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ATP-Induced Lysis of Rat Parotid Secretory Granules: Possible Role of ATP in Exocytotic Release

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Secretory vesicles isolated from a variety of mammalian tissues are known to lyse and thereby release their secretory products when exposed to ATP. This process, which will be termed ATP-induced lysis, has been studied most extensively using adrenal chromaffin-granule preparations. We report here that ATP causes the lysis of a highly purified preparation of rat parotid secretory granules. The rate of granule lysis was measured spectrophotometrically, and ATP-induced lysis was expressed as the increase in the rate of lysis (r = % lysis per min) when ATP was added. This lytic process was characterized with respect to pH, temperature, osmolarity, and the ionic composition of the media. ATP-induced lysis of parotid granules was found to have the following properties in common with the extensively characterized chromaffin-granule process:

1. It is a saturable function of ATP with half-maximal rates observed at 0.5 ± 0.1 mM ATP.
2. It is temperature dependent, eg, r = 6.1 ± 2.1%/min at 30°C vs 12.2 ± 2.5%/min at 37°C.
3. It is inhibited in hyperosmotic media, eg, r = 5.3 ± 0.3%/min at 0.3 OsM vs 0.8 ± 0.2%/min at 0.4 OsM.
4. It shows a nucleotide preference of ATP = GTP > ADP > AMP > CTP = ITP.
5. It has an anion requirement.

The above findings, combined with reports of ATP-induced lysis of cholinergic, insulin, and posterior-pituitary vesicles, imply that ATP-induced lysis may reflect an ATP-dependent property of all secretory vesicles, and as such, this vesicle property could play a similar role in each exocytotic release process. Using a model system, Miller and Racker [22] made a surprising finding that the extent to which liposomes fuse with a black lipid membrane depends on the osmotic gradient across the vesicle membrane. In view of the osmotic dependence of ATP-induced lysis in this and other secretory-vesicle preparations, we postulate that ATP may *prime* secretory vesicles for fusion with the plasma membrane by inducing and/or maintaining an osmotic gradient across the vesicle membrane.

Key words: secretory granules, ATP-induced lysis, osmotic gradient

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In recent years there have been numerous reports that ATP stimulates lysis of secretory vesicles or granules isolated from a variety of mammalian tissues [1–5]. The molecular events of this release process have been studied in the hope of gaining insight into the nature of these organelles and the intimate role they play in exocytotic release. In this regard, the adrenal chromaffin-granule process has been analyzed most extensively [5–12]. Stimulation of granule lysis in this secretory-granule preparation requires Mg^{2+}, ATP, and a high level of permeant anions, such as chloride, in the incubation media [6]. Exposure of chromaffin granules to MgATP increases the potential difference across the vesicle membrane [10], and based on studies using mitochrondrial uncouplers, it has been proposed that ATP functions as a substrate for an inwardly directed proton pumping ATPase [11, 12]. Chloride has been proposed as the permeant counterion for the proton, functioning to maintain electroneutrality [7], and catecholamine release is thought to be the result of osmotic lysis [11].

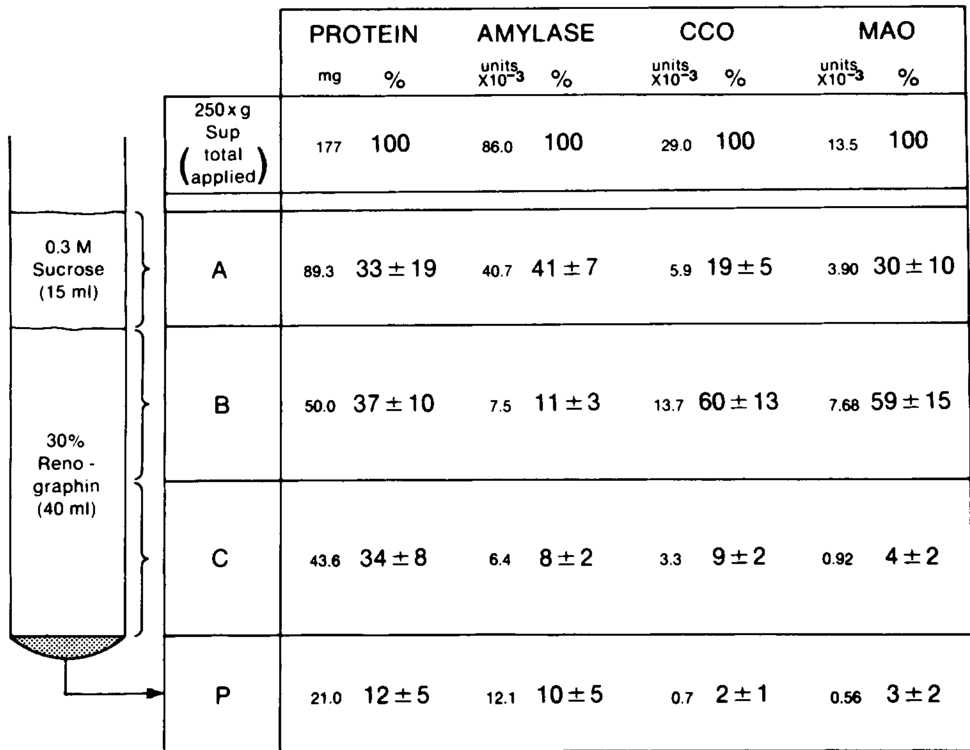
In 1971, Ishida et al [3] reported that ATP induces the lysis of a rat parotid-granule preparation. Using a more highly purified parotid-granule preparation, we analyzed various properties of the parotid-granule process and compared this phenomenon with the more extensively characterized chromaffin-granule process. We report here that the ATP-induced lysis of parotid granules has a number of properties in common with the chromaffin-granule process, which implies that both processes may have similar mechanisms of action. Portions of this study were previously reported in abstract form [13, 14].

MATERIALS AND METHODS

Isolation of Parotid Granules

Adult male Sprague-Dawley rats weighing 200–250 g were used in all experiments. The rats were housed in a light-controlled room (with a 6 am to 6 pm light period) and fed Purina Lab Chow and water, ad libitum, until the evening before the day of the experiment. For each experiment, 5 rats were fasted overnight, their parotid glands removed the next morning; a 250 g supernatant fraction was prepared from the homogenate as described previously [15]. This supernatant fluid was then sedimented through a 30% Renographin (Reno-M-60, meglumine diatrizoate, obtained from E.R. Squibb, Inc. and substituted for its European counterpart, Urographin) solution as described by Kirshner et al [16], yielding a pellet referred to as the secretary-granule fraction (fraction P of Figure 1). To assay various zones of the tube for marker enzymes and protein content, fractions were removed sequentially from the tubes using a peristaltic pump to minimize cross contamination. As shown in Figure 1, the total protein, amylase (marker of granule contents), monoamine oxidase, and cytochrome *c* oxidase (markers of outer and inner mitochondrial membranes respectively) activities were determined in each fraction. Following these determinations, the granule pellet (fraction P) was resuspended in 1 to 3 ml 0.3 M sucrose, 10 mM Hepes buffer (pH 7.2) to give a protein concentration of approximately 10 mg/ml. These granule suspensions were used in all subsequent experiments. All lysis experiments were performed within 12 h of the start of the granule isolation protocol.

To test for the intactness of the granules in each granule suspension, we determined the total amylase activity that could be pelleted by a 27,000g spin for 10 min at 0°C. For four separate granule preparations, 90% ± 7% (mean percent ± standard error of the mean) of the total amylase activity in these granule suspensions was sedimented. We concluded, therefore, that nearly all of the granules obtained by these procedures were intact and that there was little variation in granule intactness from one preparation to another.



	PROTEIN mg	PROTEIN %	AMYLASE units X10⁻³	AMYLASE %	CCO units X10⁻³	CCO %	MAO units X10⁻³	MAO %
250 x g Sup (total applied)	177	100	86.0	100	29.0	100	13.5	100
A	89.3	33 ± 19	40.7	41 ± 7	5.9	19 ± 5	3.90	30 ± 10
B	50.0	37 ± 10	7.5	11 ± 3	13.7	60 ± 13	7.68	59 ± 15
C	43.6	34 ± 8	6.4	8 ± 2	3.3	9 ± 2	0.92	4 ± 2
P	21.0	12 ± 5	12.1	10 ± 5	0.7	2 ± 1	0.56	3 ± 2

Fig. 1. Distribution total protein, amylase, cytochrome *c* oxidase and monoamine oxidase activity following centrifugation of the 250g supernatant through 30% Renographin as described in Methods. The absolute amount of protein and the units of enzyme activity are from a single granule preparation using 10 glands from 5 fasted rats. The realtive distribution is the mean % ± standard deviation of the mean for three separate granule preparations using 10 glands each. Enzyme activity measurements and units are defined in Methods section.

Preparation of a Crude Mitochondrial Fraction

In the Renographin step of the granule-isolation procedure described above, 60% of the total mitochondrial enzyme activities were found in fraction B (Fig. 1). This fraction was diluted 3-fold with 0.3M sucrose, 10 mM Hepes (pH 7.2), centrifuged at 30,000g for 15 min at 0°C; the resulting pellet was resuspended in 2 ml 0.3 M sucrose. This suspension was termed the crude mitochondrial fraction.

Assay of ATP-Induced Lysis

Incubation media contained 110 mM KCl, 5 mM $MgCl_2$, 25 mM buffers of either maleic acid or Hepes and various concentrations of disodium adenosine triphosphate (Na_2ATP) and/or sucrose to provide a final osmolarity of 0.3 OsM. Twenty μl of the granule suspension was mixed in a cuvette with 1 ml of incubation media to give an initial absorbance at 540 nm of from 0.3 to 0.5. Zero absorbance corresponded to 20 μl of granule suspension added to 1 ml of water. The cuvettes were placed in a 37°C temperature control chamber, and the change in absorbance with time was monitored using a Gilford 240 spectrophotometer with 6040 A chart recorder. The absorbance observed immediately after mixing was de-

fined as 100%. As shown in Figure 2, ATP-induced lysis was expressed as the rate of granule lysis (% per min) in Na_2ATP-containing media minus the basal rate observed when Na_2ATP was replaced with osmolar equivalents of sucrose. The basal rate of granule lysis ranged from 0.5 to 2% per min.

The initial absorbance of the incubation media was found to be directly proportional to the amount of granule suspension added (data not shown) for all absorbance readings less than 0.7. Following the addition of parotid granules to the incubation media there was a short lag of from 1 to 4 min before the rate of lysis reached a maximum value. As a result, all rates reported were the maximum rates observed after this initial lag period (Fig. 2). The extent of granule lysis (% lysis) was proportional to the total amylase activity that remained in the supernatant following centrifugation of the incubation media at 27,000g for 10 min at 0°C to pellet intact granules (eg, for three separate granule preparations, when the mean % lysis ± SD was 50 ± 2%, 55 ± 6% of total sedimentable amylase activity remained in the supernatant following incubation and centrifugation).

Additional Procedures

Protein concentrations were determined by the Lowry technique [17] as modified by Hartree [18] and crystalline bovine serum albumin was used as the standard. Amylase activity was measured as described by Schramm and Danon [19]. Monoamine oxidase activity was assayed according to the procedure of Wurtman and Axelrod [20] using 14C-tryptamine

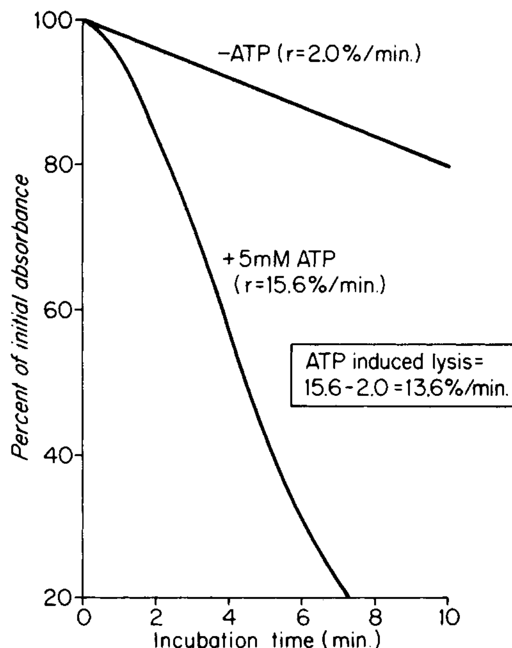


Fig. 2. Specific example of experimental protocol used to determine rate of ATP-induced granule lysis. At time 0, 20 μl aliquots of a granule preparation were added to 1 ml of media containing 110 mM KCl, 5 mM $MgCl_2$, 25 mM maleic acid buffer (pH 6.8) and either 5 mM Na_2 ATP (+ATP) or 15 mM sucrose (−ATP). The absorbance at 540 nm was monitored continuously in a temperature-controlled cuvette. In the ATP containing media there was an initial lag of from 2 to 3 min before a maximum rate of 15.6%/min was observed, whereas the −ATP control sample was linear throughout this time period. The rate of ATP-induced lysis was calculated as shown above.

as substrate, and cytochrome *c* oxidase was measured by the method of Cooperstein and Lazarow [21]. Enzyme activities were expressed in the same units used in the reference articles. All nucleotides were obtained from Sigma.

RESULTS

pH Dependence of ATP-Induced Lysis

The rate of granule lysis following the addition of 5 mM Na_2ATP was measured from pH 5–8 (Fig. 3). ATP-induced lysis was pH-dependent with maximal rates of lysis observed over a rather restricted range of pH (6.2–6.9). In addition, the pH at which maximal ATP-induced lysis was observed tended to vary from one granule preparation to another. In view of the variation in the optimal pH among preparations, the rate of granule lysis in 5 mM Na_2ATP was assayed at pH 6.4, 6.6, 6.8, and 7.0 to determine the pH optimum for each preparation before any other incubation conditions were varied. All subsequent determinations were performed at this optimal pH.

In view of the strong pH dependence of ATP-induced lysis illustrated in Figure 3, an analysis of possible variation in pH during granule lysis seemed warranted. To test for such a pH change, we determined the initial pH and the pH at 100% lysis. When this was done for 3 separate granule preparations, no change in pH was observed. ($\Delta pH < 0.01$).

ATP Stimulation of Granule Lysis

The rate of lysis of parotid granules showed a strong dependence on the ATP concentration of the incubation media. As shown in Figure 4, the rate of lysis was a saturable function of the ATP concentration with a half-maximal rate observed at 0.50 ± 0.14 mM ATP. This

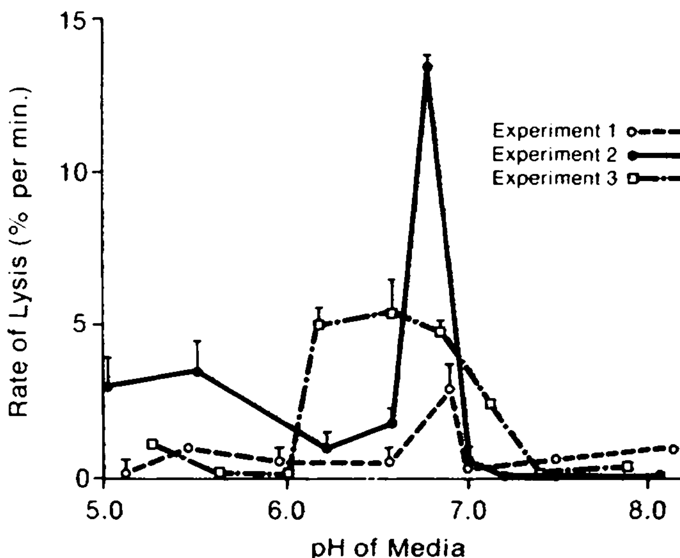


Fig. 3. Influence of pH on ATP-induced lysis of parotid granules. The incubation media contained 110 mM KCl, 5 mM $MgCl_2$, 5 M Na_2ATP or 15 mM sucrose; and either 25 mM maleic acid (pH 5 to pH 7) or 25 mM Hepes (pH 7 to pH 8). The rate of ATP-induced lysis (% lysis per min) was determined as described in Methods. The pH dependence of ATP-induced lysis is shown for three separate granule preparations. In view of the variation in the pH dependence from preparation to preparation, the rate of lysis was assayed at pH 6.2, 6.4, 6.6, 6.8, and 7.0 to determine the pH optimum for a granule preparation before any other parameters were varied.

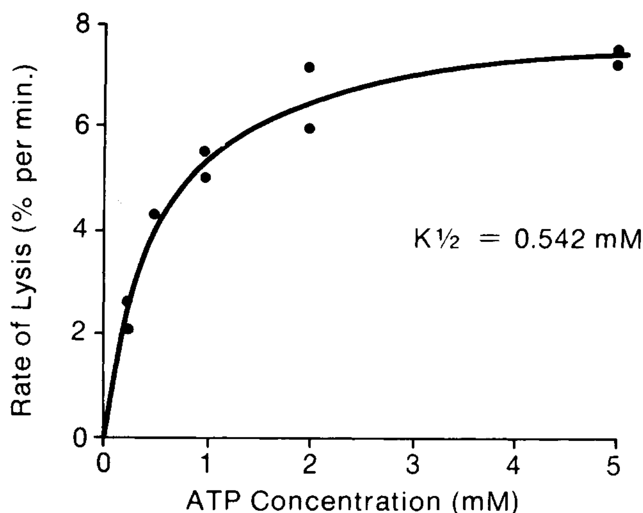


Fig. 4. The effect of increasing ATP concentration on the rate of parotid-granule lysis. The media contained 110 mM KCl, 5 mM $MgCl_2$, 25 mM maleic acid buffer (pH 6.8), and the indicated ATP concentrations plus sucrose to bring the media osmolarity to 0.3 OsM. An Eadie plot [20] of this Michaelis-Menten-like kinetic data yielded a straight line with a slope of $-K1/2$. A least square, linear regression analysis of this data resulted in the K1/2 value given above. The linear regression coefficient for the Eadie plot of this data was 0.968. The mean K1/2 ± standard deviation of the mean for three separate granule preparations was $K1/2 = 0.50 \pm 0.14$ mM.

value is consistent with the 0.75 mM ATP concentration at which Ishida et al [3] reported a half-maximal rate of ATP-induced lysis. This earlier study showed that at ATP concentrations greater than 3 mM the rate of lysis diminished. In contrast, we found that even at 5 mM ATP the rate of ATP-induced lysis was still increasing. Ishida et al [3] claimed that granule lysis was optimal when the ratio of ATP to Mg^{2+} was about one. Since our incubation media always had a 5 mM $MgCl_2$ concentration, this might account for the difference in our results. However, it is also possible that this difference might simply reflect a difference in the composition of the two parotid-granule preparations used in these studies.

The Michaelis-Menten-type kinetic relationship between the rate of lysis and the ATP concentration is analogous to the ATP dependence of the adrenal chromaffin-granule process [6]. The chromaffin-granule and parotid-granule processes show half-maximal stimulation of lysis at similar ATP concentrations of 0.2 and 0.50 mM ATP, respectively, both within the physiological range.

Dependence on Media Osmolarity

An increase in media osmolarity due to added sucrose resulted in a marked inhibition of ATP-induced lysis. An increase of from 0.3–0.4 OsM resulted in more than a 5-fold reduction (Fig. 5). A less pronounced decrease in the rate of ATP-induced lysis was noted when KCl was added to the incubation media, eg, at 0.3 OsM, $r = 5.3 \pm 0.3\%/\text{min}$ (n = 3) vs r = 1.9

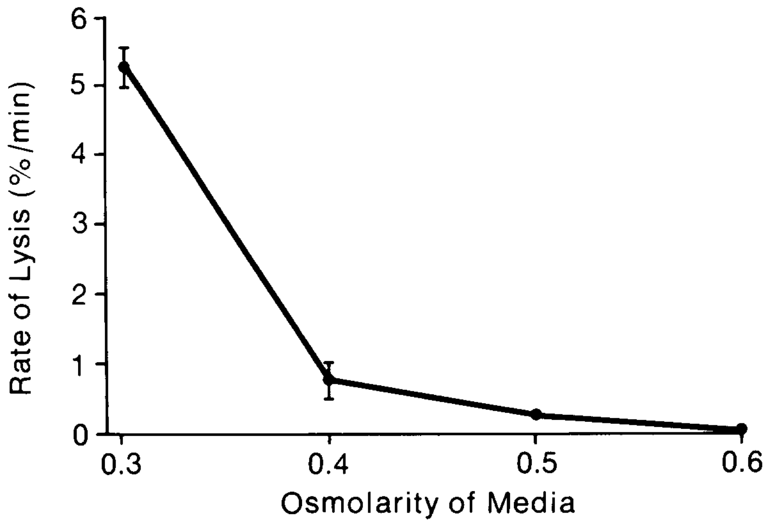


Fig. 5. The effect of elevated media osmolarity on the rate of ATP-induced parotid-granule lysis. The incubation media contained 110 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 25 mM maleic buffer (pH 6.8); and sucrose added to give the indicated osmolarity. The rate of ATP induced lysis (% lysis per min) was assayed as described in Methods.

± 0.5%/min (n = 4) at 0.4 OsM. Similar decreases in the rate of ATP-induced lysis following KCl and sucrose addition have been reported for chromaffin and numerous other secretory-vesicle preparations [1,2,4,5,24,25].

Effect of Added Mitochondria

The parotid granule preparations used in the analysis of ATP-induced lysis were found to have slight mitochondrial contamination as described in Methods. To test whether the mitochondria might play a role in ATP-induced granule lysis, we assayed the effect of adding increasing amounts of the crude mitochondrial fraction on the rate of ATP-induced lysis. An increase in the specific cytochrome *c* oxidase activity of the incubation media of up to 10-fold had no effect on the rate of ATP-induced lysis (data not shown). We conclude, therefore, that ATP-induced lysis of parotid granules is not attributable to the low level of mitochondrial contamination found in this preparation.

Temperature Dependence

The rate of ATP-induced lysis of parotid granules was measured at 30°C and 37°C. This 7° increase in the temperature of the incubation media caused a 2-fold increase in ATP-induced lysis from r = 6.1 ± 2.1%/min (n = 3) to r = 12.2 ± 2.5%/min (n = 3). We conclude from this that ATP-induced lysis of parotid granules, like the chromaffin-granule process [6], is a temperature-dependent phenomenon.

Ionic Dependence

As described in Methods, granule lysis was assayed in media containing 110 mM KCl. To test whether potassium was required for ATP-induced lysis, the media KCl was completely replaced by 2 other chloride salts, NaCl or choline chloride. As shown in Table I, there was no significant difference in the rate of ATP-induced lysis observed in any of the chloride-containing media. Therefore, neither potassium nor the other two cations were required for granule lysis.

Although lysis was stimulated to the same extent in all chloride-containing media, if the chloride salts were replaced by potassium acetate or KSCN, marked inhibition of ATP-induced lysis resulted. As shown in Table I, the rates observed in these 2 media were only 6% of the rate observed in the chloride-containing media. In addition, ATP was unable to stimulate release when KCl was replaced with an osmotic equivalent of sucrose. We conclude, therefore, that ATP-induced lysis of parotid granules has a chloride requirement. ATP-induced lysis of chromaffin granules has also been found to be a chloride-dependent process [6].

In the Ishida et al study [3], ATP-induced lysis of their parotid-granule preparation was inhibited when 0.5 mM EGTA was added to the incubation medium. However, we found that addition of 0.5 mM EGTA to the incubation medium had no effect on the rate of ATP-induced lysis (data not shown). In addition, when $MgCl_2$ was completely replaced by 5 mM $CaCl_2$, the rate of ATP-induced lysis was reduced about 14-fold (Table I). Therefore, we conclude contrary to the earlier report, that ATP-induced lysis of this parotid-granule preparation was not calcium-dependent. In addition, another divalent cation, Mn^{2+}, stimulated ATP-induced lysis to an even greater extent than Mg^{2+}. Thus, analogous to the chromaffin-granule process [5], ATP-induced lysis of the parotid-granule preparation was stimulated in Mn^{2+} and was inhibited by Ca^{2+}-containing media.

DISCUSSION

This study was stimulated by a preliminary report that ATP stimulates the lysis of a rat parotid-granule preparation [3] and an interest in whether this phenomenon is analogous to the thoroughly analyzed chromaffin-granule process. Utilizing a more highly purified and characterized parotid-granule preparation than the earlier study, ATP-induced lysis of these granules was found to have the following properties in common with the chromaffin-granule process: 1) it was a saturable function of the media ATP concentration; 2) it had a marked pH dependence; 3) it was temperature dependent; 4) it was inhibited in hyperosmotic media; 5) it had an anion requirement; and 6) it was stimulated to diminishing extents by ATP, GTP, ADP, AMP, CTP, and ITP, respectively (Table II). These findings, combined with preliminary reports of ATP-induced lysis of insulin secretory granules [2], posterior-pituitary granules [4], and cholinergic vesicles [1], imply that ATP-induced lysis may reflect an ATP-dependent property of a wide variety of secretory vesicles and as such could play a similar role in each of these exocytotic release processes.

The inhibition of ATP-induced lysis of chromaffin granules in hyperosmotic media led Casey et al [11] to propose that ATP generates an osmotic gradient across the granule membrane and that membrane rupture was due to osmotic lysis. Additional findings that mitochondrial uncouplers inhibited the process [11, 12] and that ATP increased the membrane potential [7] led to a recent proposal [8] that ATP stimulates the pumping of protons into the granules, and counterions, namely chloride, follow through specific anions

TABLE I. Dependence of ATP-Induced Granule Lysis on the Ionic Composition of the Incubation Media*

Medium	Average lysis rate (%/min)	Average rate of ATP-induced lysis (%/min)	Relative rates
KCl (normal)	5.93 ± 0.40	5.03	1.000
NaCl	6.70 ± 1.54	5.80	1.153
Choline chloride	5.69 ± 1.43	4.79	0.952
Potassium acetate	1.18 ± 0.08	0.28	0.056
KSCN	1.20 ± 0.24	0.30	0.060
Sucrose	0.95 ± 0.20	0.05	0.010
$MnCl_2$	6.63 ± 0.78	5.73	1.139
$CaCl_2$	1.27 ± 0.17	0.37	0.074
Control	0.90 ± 0.10	0	0

*The data presented in this table were obtained by the addition of 20 μl of granule suspension to 1 ml of incubation media containing: 1 mM Na_2 ATP; 25 mM maleic acid buffer (pH 6.8); 50 μM EGTA; 5 mM of either $MgCl_2$, $MnCl_2$ or $CaCl_2$; plus either 220 mM sucrose or 110 mM of one of the listed monovalent cation salts. The control medium contained no ATP, 15 mM sucrose, 25 mM maleic acid (pH 6.8), 5 mM $MgCl_2$ and 110 mM KCl and provided the basal rate of lysis. Rates of lysis were determined spectrophotometrically at 37°C and are presented as mean values ± standard deviation for three determinations.

TABLE II. Effect of ATP and Other Nucleotides on the Rate of Parotid-Granule Lysis*

Nucleotides	Average lysis rate (%/min)	Nucleotide-induced lysis rate (%/min)	Relative rates
ATP	5.93 ± 0.40	5.03	1.000
GTP	5.43 ± 0.55	4.53	0.901
ADP	2.47 ± 0.60	1.57	0.312
AMP	1.40 ± 0.30	0.50	0.099
CTP	0.97 ± 0.06	0.07	0.014
ITP	0.97 ± 0.15	0.07	0.014
None	0.90 ± 0.10	0	0

*The data presented in this table were obtained by the addition of 20 μl of a parotid-granule suspension to 1 ml of incubation media containing 110 mM KCl, 5 mM $MgCl_2$, 25 mM maleic buffer (pH 6.9) plus either 1 mM of a disodium salt of the nucleotides listed below or no nucleotide and 3 mM sucrose. Lysis rates were determined spectrophotometrically as described in Methods, and all incubations were performed at 37°C. Lysis rates are presented as means ± standard deviations for three determinations.

channels and that water influx ultimately results in osmotic lysis. The many similarities between ATP-induced lysis of parotid and chromaffin granules suggest that both processes may have similar mechanisms of action.

Despite the fact that ATP-induced lysis of both parotid and chromaffin granules might be attributed to similar membrane properties, one still must ask what the physiological significance of such an ATP-dependent process might be. In 1976, Miller and Racker [22] develop-

ed a system that permitted a precise determination of the rate at which unilamellar liposomes can be made to fuse with a black lipid membrane. A surprising finding of this model study of membrane fusion was that the extent to which fusion occurred was dependent on the osmotic gradient across the vesicle membrane. In view of these findings, we postulate that ATP serves to *prime* secretory granules for fusion with the plasma membrane by inducing and/or maintaining an osmotic gradient across the secretory granule membrane.

In this and numerous other in vitro studies, ATP has been found to initiate the osmotic lysis of secretory granules. However, in vivo the concentration of quite a number of the factors that are required for osmotic lysis are rather strongly buffered, eg, ATP, Cl^-, and pH. We conclude, therefore, that the physiological role of ATP in exocytotic release may simply be to maintain an osmotic gradient across the vesicle membrane, which indirectly facilitates fusion when the vesicle eventually contacts the plasma membrane. In other words, an osmotic gradient across a secretory vesicle may simply serve as a driving force for vesicle fusion, whereas other factors such as Ca^{2+} may be involved in actually bringing the vesicle to the membrane surface.

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