussion

Dolais-Kitabgi & Perlman (1975) observed that guinea-pig chromaffin granules release catecholamines and soluble enzymes when treated with valinomycin in the presence of certain K+ salts. They deduced that, in the presence of a membrane-permeable anion, valinomycin-mediated transport of K+ into chromaffin granules leads to osmotic lysis. The effectiveness of various anions in supporting lysis followed the order SCN−, I−, Br− > Cl− > acetate−, F−, isethionate−. They reported PO43− did not support lysis. Guinea-pig chromaffin granule membranes at 31°C are apparently permeable to these anions in this order.

Taugner (1972) made a detailed study of the effects of anions on the influx and efflux of catecholamines...
Table 1. Dependence of protein and adrenaline release on medium composition

Chromaffin granules (0.1 ml, 2.5 mg of protein) were added to 1 ml of the solutions indicated. Values shown represent the means and S.D. of four separate incubations; 100% adrenaline release = 1.97 ± 0.02 μmol and 100% protein release = 1.17 ± 0.03 mg. ATP was added as 20 μl of 50 mM-ATP/25 mM-MgSO4/5 mM-Hepes, pH 7.0. KI, KCl and KSCN were 150 mM solutions in 10 mM-Hepes, pH 7.1. Sucrose was 300 mM in 10 mM-Hepes, pH 7.1. The potassium acetate solution was made by mixing 300 mM-acetic acid with 300 mM-KOH to give a 300 mosm solution at pH 7.1. The choline chloride solution was 150 mM-choline chloride/10 mM-Tris, adjusted to pH 7.1 with HCl.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Adrenaline release (%)</th>
<th>Protein release (%)</th>
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<tbody>
<tr>
<td></td>
<td>-ATP</td>
<td>+ATP</td>
</tr>
<tr>
<td>KI</td>
<td>75.2 ± 1.8</td>
<td>95.7 ± 3.1</td>
</tr>
<tr>
<td>KCl</td>
<td>58.0 ± 2.4</td>
<td>80.7 ± 2.4</td>
</tr>
<tr>
<td>KSCN</td>
<td>87.0 ± 8.7</td>
<td>85.1 ± 4.1</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>7.8 ± 0.4</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>31.3 ± 2.1</td>
<td>29.0 ± 0.7</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>55.7 ± 3.0</td>
<td>82.3 ± 1.8</td>
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Fig. 4. Ion-dependence of extinction changes

Chromaffin granules (150 μg of protein) were added to 1 ml of the solutions described in Table 1. At time 0, 20 μl of 50 mM-ATP/25 mM-MgSO4/5 mM-Hepes, pH 7.0, was added. The data have been normalized by taking E550 at time 0 as 100% and the E550 of lysed granules as 0%. The traces shown were obtained in the following media: (a) sucrose/ATP; (b) sucrose and K2SO4; (c) choline chloride and K2SO4/ATP; (d) KCl; (e) choline chloride/ATP; (f) KSCN; (g) KSCN/ATP; (h) KCl/ATP.

However, did not correlate with effects on ATPase activity, but varied in the order SCN- > I- > Br- > Cl- > acetate-, F-. Our results (SCN- > I- > Cl- > acetate-) confirm Taunger's (1972) results for catecholamine release and show that protein release varies in the same order. This order correlates with the relative permeability of guinea-pig chromaffin-granule membranes to these anions, as discussed above. Consequently, we feel that the effectiveness of an anion in supporting release correlates with the ability of that ion to permeate the chromaffin-granule membrane. We have also found that SO42- and PO43- do not support release, but do not inhibit ATPase activity either, indicating that these ions are impermeant.

It appears that ATP-evoked release from chromaffin granules is comparable with the K+-valinomycin-evoked release observed by Dolais-Kitabgi & Perlman (1975), except that an ATP-driven proton current replaces the valinomycin-mediated K+ flux. The inhibition of ATP-induced release of protein and catecholamine by increased external osmotic pressure indicates that release occurs as a consequence of osmotic lysis. Release is decreased when chromaffin-granule ATPase activity is inhibited by N-ethylmaleimide (Poiser & Trifaro, 1967), indicating that ATP hydrolysis is required. Bashford et al. (1975a) have shown that mitochondrial uncouplers stimulate the chromaffin-granule ATPase activity, suggesting that much or all of it is attributable to a proton-translocating ATPase. That this ATPase is directly involved in ATP-dependent lysis is indicated by our finding that mitochondrial uncouplers inhibit lysis. H+ movement alone could not occur to a large extent as transmembrane electroneutrality would not be maintained. However, the presence of a permeant anion would allow a coupled proton-anion influx leading to osmotic lysis. The correlation between ATP-induced lysis and permeability of the anion provides strong evidence that this is the case. The fact
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Table 2. Uncoupler sensitivity of protein and adrenaline release

Chromaffin granules (0.1 ml, 3.2 mg of protein) were added to 0.9 ml of 150 mM-KCl/10 mM-Hepes, pH 7.0. Values shown represent the means and s.d. of four separate incubations; 100% adrenaline release = 4.6 ± 0.3 μmol and 100% protein release = 1.6 ± 0.15 mg. Dinitrophenol was added as a 100 mM solution in 10 mM-Hepes, pH 7.0. Compound S-13 was added as a 170 μM solution in ethanol. ATP was added as 20 μl of 50 mM-ATP/50 mM-MgCl2, pH 7.0.

<table>
<thead>
<tr>
<th></th>
<th>Adrenaline release (%)</th>
<th>Protein release (%)</th>
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<tbody>
<tr>
<td></td>
<td>−ATP</td>
<td>+ATP</td>
</tr>
<tr>
<td></td>
<td>39.9 ± 6.5</td>
<td>66.2 ± 8.4</td>
</tr>
<tr>
<td>No uncoupler</td>
<td>42.6 ± 4.5</td>
<td>42.0 ± 3.2</td>
</tr>
<tr>
<td>0.5 mM-Dinitrophenol</td>
<td>25.5 ± 1.8</td>
<td>19.6 ± 1.6</td>
</tr>
<tr>
<td>1.5 mM-Dinitrophenol</td>
<td>24.6 ± 2.4</td>
<td>21.5 ± 2.0</td>
</tr>
<tr>
<td>5 mM-Dinitrophenol</td>
<td>32.4 ± 2.9</td>
<td>16.5 ± 3.6</td>
</tr>
<tr>
<td>170 nM-Compound S-13 (in 0.1% ethanol)</td>
<td>34.2 ± 2.9</td>
<td>18.3 ± 1.9</td>
</tr>
<tr>
<td>0.1% Ethanol</td>
<td>65.4 ± 2.8</td>
<td>41.7 ± 1.4</td>
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</table>

Fig. 5. Uncoupler sensitivity of ATP-dependent changes in extinction

Chromaffin granules (150 μg of protein) were added to 1 ml of 150 mM-KCl/10 mM-Hepes, pH 7.0. Uncoupler was added, then 20 μl of 50 mM-ATP/25 mM-MgSO4/5 mM-Hepes, pH 7.0, was added at time 0. The data have been normalized by taking ε_{540} at time 0 as 100% and the ε_{540} of lysed granules as 0%. Uncoupler solutions were as described in Table 2. All samples without added ATP produced traces falling in band (a). These included a control and samples with 5 mM-, 15 mM-, 30 mM-dinitrophenol or 170 mM-compound S-13 in 0.1% ethanol. Also falling in this band were samples containing added ATP and either 15 mM- or 30 mM-dinitrophenol, or 170 mM-compound S-13 in 0.1% ethanol. Trace (b) was obtained with 5 mM-dinitrophenol and ATP. Trace (c) was obtained by adding either ATP or ATP and 0.1% ethanol.

that choline can replace K+ in the release process suggests that a cation (other than H+) is not required. A large H+ influx would not be expected to cause great acidification of the granule interior, because of the considerable buffering capacity provided by intragranular ATP and acidic proteins (Smith, 1968). In fact, ATP addition does cause a pH fall, but of only about 0.5 pH unit (R. P. Casey, D. Njus, G. K. Radda & P. A. Sehr, unpublished work).

Bashford et al. (1975a) discovered that the 8-anilinonaphthalene-1-sulphonate fluorescence of chromaffin-granule membranes is enhanced when ATP is added, and this enhancement is reversed by uncouplers. In mitochondria, this fluorescence is quenched on energization, whereas it is enhanced on energization of submitochondrial particles (Azzi, 1969). Since mitochondria energetically exclude H+ (Mitchell & Moyle, 1965a), whereas submitochondrial particles actively accumulate H+ (Mitchell & Moyle, 1965b), the change in fluorescence seems to be determined by the direction of proton pumping. The 8-anilinonaphthalene-1-sulphonate fluorescence enhancement observed in chromaffin granules suggests that the chromaffin-granule proton pump is inwardly directed, in agreement with the requirements of the lysis phenomenon. Bashford et al. (1975b, 1976) also showed that adrenaline uptake is inhibited by uncouplers, indicating that proton translocation is involved in adrenaline accumulation. Possible uptake mechanisms consistent with an ATP-linked H+ influx have been discussed (Bashford et al., 1976).

Lishajko (1969) found that ADP could substitute for ATP in the release process, and Izumi et al. (1975) discovered that a soluble protein seemed to stimulate release in the presence of either ATP or ADP. However, since Hillarp (1958) has reported that an adenylate kinase is present in chromaffin granule
preparations, these effects may be attributable to the conversion of 2 ADP molecules into ATP+AMP and subsequent hydrolysis of ATP.

The observation of ATP-dependent catecholamine release prompted several workers (Poisner & Trifaro, 1967; Izumi et al., 1975; Hoffman et al., 1976) to consider isolated chromaffin granules as a simple model system for studying the secretion mechanism. It appears, however, that the primary event in ATP-induced catecholamine and protein release is the translocation of protons into the chromaffin granule after ATP hydrolysis, this being accompanied or followed by an influx of anions. The consequent increase in internal osmotic pressure leads to osmotic lysis of the chromaffin granule and release of its contents. The involvement of proton influx and granule lysis in exocytotic secretion, although possible, seems unlikely.

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References


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