ATP Keeps Exocytosis Sites in a Primed State
But Is Not Required for Membrane Fusion:
An Analysis with Paramecium Cells In Vivo and In Vitro

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**Abstract.** We have tried to specify a widespread hypothesis on the requirement of ATP for exocytosis (membrane fusion). With *Paramecium tetraurelia* cells, synchronously (+1 s) exocytosing trichoeysts, ATP pools have been measured in different strains, including wild type cells, “non-discharge” (*nd*), “trichless” (*tl*), and other mutations. The occurrence of a considerable and rapid ATP consumption also in *nd* and *tl* mutations as well as its time course (with a maximum 3–5 s after exocytosis) in exocytosis-competent strains does not match the actual extent of exocytosis performance. However, from in vivo as well as from in vitro experiments, we came to the conclusion that ATP might be required to keep the system in a primed state and its removal might facilitate membrane fusion. (For the study of exocytosis in vitro we have developed a new system, consisting of isolated cortices). In vivo as well as in vitro exocytosis is inhibited by increased levels of ATP or by a nonhydrolyzable ATP analogue. In vivo–microinjected ATP retards exocytosis in response to chemical triggers, whereas microinjected apyrase triggers exocytosis without exogenous trigger. Experiments with this system also largely exclude any overlaps with other processes that normally accompany exocytosis. Our data also explain why it was frequently assumed that ATP would be required for exocytosis. We conclude (a) that membrane fusion during exocytosis does not require the presence of ATP; (b) the occurrence of membrane fusion might involve the elimination of ATP from primed fusogenic sites; (c) most of the ATP consumption measured in the course of exocytosis may be due to other effects, probably to recovery phenomena.

It has been shown with widely different secretory systems that secretory activity depends on energy supply. This holds for oocytes (49), thrombocytes (2, 45), polymorphonuclear granulocytes (57), chromaffin adrenal medullary cells (26, 54), pancreatic acinar (6), and islet cells (30). Most detailed analyses were done with mast cells (7, 11, 15, 20, 24, 28, 35, 48); with a variety of trigger agents, though not with all, energy requirement for histamine release has been reported.

Concomitantly, secretory activity was reduced by inhibitors of glycolysis (II, 30) and/or of oxidative phosphorylation (2, 6, 11, 20, 24, 26, 35, 42, 48). Also, the extent of secretory activity observed was paralleled by an increased glucose (mast cells [50]) or oxygen (different gland cells [22, 47]) consumption, respectively, as well as by a decrease of the intracellular adenosine triphosphate (ATP) pool ([46]; mast cells [24, 28, 35]).

Other evidence comes from experiments with permeabilized cells in which secretory activity can be sustained only by ATP. This was reported for chromaffin cells (3, 27), oocytes (5, 55), and pancreatic endocrine cells (33).

I. Abbreviations used in this paper: AED, aminoethyl-dextran; AIDP, adenylylimidodiphosphate; ATP, adenosinetriphosphate.

However, as we shall discuss below, there is also some evidence for an alternative view of the energy requirements for secretory activity. In most systems, secretory activity is accompanied by the transport of secretory organelles to the cell membrane and/or by exocytosis-coupled endocytosis. Both these processes, transport and endocytosis, are known to be energy dependent (1, 43, 52, 53). Energy is also required to reestablish basal Ca
textrsup>2+] concentrations. This makes it very difficult to pinpoint the site where ATP would be eventually required.

A reliable answer as to whether ATP would be required for exocytosis per se could come from “simple” systems involving exocytotic membrane fusion only. The *Paramecium* cell, a ciliated protozoon, represents such a system with 1,000 secretory organelles ("trichocysts") docked to the cell membrane for immediate release (36, 39). As we show here, the cortex of these cells can be isolated to study exocytosis in vitro and, thus, to determine minimum conditions for exocytosis, including its ATP requirements. We also demonstrate that no other processes, except for exocytosis in the proper sense, take place in this system. These experiments were supplemented by studies with intact cells in which we manipulated ATP levels by microinjections of ATP or of
apyrase, as well as by metabolic inhibitors. We shall see that our data are in line with data from other "simple" systems, though interpretations in the literature are almost in unison to the opposite.

We also took advantage of the possibility to induce synchronous exocytosis (within ~1 s) in our system (36,39) and of the availability of a battery of mutations (38,41) of which some cannot perform exocytic membrane fusion. All this facilitates the elucidation of the possible role of ATP in exocytosis performance.

Materials and Methods

Cell Cultures

Paramecium tetraurelia cells were grown monoxenically, with Enterobacter aerogenes added, in a lettuce medium and harvested at early stationary phase. Strains used were (a) normally secreting cells including wild type (7S) and closely related strains (K401); (b) normal secretors but with defective ciliary reversion reaction (d4-500r; [21]); (c) the non-discharge mutations nd6 and the conditional nd9 mutation (nd9-18°C secretes, nd9-28°C does not secrete; cf. references 38, 41); (d) strain lam38 with a few trichocysts that cannot be transferred to the cell membrane and strain trichless (tl; cf. references 38, 41).

Synchronous Exocytosis Induction with Cells

Trigger experiments were performed with the use of aminophenylhexaestran (AED) as a trigger agent (36,39) and with ~10^-2 M [Ca^{2+}] present. During a period of ~1 s, cells capable of exocytosis expel up to ~1,000 trichocysts, depending on the strain (for detailed numbers see reference 39).

Measurements of ATP Pools in Cells

Cells were concentrated to a density of 2 x 10^9/ml. AED was added and at different time points afterwards cells were inactivated by lysing in 6.7% trichloracetic acid (TCA), 0°C, for 10 min. According to thermocouple measurements, cells attain 0°C within ~0.2 s. Samples were brought to pH 7.0 by adding 1.0 M Tris. A 100-μl sample, diluted 1:50 with distilled water, was mixed with 100 μl Lumit (commercial luciferase with adenylylimidodiphosphate (AIDP), a nonhydrolyzable ATP analogue from Sigma Chemical Co.) and adjusted to 10^-5 M. Cortices were used for up to 30 min or only slightly more, since after 60 min their reaction was diminished. The addition of a protease inhibitor cocktail (51) had no effect on our results and, therefore, was omitted in most experiments. About 20 to 30 preparations were evaluated per data point presented in Table I.

The trigger procedure outlined above entails the fusion of the trichocyst membrane with the cell membrane (as visualized repeatedly by electron microscopy). The trichocyst contents elongate without being completely discharged. The number of trichocysts undergoing exocytosis this way was counted in focus series under a phase-contrast microscope. To achieve reliable data, all cortex specimens were evaluated by counting trichocysts always only along the spherical end (e.g., at the lower left in Fig. 2 b). The release rate thus achieved under maximal trigger conditions was rated 100%, although only approximately half of the proportion of trichocysts was released when compared with experiments in vivo. This might be due to loss or damage of some of the trichocysts; both side effects were actually observed to occur.

To ascertain the occurrence of true exocytotic membrane fusion in the isolated cortex preparations, we also prepared cortices from nd9-28°C cells which are unable to fuse their trichocyst membrane with the cell membrane. We thus found only a small percentage of "false" exocytosis (see Results).

Electron Microscopy

Standard methods were used as in our previous work (36,37).

Results

In Vivo Effect of the ATP Concentration

We have manipulated intracellular ATP levels in different ways. When we injected 1 mM (final concentration) of Tris-ATP, this retarded AED-mediated exocytosis (that normally takes place within only ~1 s) for ~2 min (Table I). Only then exocytosis started and was completed within ~3 min. With Mg-ATP, the inhibition effect was somewhat less immediate, but it lasted over longer time periods (Table I). This difference might be explained by the fact, that ATP can bind some of the intracellular Mg^{2+}.

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trichocysts over much longer time periods, i.e., for up to 15 min.

The alternative experiment was the injection of apyrase that caused massive exocytosis (Fig. 1, Table I). Evidently it takes some time for the enzyme to diffuse to the cell periphery (as observed also with various fluorescent probes of similar size; our unpublished observations). Since the only apyrase isoform available was Ca$^{2+}$ dependent, Ca$^{2+}$ had to be co-injected ($10^{-5}$ M). Controls were as follows. (a) When only Ca$^{2+}$ was injected, this did not induce any exocytosis (although Ca$^{2+}$ is required for trichocyst release; see references 29, 39); the negative response might be due to rapid Ca$^{2+}$ sequestration. (b) Cells were fully viable during their actual experiment. (c) When other proteins (see Materials and Methods) were injected, this did not provoke trichocyst release.

ATP depletion could also be produced by inhibitors of energy metabolism. In these experiments, it was much more difficult than in experiments with apyrase to preserve the cells from cell damage. In a procedure outlined in Materials and Methods (20 min NAN3, supplemented for 5 min with trichocysts over much longer time periods, i.e., for up to 15 min. trichocysts (though they expand from a condensed to a rod-like form, as they do in vivo), remain lodged in the exocytotic like form, as they do in vivo), remain lodged in the exocytotic

**Table I. Effect of the Manipulation of ATP Concentration on Exocytosis In Vivo and In Vitro**

| Type of preparation | Type of manipulation | Mode of exocytosis triggering | Exocytotic response† | Remarks—including reaction after different time periods‡ |
|---------------------|----------------------|------------------------------|----------------------|--------------------------------------------------------|
| Cells without additives | None | AED | 100% | No injection; immediate exocytotic response (~1 s) |
| | Microinjection of 1 mM Tris-ATP | AED | 10 s p.i.§ | (a) 0% → (b) 10% → (c) 100% | (a) 0-1 min p.i. → (b) 1-2 min p.i. → (c) up to 3 min p.i. (delayed reaction) |
| | Microinjection of 1 mM Mg$^2+$-ATP | AED | 1 min p.i. | (a) 100% | (a) 1 min p.i. → (b) 1-3 min p.i. → (c) prolonged inhibition |
| | Microinjection of 1 mM AIDP | AED 30 s p.i. | 100% | (a) 100% | (a) 100% → (b) delayed and prolonged inhibition |
| | Microinjection of apyrase (~100 μg/ml) | see Type of manipulation | 100% | Exocytosis starting at injection site; completion within 90 s (Fig. 1) |
| Cells with metabolic inhibitors | NaN$_3$ → salicylhydroxamic acid (20 min → 5 min; 5 mM each) | see Type of manipulation | 25% | 29% ATP remaining; for side effects of drugs, see text |
| Cortices (in vitro exocytosis) | None | None | 0% | No exocytosis (Fig. 2 a) |
| | None (ATP <10$^{-6}$M) | None | 0% | 
| | 0.33 mM ATP | High Ca$^{2+}$/low Mg$^{2+}$ | 100% | Maximal exocytosis (Fig. 2 b) |
| | 1.0 mM ATP | High Ca$^{2+}$/low Mg$^{2+}$ | 50% | Half-maximal exocytosis† |
| | 2.0 mM ATP | High Ca$^{2+}$/low Mg$^{2+}$ | 0-10% | Almost total exocytosis inhibition |
| | 1.0 mM AIDP | High Ca$^{2+}$/low Mg$^{2+}$ | 0% | Total exocytosis inhibition |

* 75 and K401 cells were used for these experiments. Concentrations of microinjected materials are estimated final intracellular values.

† Full response (~90% of trichocyst population releasable from cells under optimal conditions [39]). For cortex preparations, the maximal number of releasable trichocysts was smaller than expected (see text); for these preparations, too, 100% indicates maximal values achieved. Numbers given here for exocytotic response are estimates valid within an estimated uncertainty of ± 10% due to the counting procedure. Within this limit data were also well reproducible from one experiment to another. For numbers of experiments (n), see Materials and Methods; for metabolic inhibitor studies, n = 17.

‡ Reaction time, ~1 min.

The conclusion from these in vivo experiments is that ATP exerts a negative modulatory effect on exocytosis at physiological concentrations (Table I) which are ~<1.3 mM (29).

**In Vitro Exocytosis and the Effect of the ATP Concentration**

We tried to substantiate this assumption by experiments in vitro, using isolated surface complexes ("cortices") (Figs. 2-4). These consist of cell membranes with trichocysts attached; they are devoid of cilia, but contain cortical microfilaments and microtubules, as well as cortical compartments of unknown function ("alveolar sacs") and some mitochondria. It was, therefore, important to ascertain that, in the absence of substrates, ATP levels were as low as ~<10$^{-6}$ M.

These cortices retain their exocytosis capacity for a maximum of 30-60 min after isolation. Upon changing the Mg$^{2+}$/Ca$^{2+}$ ratio from 10:0.01 (mM) during isolation to 0.5:0.01 (mM), trichocysts are being liberated over a period of 1 min (Fig. 2). It was crucial to show that (a) this process involves membrane fusion, i.e., real exocytosis (Fig. 4), and (b) endocytosis does not occur (Figs. 2 and 4). Evidently, endocytosis of trichocyst membranes is impeded by the fact that trichocysts (though they expand from a condensed to a rod-like form, as they do in vivo), remain lodged in the exocytotic opening (Figs. 2 and 4) for unknown reasons. (In contrast, there occurs very rapid exo-endocytosis coupling during
Figure 1. Phase-contrast micrographs of a cell. (a) 30, (b) 50, (c) 70, and (d) 90 s after injection of apyrase (0.1 mg/ml estimated final concentration in the cell). While the cell remains in constant motion, it discharges an increasing number of trichocysts, visible as rodlike structures in the medium. Bar, 100 μm.

As in vivo, ATP or AIDP clearly inhibit exocytosis in vitro as well (Table I). From a set of data using different ATP concentrations, we estimated concentrations required for maximal or half-maximal inhibition (Table I) to be 1.0 and 0.33 mM ATP, respectively. 1 mM AIDP inhibits exocytosis in vivo by 90%.

Since cortices prepared from exocytosis-incompetent nd9-28°C cells displayed a leakage rate of only ~5%, preparative artifacts can be largely excluded from these in vitro experiments.
Figure 2. Phase-contrast micrographs of isolated cortices (a) in the resting stage and (b) in a stage showing exocytosis. a shows resting trichocysts as rods inside a cortex (arrows). Note that trichocysts are not fully expelled but remain inserted in the cortex preparation (arrows in b). The limited depth of focus allows one to recognize only some of the extruding trichocysts. Bar, 10 μm.

Figure 3. Ultrathin sections of a cortex preparation, showing the presence of the cell membrane, underlying alveolar sacs (AS) and microfilamentous elements (MF). The occurrence of trichocysts, docked to the cell membrane, is evident particularly in median sections (T₁, T₂). Note also the absence of cilia and the presence of mitochondria, e.g., in a cluster around M. Bar, 1 μm.
Figure 4. Ultrathin section of trichocysts (T) contained in a cortex preparation that also shows the cell membrane (CM) and underlying alveolar sacs (AS). a is at rest, displaying the triehocyst tip (TT) and the trichocyst body (TB). b is after exocytosis in vitro, showing the fusion of the trichocyst membrane (TM) with the cell membrane (CM). Note also the decondensation of the periodically banded contents of the trichocyst body when it is released. Bar, 0.1 μm.

Decay of ATP Pools in the Course of Trichocyst Release

We also analyzed the time course of ATP pools in different strains in response to AED (Fig. 5) that causes exocytosis of up to ~1,000 trichocysts per cell (wild type) within ~1 s without any recognizable cell damage (36, 39). ATP pools reached a minimum 3–5 s after actual exocytosis performance. Hence, the decay of ATP pools actually measured cannot be due to exocytosis induction. Also, ATP is reduced maximally by 30%, whereas ATP must be reduced much more (see above and Table I) to achieve a massive release of trichocysts. The rapid reversal of ATP pools to normal values within ~30 s also agrees with previous estimates of the ATP turnover in these cells (29). The extent of ATP consumption found here, when referred to individual exocytosis events, also matches our previous data (29). However, experiments with different mutations showed that ATP consumption occurs also in nd6, nd9–28°C, and d1 mutations (Fig. 5). From these data and from the time course of ATP decay, we conclude that ATP is used for processes other than exocytosis (see below).

It is well established that Ca²⁺ is required not only for trichocyst exocytosis (29, 39), but that it also induces ciliary reversal (31) in all strains analyzed except for d4-500r (a "pawn" mutation [21]). In response to AED d4-500r cells also display a considerable decay of ATP concentrations, not different from other strains (Fig. 5). In fact, when d4-500r cells were triggered by AED to expel their trichocysts and when a second AED trigger was applied some time afterwards, the same ATP decay was obtained again (Fig. 6). We assume, therefore, that the ATP consumption measured in all these cases might possibly be due to an energy requirement for reestablishing the status quo, e.g., to reestablish resting Ca²⁺ concentrations. These data also explain the dilemma...
of an interpretation relating ATP measurements to secretory activity in many other systems.

Discussion

Possible Sites of ATP Involvement in Exocytosis

Our experiments aimed at specifying the possible role of ATP for exocytosis. It turned out that a decay of ATP concentrations or effects of energy metabolic inhibitors cannot be directly correlated with exocytotic membrane fusion (i.e., exocytosis in the proper sense). Precisely this had been inferred in many publications (see the introduction). Our system is one of the simplest available to analyze exocytosis, since trichocysts are arrested just before membrane fusion and can be expelled within \( \sim 1 \) s (36, 39). Exocytosis-coupled endocytosis occurring in vivo (37) is suppressed in the isolated cortices (Fig. 4).

The results obtained here with changing ATP concentrations in vivo and in vitro could be interpreted in line with a hypothesis for an indirect role of ATP for membrane fusion (5, 40, 54). Yet Poste and Allison (40), who propagated this
We should finally like to focus our attention on systems without large scale vesicle transport. Such "simple" systems are, e.g., (a) oocytes with their cortical granules aligned along the cell membrane and without the occurrence of membrane retrieval (19); (b) mast cells with their capacity to perform compound exocytosis (28); and (c) blood platelets with their tubular extensions of the surface membrane that allow for a rapid release of granule contents (34). Although these are "rapid secretors," they require up to 1 min to release most of their contents. Thus, they are by far slower than paramecia. It was found that mast cells can secrete, in principle, also without any ATP consumption (20, 24, 35). When, nevertheless, a reduction of the endogenous ATP pool is observed, this could be explained by several alternatives. (a) ATP consumption occurs only with some delay after exocytosis, i.e., in a recovery phase (24). (This corresponds to our findings in Fig. 5.) Accordingly glucose metabolism is delayed in a concomitant fashion (50). Beaven et al. (7) argue that ATP would be required for maintaining the Ca\textsuperscript{2+} signal for sustained histamine secretion. (b) Metabolic inhibitors reduce secretory activity only to an extent as anti-actin drugs do (15, 48), so that reactions of contractile elements rather than exocytosis per se could account for ATP consumption. These elements are not assumed, however, to cause exocytosis in mast cells (28), precisely as we assume for paramecia (25, 37). With thrombocytes, cell contraction accompanies secretory activity (34), which again could account for the ATP consumption, and metabolic inhibitors reduce secretion by only 50\% (45). As to oocytes, Whitaker and Baker (55) as well as Sasaki (44) present data showing considerable cortical granule discharge from permeabilized cells in the absence of exogenous ATP.

In retrospect, even when the simplest exocytosis systems, (i.e., mast cells, oocytes, and thrombocytes) or permeabilized cells were analyzed, no unequivocal evidence for a requirement of ATP for exocytosis per se could be obtained.

**Main Conclusions**

The widespread assumption of the requirement of ATP for secretory activity should be specified as follows. (a) ATP is likely to be required for the transport of organelles to/from the cell membrane or for different phosphorylation phenomena. Although protein phosphorylation is considered a crucial phenomenon for secretory activity, trichocyst secretion, which takes only ~1 s, is accompanied by reversible phosphorylation phenomena (59), and the time course of all these phenomena is not related at all to that of ATP pools. (b) In our opinion the major proportion of the ATP consumed during synchronous trichocyst release may be accounted for by ATP-dependent removal of Ca\textsuperscript{2+} (10) from the cytosol (since ATP decays in response to AED even in the absence of exocytosis or of ciliary reversal, the predominant, immediately visible Ca\textsuperscript{2+}-dependent reactions in paramecia). This could also explain the ATP consumption accompanying secretory activity in other systems. (c) From our point of view, the presence of ATP is not required for membrane fusion during exocytosis. ATP consumption during secretion does not logically infer that it would have to be present for fusion to occur. On the contrary, ATP could help to keep the system in a primed state. ATP hydrolysis (though it can account only for undetectably small amounts of the ATP decay measured in our experiments) could then represent one among several

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**Comparison with Other Systems**

How would this fit into the wide-spread assumption that ATP were required for exocytosis? As stated in the introduction, one has inferred from experiments with quite different cell types that "ATP is required for exocytosis." Although experiments with electrically permeabilized chromaffin cells, which allow for a deliberate manipulation of the ATP concentrations, seemed to confirm this claim (3, 27), Baker and his associates clearly warned that other processes might account for the ATP requirement for the overall response. Among them are granule transport and ghost retrieval that occur also in permeabilized cells (3, 27), as well as reversible ATP-dependent binding of some cytosolic proteins (14). At least the energy requirement for intracellular vesicle transport appears well established from studies with widely different systems (1, 43, 52, 53). Alternatively, it had been noted with differently permeabilized chromaffin (18, 56) or pancreatic island cells (33) that secretory activity can be induced to a considerable extent without exogenous ATP added.

Figure 6. ATP consumption in strain d4-500r measured 3–5 s after adding AED. Trichocysts are expelled only in response to the first stimulus, but not to the second one (applied 1 h later after thorough washes). Since this strain is unable to perform ciliary reversal (another Ca\textsuperscript{2+}-requiring, ATP-consuming phenomenon occurring in other strains; see text), the ATP consumption (at least in response to the second trigger) cannot be due to either exocytosis or ciliary reversal. ep, error probability; N, number of experiments.
possible factors relevant for the initiation of membrane fu-
mains unknown so far, but possible aspects arising from the
literature are (a) de-inhibition of ATP-induced cation chan-
bservations leading to (b) reversible binding of Ca\(^{2+}\) to the
membrane depending on ATP (13); (c) a possible fusogenic
effect of free phosphate generated by ATP hydrolysis (23,
58); (d) a general destabilizing effect achieved by removing
ATP from membranes (40). (e) Other mechanisms could
operate via proton-pumping ATPases of secretory granules
(see reference 4).

It remains to be seen whether any of these mechanisms
cooperate with other fusogenic processes and whether
membrane fusion during exocytosis follows the same prin-
ciples in all the different systems. Baker and Knight (4) find it
... necessary to take very seriously the possibility that ATP is
required ... perhaps not at all for exocytosis.” This suspi-
cion at least can now be fully supported by our study.

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