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

Jeannine Vilmart-Seuwen, Helmut Kersken, Renate Stürzl, and Helmut Plattner

Faculty of Biology, University of Konstanz, D-7750 Konstanz, Federal Republic of Germany

Abstract. We have tried to specify a widespread hypothesis on the requirement of ATP for exocytosis (membrane fusion). With *Paramecium tetraurelia* cells, synchronously ($\sim$1 s) exocytosing trichocysts, 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 vitro exocytosis is facilitated in ATP-free media. 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.

I T 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 (11, 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)[1] 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).

1. *Abbreviations used in this paper:* AED, aminoethyldextran; 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^{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 $\geq$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 exocytotic 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 reversal 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 tam38 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 aminoethyldextran (AED) as a trigger agent (36, 39) and with ~10^{-4} 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 × 10^4/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 luciferin–luciferase with adequate Mg^{2+} content) from Lumac (Medical Products Division, St. Paul, MN; and Abimed, Düsseldorf, Federal Republic of Germany). Calibration was performed with 25–500 pg ATP/100-μl sample. To follow the time course of ATP consumption during synchronous exocytosis more easily, all data were normalized and expressed as percentage of the value before triggering.

In a similar way we also estimated the concentration of ATP in isolated cortex preparations.

Application of Metabolic Inhibitors to Cells

The effect of the following metabolic inhibitors on exocytosis performance was tested. We applied 5 mM NaN_3 (10–20 min, followed by 5 min addition of 5 mM salicylhydroxamic acid (Sigma Chemical Co., St. Louis, MO) to block the cyanide-insensitive respiratory shunt in these cells (16, 17, 32). We tested in parallel the vitality of the cells, the actual ATP pool, and spontaneous as well as AED-mediated exocytosis. Aliquots were washed and the same parameters were analyzed again. It was attempted to maintain vitality at >90% during these manipulations and samples below this limit were not further evaluated.

Quantitation of Exocytosis Performance by Cells

This can easily be done with individual cells by counting the number of trichocysts released under a phase-contrast microscope (36, 39). We also used our previous method (36, 39) to determine the total numbers of trichocysts.

Microinjection Experiments

The method used was as described by Kersken et al. (25). The volume injected into 7S or K401 cells was ~10% of the cell volume. Apyrase (ATP diphosphohydrolase, Ca^{2+}-dependent form EC 3.6.1.5) from Sigma Chem-

ical Co. (type A6132) was microinjected in a final concentration of 100 μg/ml, together with 10^{-5} M Ca^{2+}. For controls, we injected Ca^{2+} alone or other proteins of widely different size and charge (microperoxidase; horseradish peroxidase [Sigma type VII]; bovine serum albumin; preimmune sera from rabbits; each at a concentration of 100 μg/ml). All this remained without any immediate or retarded biological effects pertinent to this study.

Alternatively, Tris-ATP or Mg-ATP (Sigma Chemical Co.) was injected to double the endogenous ATP concentration (~1 mM; reference 29) and AED was added to the outside medium 10 s to ≥1 min afterwards to observe a retardation effect of injected ATP. Similar experiments were conducted with adenylylimidodiphosphate (AIDP), a nonhydrolyzable ATP analogue from Sigma Chemical Co.

All injected compounds were dissolved in 10 mM Tris-HCl buffer, pH 7.4. This "injection buffer" showed no effects when injected alone. Up to 10 experiments were performed to obtain one data point presented in Table I.

Isolation of Cortices and Exocytosis In Vitro

Cortex preparations were obtained from wild-type (7S) cells by gentle homogenization in a glass homogenizer with a loosely fitting glass pestle. The homogenizing medium consisted of Tris-maleate buffer (5 mM, pH 7.0) with 10 mM $MgCl_2$ and 10^{-5} M $CaCl_2$ added. Isolation was carried out by centrifugation (800 rpm, 10 min at 4°C) in a "Minifuge" from Heraeus Christ (Osterode, FRG), equipped with a swing-out rotor.

Exocytosis was induced in these in vitro preparations by changing the Ca^{2+}/Mg^{2+} ratio to 10^{-5} M Ca^{2+} and 0.5 mM Mg^{2+}, in the same buffer, at room temperature. In these assays Tris-ATP, or eventually AIDP, was added in concentrations between 0 and 2 mM (pH adjusted to 7.0). The final free Ca^{2+} concentration was always controlled with a Ca^{2+}-selective electrode (Radiometer type 2112) 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-induced 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+}). By and large the response to ATP correlates approximately with the endogenous concentration of ATP and its turnover time estimated from O_2 consumption (29). In contrast, microinjected AIDP inhibited, with some delay (see Table I), the release of

*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–ATP	AED } 1 min p.i.	(a) 30% → (b) 50%	(a) 1 min p.i. → } prolonged (b) 1-3 min p.i. } inhibition
	Microinjection of 1 mM AIDP	AED 30 s p.i. AED 150 s p.i.	100% 0%	} Delayed but prolonged inhibition up to 15 min (tested)
	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 } (ATP <10^{-6}M)	None	0%	No exocytosis (Fig. 2 a)
		High Ca^{2+}/low Mg^{2+}	100%	Maximal exocytosis (Fig. 2 b)
	0.33 mM ATP	High Ca^{2+}/low Mg^{2+}	50%	Half-maximal exocytosis‖
	1.0 mM ATP	High Ca^{2+}/low Mg^{2+}	0-10%	Almost total exocytosis‖ inhibition
	2.0 mM ATP	High Ca^{2+}/low Mg^{2+}	0%	Total exocytosis inhibition
	1.0 mM AIDP	High Ca^{2+}/low Mg^{2+}	0%	Total exocytosis inhibition

* 7S and K401 cells were used for these experiments. Concentrations of microinjected materials are estimated final intracellular values.
‡ 100% = 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.
§ p.i., post injection.
‖ Reaction time, ~1 min.

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 exocytotic response period, since all cell functions recognizable in the microscope were maintained over a period exceeding the experiment; also trypan blue was excluded from such cells over a time period much longer than required for the 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 NaN_3, supplemented for 5 min with salicylhydroxamic acid) we could combine the least cell damage with the maximum effect on cells (percentage of cells showing ciliary arrest and exocytosis). We obtained 90-95% viable cells, 55% being immobilized. As indicated in Table I, cellular ATP was reduced to 29% (± 1; n = 17); this was paralleled by an exocytosis rate of ~25%. Aliquots of these

samples could replenish their ATP pool to 85%, when inhibitors were washed out. Higher concentrations or longer time periods could not be used in these inhibitor studies because of increasing irreversible cell damage.

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 rodlike 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
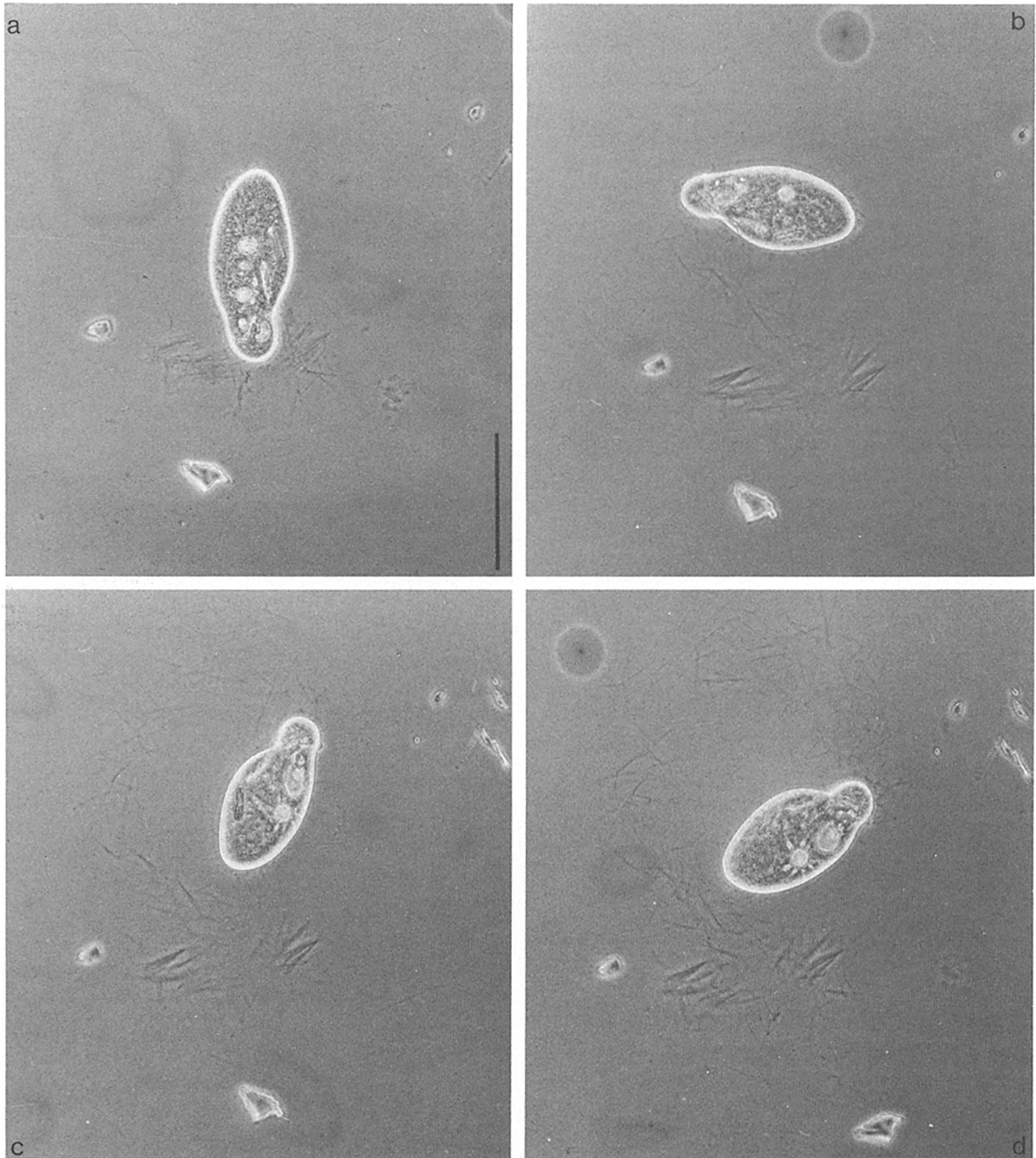
Figure 1. Phase-contrast micrographs of a cell. (*a*) 30, (*b*) 50, (*c*) 70, and (*d*) 90 s after injection of apyrase (0.1 mg/ml estimated final concentration in the cell). While the cell remains in constant motion, it discharges an increasing number of trichocysts, visible as rodlike structures in the medium. Bar, 100 μm.

trichocyst release in vivo [37] that would complicate the interpretation of such experiments).

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.
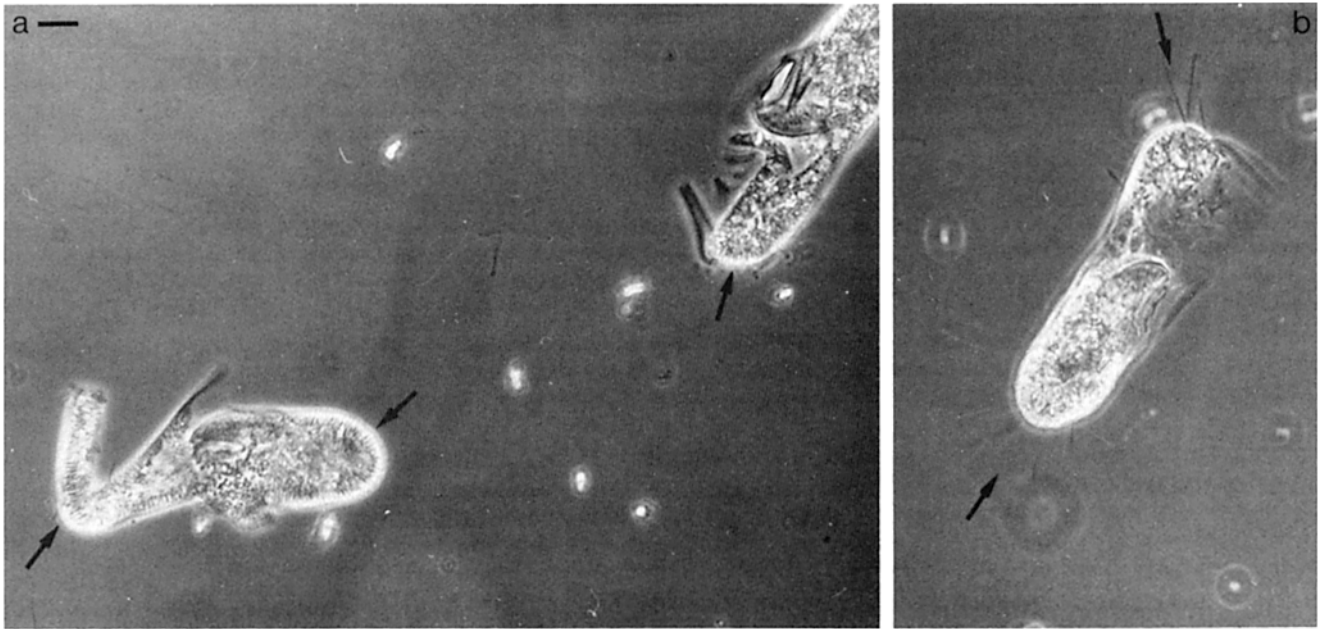
Figure 2. Phase-contrast micrographs of isolated cortices (*a*) in the resting stage and (*b*) in a stage showing exocytosis. *a* shows resting trichocysts as rods inside a cortex (*arrows*). Note that trichocysts are not fully expelled but remain inserted in the cortex preparation (arrows in *b*). The limited depth of focus allows one to recognize only some of the extruding trichocysts. Bar, 10 μm.
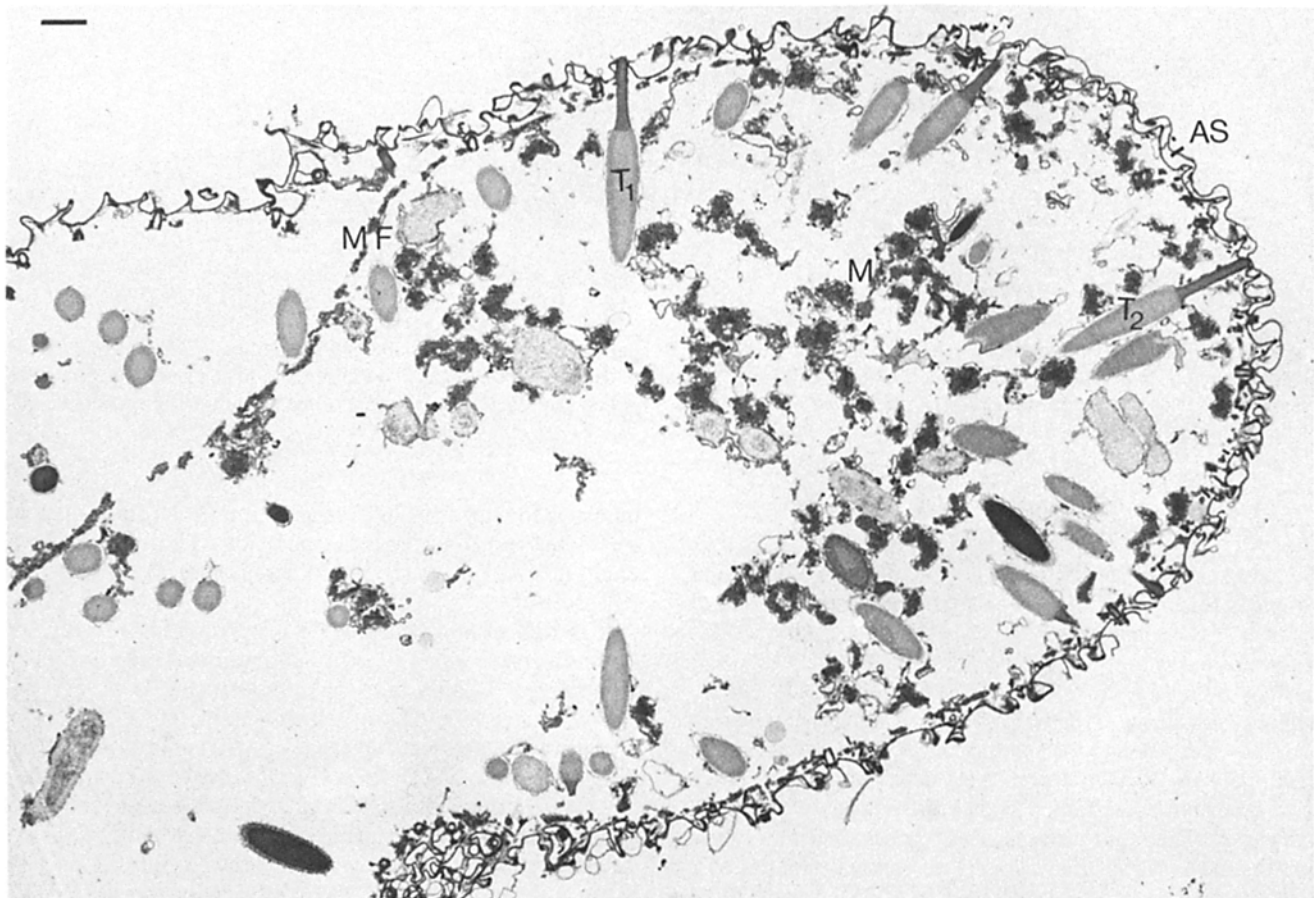

Figure 3. Ultrathin sections of a cortex preparation, showing the presence of the cell membrane, underlying alveolar sacs (*AS*) and microfilamentous elements (*MF*). The occurrence of trichocysts, docked to the cell membrane, is evident particularly in median sections (T_1, T_2). Note also the absence of cilia and the presence of mitochondria, e.g., in a cluster around *M*. Bar, 1 μm.
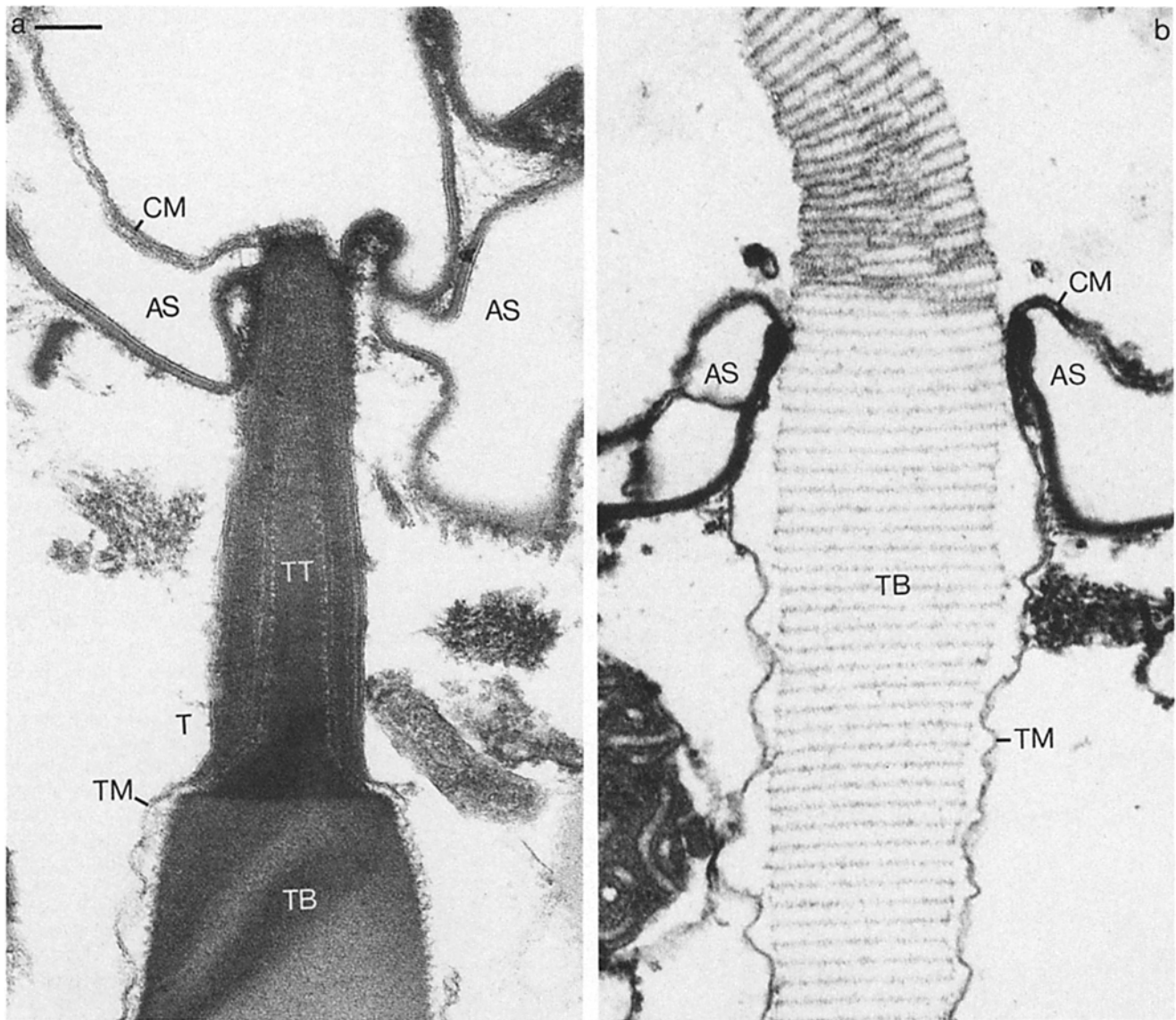
Figure 4. Ultrathin section of trichocysts (*T*) contained in a cortex preparation that also shows the cell membrane (*CM*) and underlying alveolar sacs (*AS*). *a* is at rest, displaying the trichocyst tip (*TT*) and the trichocyst body (*TB*). *b* is after exocytosis in vitro, showing the fusion of 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 oc-

curs also in *nd6*, *nd9–28° C*, and *tl* 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 Ca^{2+} 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 Ca^{2+} concentrations. These data also explain the dilemma
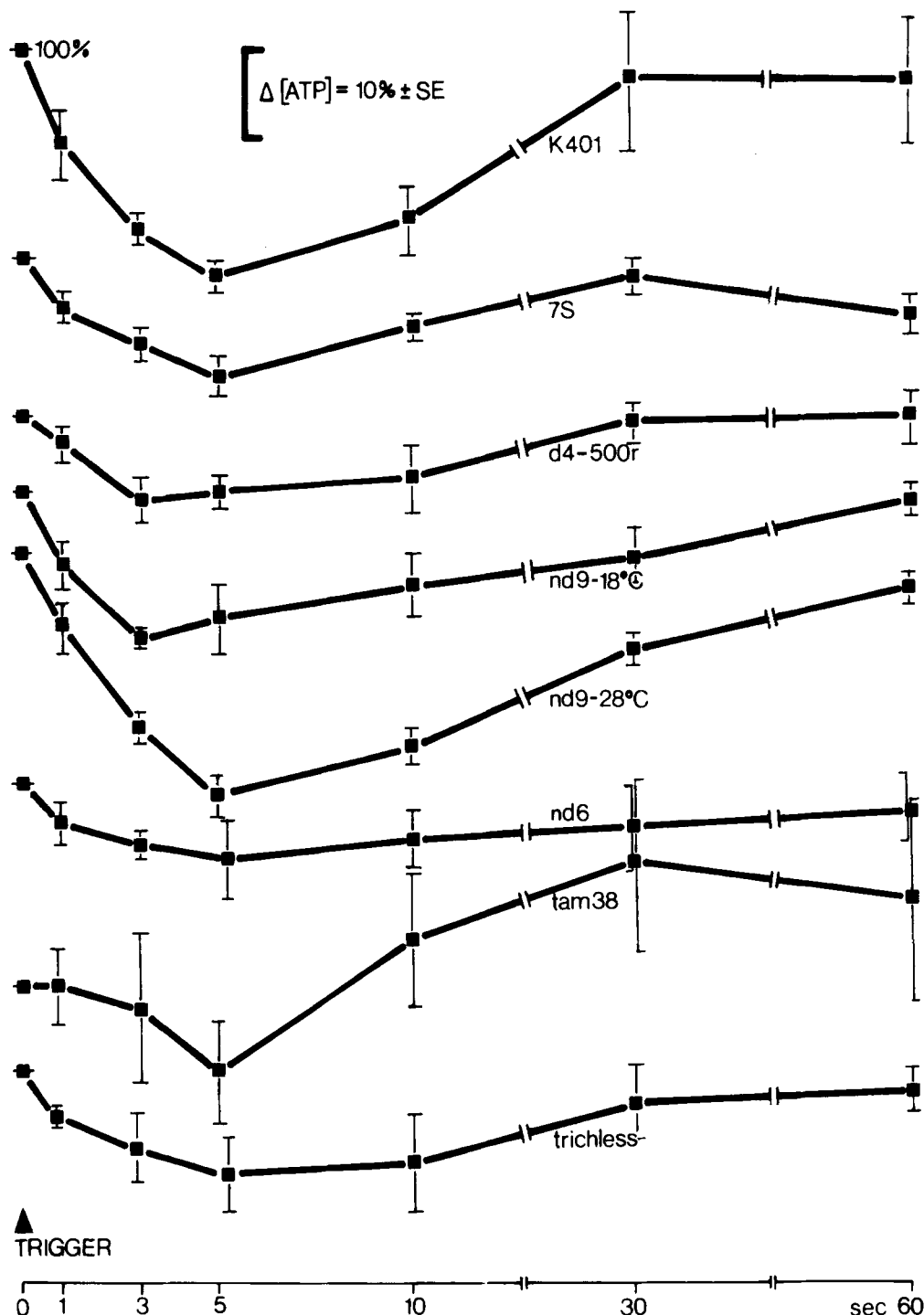
Figure 5. Reversible decay of ATP pools after synchronous exocytosis in different strains of *Paramecium tetraurelia* (see Materials and Methods). The actual time required for the release of trichocysts—occurring in strains *K401, 7S, d4-500r,* and *nd9-18°C* (but not in others)—is in the range of 1 s. For the sake of better comparison ATP concentrations were normalized, so that data point at 0 s has no standard error indicated. 10% decay is indicated by the bar on top. Individual data points are from 12-18 experiments each. The most extensive decay of ATP pools occurs with strains *K401* and *nd9-28°C*; these are strains with somewhat lower ATP pools. Note that in all strains ATP pools decay over 5 s and are replenished after 30-60 s. Only strain *tam38* displays considerable overcompensation. Important aspects are (*a*) the occurrence of ATP decay also in strains without exocytosis performance and (*b*) its time course that by far exceeds the time required for exocytosis (1 s) in competent strains.

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 ~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
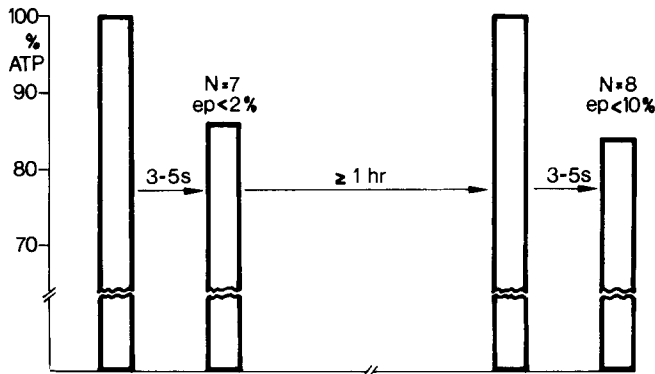
Figure 6. ATP consumption in strain *d4-500r* measured 3–5 s after adding AED. Trichocysts are expelled only in response to the first stimulus, but not to the second one (applied 1 h later after thorough washes). Since this strain is unable to perform ciliary reversal (another Ca^{2+}-requiring, ATP-consuming 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.

hypothesis, also considered other ATP-consuming steps, such as actomyosin activation in exocytosis regulation—a hypothesis disproved for paramecia (25, 37). The possible role of ATP as a negative modulator for exocytotic membrane fusion is further supported by the inhibitory effect of AIDP (Table I).

In support of a hypothesis that assumes ATP hydrolysis as a fusogenic step, we had previously found cytochemical evidence for a Ca^{2+}-ATPase at trichocyst extrusion sites in exocytosis-competent strains (38). (This has been corroborated by spectrophotometric Ca^{2+}-ATPase measurements [8, 51]). Due to possible overlaps this might include a variable protein kinase–phosphatase system, since non-exocytosing strains selectively do not show such a dephosphorylation step (59). Concomitantly, γ-thiophosphorylation, which is not accessible for phosphatase activity, inhibits secretory activity in chromaffin cells, possibly by keeping it in a primed state (9). It thus agrees well that both factors, absence of ATP and protein dephosphorylation, stimulate exocytosis in paramecia.

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.

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^{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 only by 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 wide-spread 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 dephosphorylation 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^{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^{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

possible factors relevant for the initiation of membrane fusion during exocytosis.

How ATP removal could promote membrane fusion remains unknown so far, but possible aspects arising from the literature are (a) de-inhibition of ATP-induced cation channel blockage (12); (b) reversible binding of Ca^{2+} to the cell 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 principle 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 suspicion at least can now be fully supported by our study.

References

1. Adams, R. J. 1982. Organelle movement in axons depends on ATP. *Nature (Lond.).* 297:327-329.

2. Akkerman, J. W. N., G. Gorter, L. Schrama, and H. Holmsen. 1983. A novel technique for rapid determination of energy consumption in platelets. Demonstration of different energy consumption associated with three secretory responses. *Biochem. J.* 210:145-155.

3. Baker, P. F., and D. E. Knight. 1981. Calcium control of exocytosis and endocytosis in bovine adrenal medullary cells. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 296:83-103.

4. Baker, P. F., and D. E. Knight. 1984. Calcium control in bovine adrenal medullary cells. *Trends Neurosci.* 7:120-126.

5. Baker, P. F., and M. J. Whitaker. 1978. Influence of ATP and calcium on the cortical reaction in sea urchin eggs. *Nature (Lond.).* 276:513-515.

6. Bauduin, H., M. Colin, and J. E. Dumont. 1969. Energy sources for protein synthesis and enzymatic secretion in rat pancreas in vitro. *Biochim. Biophys. Acta.* 174:722-733.

7. Beaven, M. A., J. Rogers, J. P. Moore, T. R. Hesketh, G. A. Smith, and J. C. Metcalfe. 1984. The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *J. Biol. Chem.* 259:7129-7136.

8. Bilinski, M., H. Plattner, and R. Tiggemann. 1981. Isolation of surface membranes from normal and exocytotic mutant strains of *Paramecium tetraurelia*. Ultrastructural and biochemical characterization. *Eur. J. Cell Biol.* 24:108-115.

9. Brooks, J. C., S. Treml, and M. Brooks. 1984. Thiophosphorylation prevents catecholamine secretion by chemically skinned chromaffin cells. *Life Sci.* 35:567-574.

10. Browning, J. L., and D. L. Nelson. 1976. Biochemical studies of the excitable membrane of *Paramecium aurelia*. I. $^{45}Ca^{2+}$ fluxes across resting and excited membrane. *Biochim. Biophys. Acta.* 448:338-351.

11. Chakravarty, N. K. 1979. The metabolism of mast cells and biochemical changes associated with histamine release. *In* The Mast Cell, Its Role in Health and Disease. J. Pepys and A. M. Edwards, editors. Pitman Medical Publishing Co., Kent, UK. 38-46.

12. Cook, D. L., and C. N. Hales. 1984. Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature (Lond.).* 311:271-273.

13. Corpus, V., and A. Y. Sun. 1983. Effects of ATP on calcium binding to synaptic plasma membrane. *Neurochem. Res.* 8:501-520.

14. Creutz, C. E., L. G. Dowling, J. J. Sando, C. Villar-Palasi, J. H. Whipple, and W. J. Zaks. 1983. Characterization of the chromobindins. Soluble proteins that bind to the chromaffin granule membrane in the presence of Ca^{2+}. *J. Biol. Chem.* 258:14664-14674.

15. Douglas, W. W., and Y. Ueda. 1973. Mast cell secretion (histamine release) induced by 48-80: calcium-dependent exocytosis inhibited strongly by cytochalasin only when glycolysis is rate-limiting. *J. Physiol.* 234:97P-98P.

16. Doussière, J. 1977. La respiration cyano-résistante chez *Paramecium tetraurelia*. Ph.D. thesis. Université Scientifique et Médicale de Grenoble. 1-200.

17. Doussière, J., and P. V. Vignais. 1984. AMP-dependence of the cyanide-insensitive pathway in the respiratory chain of *Paramecium tetraurelia*. *Biochem. J.* 220:787-794.

18. Dunn, L. A., and R. W. Holz. 1983. Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J. Biol. Chem.* 258:4989-4993.

19. Epel, D., and V. D. Vacquier. 1978. Membrane fusion events during invertebrate fertilization. *In* Membrane Fusion. G. Poste and G. L. Nicolson, editors. Elsevier/North-Holland, Amsterdam, New York, Oxford. 1-63.

20. Foreman, J. C., J. L. Mongar, and B. D. Gomperts. 1973. Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. *Nature (Lond.).* 245:249-251.

21. Haga, N., M. Forte, Y. Saimi, and C. Kung. 1982. Microinjection of cytoplasm as a test of complementation in *Paramecium*. *J. Cell Biol.* 92:559-564.

22. Herzog, V., H. Sies, and F. Miller. 1976. Exocytosis in secretory cells of rat lacrimal gland. Peroxidase release from lobules and isolated cells upon cholinergic stimulation. *J. Cell Biol.* 70:692-706.

23. Hoekstra, D., J. Wilschut, and G. Scherphof. 1985. Fusion of erythrocyte ghosts induced by calcium phosphate and calcium-phosphate complexes. *Eur. J. Biochem.* 146:131-140.

24. Johansen, T. 1983. Utilization of adenosine triphosphate in rat mast cells during and after secretion of histamine in response to compound 48/80. *Acta Pharmacol. Toxicol.* 53:245-249.

25. Kersken, H., M. Momayezi, C. Braun, and H. Plattner. 1986. Filamentous actin in *Paramecium* cells: functional and ultrastructural changes correlated with phalloidin affinity labeling in vivo. *J. Histochem. Cytochem.* 34:455-465.

26. Kirshner, N., and W. J. Smith. 1966. Metabolic requirements for secretion from the adrenal medulla. *Science (Wash. DC).* 154:422-423.

27. Knight, D. E., and P. F. Baker. 1982. Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J. Membr. Biol.* 68:107-140.

28. Lagunoff, D., and E. Y. Chi. 1980. Cell biology of mast cells and basophils. *In* Cell Biology of Inflammation. G. Weissmann, editor. Elsevier North Holland Biomedical Press. Amsterdam. 217-265.

29. Matt, H., M. Bilinski, and H. Plattner. 1978. Adenosinetriphosphate, calcium and temperature requirements for the final steps of exocytosis in *Paramecium* cells. *J. Cell Sci.* 32:67-86.

30. Matthews, E. K. 1975. Calcium and stimulus-secretion coupling in pancreatic cells. *In* Calcium Transport in Contraction and Secretion. E. Garafoli, F. Clementi, W. Drabikowski, and A. Margreth, editors. Elsevier/North-Holland, Amsterdam, Oxford. 203-210.

31. Naitoh, Y., and H. Kaneko. 1972. Reactivated Triton-extracted models of *Paramecium*: modification of ciliary movement by calcium ions. *Science (Wash. DC).* 176:523-524.

32. Ogura, T., T. Irie, and I. Usuki. 1985. Respiratory inhibition by cyanide and salicylhydroxamic acid on the three species of *Paramecium* in stationary growth phase. *Comp. Biochem. Physiol.* 80A:167-171.

33. Pace, C. S., J. T. Tarvin, A. S. Neighbors, J. A. Pirkle, and M. H. Greider. 1980. Use of a high voltage technique to determine the molecular requirements for exocytosis in islet cells. *Diabetes.* 29:911-918.

34. Painter, R. G., and M. H. Ginsberg. 1984. Centripetal myosin redistribution in thrombin-stimulated platelets. Relationship to platelet factor 4 secretion. *Exp. Cell Res.* 155:198-212.

35. Peterson, C., and B. Diamant. 1974. Increased utilization of endogenous ATP in isolated rat mast cells during histamine release induced by compound 48/80. *Acta Pharmacol. Toxicol.* 34:337-346.

36. Plattner, H., H. Matt, H. Kersken, B. Haacke, and R. Stürzl. 1984. Synchronous exocytosis in *Paramecium* cells. I. A novel approach. *Exp. Cell Res.* 151:6-13.

37. Plattner, H., R. Pape, B. Haacke, K. Olbricht, C. Westphal, and H. Kersken. 1985. Synchronous exocytosis in *Paramecium* cells. VI. Ultrastructural analysis of membrane resealing and retrieval. *J. Cell Sci.* 77:1-17.

38. Plattner, H., K. Reichel, H. Matt, J. Beisson, M. Lefort-Tran, and M. Pouphile. 1980. Genetic dissection of the final exocytosis steps in *Paramecium tetraurelia* cells: cytochemical determination of Ca^{2+}-ATPase activity over preformed exocytosis sites. *J. Cell Sci.* 46:17-40.

39. Plattner, H., R. Stürzl, and H. Matt. 1985. Synchronous exocytosis in *Paramecium* cells. IV. Polyamino compounds as potent trigger agents for repeatable trigger-redocking cycles. *Eur. J. Cell Biol.* 36:32-37.

40. Poste, G., and A. C. Allison. 1973. Membrane fusion. *Biochim. Biophys. Acta.* 300:421-465.

41. Pouphile, M., M. Lefort-Tran, H. Plattner, M. Rossignol, and J. Beisson. 1986. Genetic dissection of the morphogenesis and dynamics of exocytosis sites in *Paramecium*. *Biol. Cell.* 56:151-162.

42. Ranadive, N. S., and C. G. Cochrane. 1971. Mechanism of histamine release from mast cells by cationic protein (band 2) from neutrophil lysosomes. *J. Immunol.* 106:506-516.

43. Riezman, H. 1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell.* 40:1001-1009.

44. Sasaki, H. 1984. Modulation of calcium sensitivity by a specific cortical protein during sea urchin egg cortical vesicle exocytosis. *Dev. Biol.* 101:125-135.

45. Sanchez, A. 1985. Ca^{2+}-independent secretion is dependent on cytoplas-

mic ATP in human platelets. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 191:283–286.

46. Santos-Sacchi, J., and M. Gordon. 1981. Depletion of ATP is associated with the acrosome reaction in guinea pig sperm. *J. Cell Biol.* 91(2, Pt. 2):168a. (Abstr.)

47. Schmidt, H. W., V. Herzog, and F. Miller. 1980. Oxygen consumption of isolated acini from rat parotid gland. *Eur. J. Cell Biol.* 20:201–208.

48. Schnitzler, S., H. Renner, and U. Pfüller. 1981. Histamine release from rat mast cells induced by protamine sulfate and polyethylene imine. *Agents & Actions.* 11:73–74.

49. Schön, E. A., and G. L. Decker. 1981. Ion-dependent stages of the cortical reaction in surface complexes isolated from *Arbacia punctulata* eggs. *J. Ultrastruct. Res.* 76:191–201.

50. Svendstrup, F., and N. Chakravarty. 1977. Glucose metabolism in rat mast cells during histamine release. *Exp. Cell Res.* 106:223–231.

51. Tiggemann, R., and H. Plattner. 1982. Possible involvement of a calmodulin-regulated Ca^{2+}-ATPase in exocytosis performance in *Paramecium tetraurelia* cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 148:226–230.

52. Tolleshaug, H., S. O. Kolset, and T. Berg. 1985. The influence of cellular ATP levels on receptor-mediated endocytosis and degradation of asialoglycoproteins in suspended hepatocytes. *Biochem. Pharmacol.* 34:1639–1645.

53. Vale, R., B. J. Schnapp, T. S. Reese, and M. P. Sheetz. 1985. Organelle,

bead, and microtubule translocations promoted by soluble factors from the squid giant axon. *Cell.* 40:559–569.

54. Viveros, O. H. 1975. Mechanism of secretion of catecholamines from adrenal medulla. *In* Handbook of Physiology, Section 7, Vol. 6. H. Blaschko, G. Sayers, and A. D. Smith, editors. Am. Physiol. Soc. Washington, D. C. 389–426.

55. Whitaker, M. J., and P. F. Baker. 1983. Calcium-dependent exocytosis in an *in vitro* secretory granule plasma membrane preparation from sea urchin eggs and the effects of some inhibitors of cytoskeletal function. *Proc. R. Soc. Lond. B Biol. Sci.* 218:397–413.

56. Wilson, S. P., and N. Kirshner. 1983. Calcium-evoked secretion from digitonin-permeabilized adrenal medullary chromaffin cells. *J. Biol. Chem.* 258:4994–5000.

57. Woodin, A. M., and A. A. Wieneke. 1964. The participation of calcium, adenosine triphosphate and adenosine triphosphatase in the extrusion of the granule proteins from polymorphonuclear leucocytes. *Biochem. J.* 90:498–509.

58. Zakai, N., R. G. Kulka, and A. Loyter. 1977. Membrane ultrastructural changes during calcium phosphate-induced fusion of human erythrocyte ghosts. *Proc. Natl. Acad. Sci. USA.* 74:2417–2421.

59. Zieseniss, E., and H. Plattner. 1985. Synchronous exocytosis in *Paramecium* cells involves very rapid (≤ 1 s), reversible dephosphorylation of a 65-kD phosphoprotein in exocytosis-competent strains. *J. Cell Biol.* 101:2028–2035.