Cell surface complexes ('cortices') isolated from *Paramecium tetraurelia* cells as a model system for analysing exocytosis *in vitro* in conjunction with microinjection studies

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Cortex preparations isolated from *Paramecium tetraurelia* cells consist of surface membranes with secretory organelles (trichocysts) still attached. In the absence of nucleotides, in media with a pCa of 5–5.5 and a pH of ≥ 6.5 , maximal exocytosis occurred when the Mg^{2+} concentration was lowered from 10 to 0.5 mM. ATP, as well as its non-hydrolysable analogues adenosine 5'-[γ -thio]triphosphate (ATP[S]) and adenosine 5'-[$\beta\gamma$ -imido]triphosphate (App[NH]p), inhibited exocytosis at a concentration equivalent to that occurring *in vivo* (as determined by h.p.l.c.), but preincubation with ATP augmented the exocytotic response. GTP and its analogues only slightly stimulated exocytosis *in vitro*, but sensitivity to Ca^{2+} was increased significantly, in particular with GTP. These effects of nucleotides were rapidly reversible. Intracellular GTP concentrations (0.35 mM) would suffice for full activation with the pCa_i values assumed to occur in these cells during activation. On microinjection, ATP inhibited the secretagogue response in intact cells. Whereas microinjected GTP stimulated exocytosis (membrane fusion) without a secretagogue added, Gpp[NH]p remained without any effect; GTP[S] permanently abolished any triggered secretory response. Concomitantly, h.p.l.c. analysis of triggered and untriggered cells showed that GTP hydrolysis occurs immediately after synchronous (1 s) exocytosis *in vivo*. The precise site(s) of action of GTP during signal transduction in *Paramecium* cells remain to be determined.

INTRODUCTION

Regulation of triggered exocytosis depends on various cytosolic factors including proteins, Ca^{2+} and nucleotides (for review, see [1,2]). With many cells, the techniques of electroporation and chemical permeabilization have been applied to allow such regulatory compounds or ions to gain access to exocytosis sites [1]. In these cases, however, these components have access to not only the internal but also the external side, and some effects might be difficult to interpret. For instance, Ca^{2+} in many cases is considered to be involved not only in membrane fusion regulation [2–4], but also in the discharge of secretory contents [5]. This is the case in *Paramecium* cells [6], in which we also found a cytotoxic effect of exogenous ATP or GTP. Since patch-clamp procedures are also not applicable to all cell types, e.g. to *Paramecium* cells, use of two different methods of compounds to be tested, by permeabilization or microinjection, appears to be feasible.

The *Paramecium* cell is a favourable system for analysing the final steps in exocytosis for various reasons. Most secretory organelles (trichocysts) are firmly attached to the cell membrane, ready for immediate release in response to polyamine secretagogues (for review, see [6]). The cell surface complex ('cortex'), with trichocysts and subplasmalemmal compartments (alveolar sacs, probably Ca^{2+} stores) still attached, can be isolated for studies *in vitro* [7]. In addition, substances can be easily microinjected into *Paramecium* cells [8]. Trichocyst contents are released as thin needles that can be counted in the light microscope for quantification of exocytosis [7].

Therefore we have now used the *Paramecium* system for analysis of regulation of exocytosis both *in vitro* and in microinjection studies. Since ATP and GTP are seen to be important, we have also determined their concentrations *in vivo* by h.p.l.c., both before and immediately after exocytosis which in this system takes place synchronously within 1 s [6]. This allows

the estimation of GTP consumption per exocytotic event and thus may give some clues to possible mechanisms involved in signal transduction.

It is also important to compare secretory cells of a different evolutionary level to understand basic mechanisms in common to all eukaryotes. Only in a limited number of higher eukaryotic cells, like egg cells [9], can the majority of secretory vesicles also be released without any interference of intracellular transport phenomena, as in *Paramecium*. So far, cortex preparations comparable to ours have been obtained only from some egg cells [9,10].

MATERIALS AND METHODS

Preparation of cortices

Cultivation of *Paramecium tetraurelia* strain 7S (wild type), counting of cell numbers and processing of cortices was as described before [7]. Briefly, cells were disrupted in a glass Potter–Elvehjem homogenizer with a loosely fitting Teflon pestle (clearance 0.1 mm, 20–30 strokes) at room temperature in 5 mM-Tris/maleate/10 mM-MgCl₂, pH 7.0. Cell disruption was assessed by light microscopy and cortices were isolated at 4 °C by centrifugation (50 g, 2 min). Exocytosis was triggered *in vitro* by diluting the MgCl₂ concentration from 10 to 0.5 mM with 5 mM-Tris/maleate/50 mM-KCl in the presence of 10 μ M- Ca^{2+} . The medium was thus roughly adjusted to mimic intracellular conditions (Table 1) (the omission of Na⁺ had no effect in pilot experiments). Quantification of exocytosis *in vitro* in the phase-contrast microscope was also as described before [7].

In some experiments, KCl was replaced by potassium glutamate or potassium gluconate and MgCl₂ by MgSO₄ for reasons indicated by Baker & Knight [3]. However, in this case the exocytotic response was lower than with the standard conditions indicated above.

Abbreviations used: AED, aminoethyl-dextran; App[NH]p, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; Gpp[NH]p, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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Measurement of pCa

Actual pCa values were determined using a Ca^{2+} -carrier type ETH 129 electrode [19]. Calibration curves were obtained with pCa values between 10 and 2 in the following buffers [20]: EGTA (pCa = 7–10), HEEDTA (*N*-[2-hydroxyethyl]ethylenediamine-*NN'*-triacetic acid; pCa = 6) or nitrilotriacetate (pCa = 4–5). Over the whole pCa range the readings (in mV) increased linearly with pCa. In every experiment (and also after adding nucleotides) the actual free Ca^{2+} concentration was determined in the applied medium. Since some side-effects of Ca^{2+} buffers are difficult to control [21], we used EGTA only for experiments with cortices at pCa < 5.

Measurement of endogenous nucleotides

To determine endogenous nucleotide concentrations a 1.5 ml portion containing 3×10^5 cells in 5 mM-Pipes (disodium salt)/1 mM- CaCl_2 /1 mM-KCl, pH 7.0, was pipetted into 3 vol. of 20% (v/v) trichloroacetic acid (0 °C) and homogenized vigorously at 4 °C until all cell structures were destroyed. After centrifugation (10000 g; 10 min), [^3H]GTP (4.5×10^5 dpm/20 pmol; (Amersham-Buchler, Braunschweig, Germany) was added to the supernatant as an internal standard. Samples were extracted five times with water-saturated diethyl ether, which was then evaporated prior to freeze-drying and subsequent resuspension in the 'mobile phase' (see below) for controls for liquid scintillation counting and for h.p.l.c. analysis.

H.p.l.c. separations

These were carried out using a Nucleosil C_{18} 5 μm reversed-phase column (4.6 mm internal diam. $\times$ 250 mm) fitted with a guard column (4.6 mm $\times$ 10 mm) filled with the same material (Machery-Nagel, Düren, Germany). The column was isocratically eluted at 1.5×10^7 Pa at room temperature with the mobile phase composed of propan-2-ol/triethylamine/phosphoric acid (85%)/water (0.6:1.0:0.3:98.1, by vol.) at pH 7.2 [22]. U.v. absorbance was recorded continuously at 254 nm and radioactivity was determined with a Raytest Philips Liquid Scintillation counter PW 4700 (Isomess, Straubenhard, Germany).

GDP, GTP, ADP and ATP were eluted at 10.93, 13.56, 22.38 and 31.51 min respectively. Identification of nucleotides and calculation of their concentration was done by using calibration chromatograms obtained under identical conditions with 0.1 mg/ml added to the mobile phase; 200–700 ng were used for calibration. Retention times of biological samples were compared with standard chromatograms and a linear regression line was calculated for calibration. Intracellular concentrations were calculated for a cell volume of 10^{-10} litres as determined below.

Nucleotides

Nucleotides used for trigger experiments *in vitro* or for microinjection experiments were: ATP, Tris salt (Sigma, Deisenhofen, Germany); ATP, magnesium salt (Sigma); adenosine 5'-[γ -thio]triphosphate (ATP[S]), tetralithium salt (Boehringer, Mannheim, Germany); adenosine 5'-[β -imido]triphosphate (App[NH]p), tetralithium salt (Boehringer, Sigma); ADP, disodium salt (Boehringer, Sigma); AMP, sodium salt (Boehringer, Sigma); GTP, Tris salt Type VI (Sigma); GTP[S], tetralithium salt (Sigma); Gpp[NH]p, sodium salt (Sigma); GDP, dilithium salt (Boehringer); GMP, disodium salt (Sigma).

With the nucleotide analogues used, ATP or GTP contamination was < 1%, which cannot therefore account for the effects reported in the Results section. (In controls, equivalent concentrations of Li^+ were also without effect in microinjection studies.)

Other techniques

Cell triggering was performed *in vivo* using 0.005% (w/v) aminoethyl-dextran (AED), as indicated previously [6].

Microinjections were performed according to the same methodology and with the identical instrumentation as described previously [8]. Cells were then exposed to AED as indicated above. Trichocyst release was monitored over different time periods.

Cell volume was estimated from light micrographs to be 10^{-10} litres, as a reference volume for calculating intracellular concentrations.

RESULTS

Establishment of the *in vitro* system and effects of Ca^{2+} , Mg^{2+} and pH

To study exocytosis *in vitro*, cortices were isolated in a medium roughly adjusted to resemble the estimated internal milieu (Table 1). A pMg value of 2 was used for isolation in an inhibited state in the presence of a pCa of 5. Dilution of Mg^{2+} from 10 to 0.5 mM, a level close to physiological conditions, sufficed to trigger exocytosis *in vitro*. Exocytosis was maximal with a pCa between 5.5 and 5 (without nucleotides added) and declined at lower or higher values (Fig. 1). Dependency on pH showed a plateau at a value of around 6.5, with half-maximal activity at pH 6.0 and total inhibition at pH 5.0 (Fig. 2). Throughout this study, 100% exocytosis means the amount of exocytosis observed under standard trigger conditions (pCa = 5; Mg^{2+} concentration shift from 10 to 0.5 mM; pH 7.0; without nucleotides added).

Nucleotide contents *in vivo*

To adjust the system to physiologically relevant concentrations, nucleotide concentrations occurring *in vivo* were determined by h.p.l.c. before and immediately (1 s) after exocytosis triggering by the polyamine secretagogue AED. Fig. 3 is a representative h.p.l.c. chromatogram. Fig. 4 shows ATP concentrations (1.25 mM) which are comparable with previous data (Table 1), with no statistically significant change at 1 s after exocytosis; the same holds for ADP (1.1 mM). During exocytosis, however, GTP

Table 1. Intracellular concentrations of cations and nucleotides in the cytosol of *Paramecium* and related species

The pH of the cytosol of *P. caudatum* cells is 6.8 [18].

	Concentration (mM)	Species	Source/reference
Ions:			
Na^+	3–4	<i>P. tetraurelia</i>	[11]
	2	<i>Tetrahymena pyriformis</i>	[12]
K^+	20	<i>P. tetraurelia</i>	[11]
	34	<i>P. caudatum</i>	[13]
	40	<i>P. caudatum</i>	[14]
Mg^{2+}	1	<i>Stylonychia</i>	[15]
		<i>Paramecium</i>	No data available
Ca^{2+} (free):			
Resting	6×10^{-4}	<i>P. caudatum</i>	[14]
Activated	2×10^{-3}	<i>P. caudatum</i>	[14]
Nucleotides:			
ATP	0.9	<i>P. caudatum</i>	[16]
	1.25	<i>P. tetraurelia</i> K401	[17]
	1.1	<i>P. tetraurelia</i> K401	This paper
	1.25	<i>P. tetraurelia</i> 7S	This paper
GTP	0.35	<i>P. tetraurelia</i> 7S	This paper

Fig. 1. Ca^{2+} -dependency of exocytosis *in vitro*

For trigger conditions, see the Materials and methods and Results sections. The pH was corrected by KOH to 7.0; the pCa was measured by using a Ca^{2+} electrode ETH 129. Bars indicate s.d. values from three experiments.

Fig. 2. Dependency of exocytosis *in vitro* on pH

The pH was adjusted by KOH or HCl, and the pCa was 5; $n = 3$.

concentrations decreased significantly by one third, from 0.35 to 0.23 mM (Fig. 4); GDP concentrations change inversely; in both cases statistical significance is achieved ($P < 0.05$) (Wilcoxon test).

Combined nucleotide and ion effects *in vitro*

Fig. 5 shows the inhibitory effect of ATP and of non-hydrolysable analogues ATP[S] and App[NH]p (when added immediately before exocytosis triggering *in vitro*) at physiological concentrations of ATP. Only concentrations of less than $10 \mu\text{M}$ were not inhibitory. GTP and its analogues were slightly stimulatory (Fig. 5), but barely so at physiological concentrations (Table 1). However, GTP caused a significant left shift of the Ca^{2+} -sensitivity curve (Fig. 6). GTP[S] was slightly less and Gpp[NH]p considerably less efficient. With GTP concentrations of 0.3 mM, equivalent to those in the cell (Fig. 4), sensitization for physiological Ca^{2+} levels (pCa approx. 6–7; Table 1) can be achieved; this is shown in Table 2, with values derived from [GTP] versus pCa curves (only partly shown in Fig. 6).

Fig. 3. Representative h.p.l.c. chromatogram obtained for nucleotides from non-stimulated cells

Some peaks are unidentified.

Fig. 4. Adenine and guanine nucleotide concentrations determined by h.p.l.c. for non-stimulated cells (□) and cells 1 s after synchronous exocytosis (■)

Only changes in GTP and GDP concentrations (but not ATP or ADP) are statistically significant at a 95% level (Wilcoxon test). Results are means $\pm$ s.d. ($n = 6$).

Since a shift in Mg^{2+} concentration from 10 to 0.5 mM (in the presence of a pCa of 5) was routinely used to trigger exocytosis *in vitro* (standard trigger conditions), the effects of GTP and pMg were analysed (Fig. 7). Regardless of whether GTP was present or absent, 10 mM- Mg^{2+} caused total inhibition, whereas 0.5 mM- Mg^{2+} allowed for maximal exocytosis.

Reversibility of the effects of ATP and GTP and their interaction

When isolated without nucleotides, cortices remained fully reactive for 1 h; stimulation by GTP was slightly decreased by 2 h, whereas the inhibitory effect of ATP persists for a long time (Table 3a,b). When ATP or GTP were applied to cortices for different incubation periods and then removed, the 100% standard response was maintained for 1 h (Table 3c). It is evident from Table 3 that effects of ATP and GTP can be rapidly reversed. However, when GTP effects are tested after different periods of preincubation without nucleotides (Table 3d), the

Fig. 5. Effects of nucleotide triphosphates on exocytosis *in vitro*

Experiments were carried out under standard conditions (pH 7.0, pCa 5). □, ATP; ○, ATP[S]; △, App[NH]p; ■, GTP; ●, GTP[S]; ▲, Gpp[NH]p. Results are means $\pm$ S.D. ($n = 6$).

Fig. 6. Dependence of exocytosis *in vitro* on pCa (pH 7.0) in the presence of guanine nucleotides

○, Control without nucleotides added; ●, GTP; □, GTP[S]; ■, Gpp[NH]p. Results are means $\pm$ S.D. ($n = 3$).

Table 2. Dependence of Ca^{2+} -stimulated exocytosis *in vitro* on GTP concentration

Exocytosis was induced by an Mg^{2+} shift from 10 to 0.5 mM.

GTP (mM)	pCa required for:		
	Minimal activation (threshold)	Half-maximal activation	Maximal activation
0	6.7	6.1	5.4
0.3	6.8	6.4	5.9
2.0	9.0	7.7	6.7

Fig. 7. Influence of $[\text{Mg}^{2+}]$ on exocytosis *in vitro* under standard conditions

Conditions were: pCa 5; pH 7.0. ○, Without GTP; ●, with 2 mM-GTP. Results are means $\pm$ S.D. ($n = 3$).

exocytotic response declined markedly between 30 and 60 min. Therefore aging phenomena were avoided in our analysis by using cortices for up to 30 min only.

Though ATP was inhibitory and GTP was stimulatory (Fig. 5, Table 3), the presence of ATP or GTP during the whole cortex preparation and isolation procedure increased the exocytotic responsiveness of the system to GTP (Table 4). This was particularly evident for physiological ATP levels (1 mM), which

Table 3. Long-term incubation of cortices with nucleotides (2 mM) for analysing reversibility of effects achieved and aging phenomena

Exocytosis was triggered by diluting Mg^{2+} from 10 to 0.5 mM in the presence of a pCa of 5. Values are means $\pm$ S.D. ($n = 3$). (a) No nucleotides added; (b) nucleotides added after incubation times indicated; (c) no nucleotides present during cortex isolation, but added for different incubation times and then removed before triggering; (d) no nucleotides present during cortex isolation, but added for different incubation times, washed out again and added again during triggering. For 100% reference value, see text. n.d., not done.

Incubation time (min)	Exocytosis achieved <i>in vitro</i> (%)					
	(a)	(b)		(c)		(d)
		ATP	GTP	ATP	GTP	ATP GTP
0	100	5 $\pm$ 7	133 $\pm$ 5	100	n.d.	7 135 $\pm$ 8
5	100 $\pm$ 4	n.d.	n.d.	n.d.	102 $\pm$ 3	n.d. 126 $\pm$ 3
30	103 $\pm$ 3	n.d.	n.d.	98	101 $\pm$ 5	5 131 $\pm$ 11
60	103 $\pm$ 11	n.d.	n.d.	97	99 $\pm$ 4	5 110 $\pm$ 6
120	94 $\pm$ 2	5	108 $\pm$ 7	88	93 $\pm$ 2	3 110 $\pm$ 4

Table 4. Effect of presence and absence of nucleotides during preparation of cortices and/or during exocytosis triggering

Exocytosis was triggered by changing $[Mg^{2+}]$ from 10 to 0.5 mM in the presence of a pCa of 5. Stimulation occurred in the presence of millimolar ATP or GTP. n.d., not done.

Nucleotide present during cortex preparation	Stimulation achieved with nucleotides added after isolation (%)		
	No nucleotides added*	ATP	GTP
None	100	10	135
1 mM-ATP	150	10	180
0.2 mM-GTP	120	n.d.	n.d.
2 mM-GTP	150	10	190

* Residual nucleotide concentrations were 50 μ M-ATP and 10 or 100 μ M-GTP respectively, depending on their concentration during isolation.

are inhibitory *per se* (Table 3) unless diluted during triggering of exocytosis (Table 4).

Microinjection studies

We observed that ATP or GTP added to whole cells in concentrations equivalent to those occurring within the cells impaired cell viability. When microinjected, ATP, GTP and analogues, as well as ADP and AMP, in amounts equivalent to (adenosine nucleotides) or somewhat higher than (guanosine nucleotides) the respective endogenous concentrations, caused no cell damage. We then looked for a direct trigger effect or, at different time periods after injection, for an inhibition of AED-triggered exocytosis (Table 5).

Retardation of exocytosis occurred within 1 min with Tris- or Mg^{2+} -ATP. This corresponds roughly to the time required for

ATP pool turnover [7,17]. Interestingly ATP[S] had no effect, whereas App[NH]p was ineffective over short (30 s) periods, but most effective over longer periods of time (≥ 5 min). ADP and AMP had no effect (Table 5).

GTP caused slight (10%) exocytosis by 30 s, which slowly increased ($\leq 80\%$ at 5–10 min) in the absence of a secretagogue. Table 5 also shows permanent inhibition by microinjected GTP[S], whereas Gpp[NH]p had no effect.

Microinjected Ca^{2+} does not trigger exocytosis in *Paramecium*, probably due to rapid sequestration. Microinjected pyrophosphate or phosphate (up to 1 mM tested) was also without any effect (results not shown).

DISCUSSION

Comments on the system used

By adjusting our system *in vitro* to ion and nucleotide concentrations occurring *in vivo* (Table 1) we tried to obtain physiologically relevant data. Values for pCa, at rest or in the activated state are derived from electrophysiological analyses of the 'ciliary reversal reaction' (see Table 1). Direct pCa measurements were not possible, since *Paramecium* does not easily take up and/or activate the usual fluorochromes.

With cortices, it suffices to dilute the Mg^{2+} concentration from 10 to 0.5 mM in the presence of a pCa of 5 at pH 7.0 to induce exocytosis via membrane fusion [7]. When compared with these standard conditions (without nucleotides added; see above), additional stimulation can be achieved by GTP (Tables 2 and 3). This might be the reason why, in our previous work [7], only less than half of the expected trichocysts could be released *in vitro*.

Only with hydrolysable ATP or GTP were identical results achieved *in vivo* and *in vitro* (Table 5, Fig. 5), thus stressing their physiological relevance. With systems *in vitro* one has to consider that any added compound is in contact with both the intra- and the extracellular sides. In our case, trinucleotides impaired cell

Table 5. Influence of microinjected nucleotides on AED-triggered exocytosis

On average, at least five injections were made with every nucleotide, resulting consistently in the same effects. Quantitative indications 'response (%)' are estimates which are as precise as possible under the imaging conditions used.

Nucleotide	Nucleotide concentration (mM)	Exocytotic response without AED (%)	Time period between microinjection and AED-application	Exocytotic response observed		
				Response (%)	Time period required for response	Interpretation
None	–	–	No injection	100	1 s	Immediate effect of AED
Tris-ATP	1	0	10 s	0	0–1 min	Retardation
	1	0	10 s	10	1–2 min	Retardation
	1	0	10 s	100	≥ 3 min	Retardation
Mg^{2+} -ATP	1	0	30 s	50–60	4–5 min	Retardation
	1	0	60 s	30	1 min	Decreased retardation
ATP[S]	0.5	0	30 s	100	Immediate	No retardation
	1	0	4 min	100	Immediate	No retardation
App[NH]p	1	0	30 s	100	Immediate	No short-term effect
	1	0	150 s	0	≥ 5 min	Long-term retardation
ADP	1	0	15 s	100	Immediate	No effect
	1	0	120 s	100	Immediate	No effect
AMP	1	0	15 s	100	Immediate	No effect
	1	0	120 s	100	Immediate	No effect
Tris-GTP	1	10	No AED	–	10–30 s	Slight triggering
	1	50–80	No AED	–	~ 5 –7 min	Massive exocytosis
GTP[S]	1	0	5 min	0	≥ 10 min	Permanent inhibition
	1	0	10 min	0	≥ 10 min	Permanent inhibition
Gpp[NH]p	1	0	30 s	100	Immediate	No effect
	1	0	2 min	100	Immediate	No effect

viability when added to the outside, but not when microinjected. Therefore, a mutual control appeared necessary.

Since trichocysts are not released from cortices prepared from non-discharge mutants [7], this precludes a direct fusogenic effect of Ca^{2+} . In our case, the pCa required for exocytosis *in vitro* (Table 2) was 6.1–7.7 (half maximal) or 5.4–6.7 (maximal), depending on the GTP concentration present. This is within the pCa range occurring in these cells (Table 1) and within the range required for the same response in cortices derived from egg cells [9].

Priming effect of ATP and trigger effect of GTP

In accordance with previous data [7] ATP was inhibitory when present during exocytosis (Tables 3 and 4). This effect must take place at the level of membrane fusion, since microinjected ATP also retards exocytosis (Table 5) over a time period comparable with the ATP pool turnover time [17]. However, as postulated before [7], we found that ATP exerts a stimulating (priming) effect when used for preincubation of cortices before triggering of exocytosis (Table 4). Although discussions on the requirements of ATP for exocytosis have been quite controversial [1], it has become increasingly accepted that ATP might prime exocytosis sites in a variety of systems [1,2,23–25]. This priming effect of ATP becomes particularly clear when GTP is added during exocytosis triggering (Tables 3 and 4), just as in some other systems [23].

Effects of nucleotide analogues

Results obtained with ATP[S] or App[NH]p in other systems are quite variable. ATP[S] abolishes secretion in permeabilized chromaffin cells [26] or, after microinjection, in oocytes [27], whereas App[NH]p has no effect on permeabilized mast cells [24]. In our system we found a discrepancy only between *in vivo* (intracellular) and *in vitro* (intra- and extracellular) application of ATP[S] (Table 5, Fig. 6), showing no effect or an inhibitory effect respectively, whereas ATP and App[NH]p retarded or inhibited exocytosis *in vivo* as well as *in vitro*. The reason for these discrepancies remains unclear. Adenosine (results not shown), ADP and AMP (Table 5) had no effect.

Possible implications for stimulus–secretion coupling

The published ATP decay curves during trichocyst exocytosis *in vivo*, with a minimum at 5 s and full replenishment of the ATP pool 30 s after synchronous (1 s) exocytosis [7], make it plausible that we see no changes 1 s after triggering (Fig. 4), when samples were taken. This is unlike the situation with GTP, which has a smaller pool, and whose involvement in exocytosis regulation can be seen from its effects both *in vivo* and *in vitro* (Figs. 5–7) and from the rapid GTP hydrolysis observed (Fig. 4). The additive effects of sequential application of ATP and GTP (Table 4) still have to be elucidated in more detail.

In our system, guanosine triphosphates cause a left shift of the Ca^{2+} sensitivity curve *in vitro* ($\text{GTP} > \text{GTP[S]} > \text{Gpp[NH]p}$; Fig. 6) as in most other systems (e.g. mast cells [23,28], neutrophils [29], platelets [30] and oocytes [31,32]). In contrast with some of the work quoted, microinjected GTP triggered trichocyst exocytosis, whereas AED-triggered exocytosis was inhibited by GTP[S] but not by Gpp[NH]p (Table 5). A similar discrepancy of the effects of GTP[S] was found in the literature: only α -toxin-permeabilized PC12 cells [33] and digitonin-treated isolated chromaffin cells [25] are inhibited by GTP[S], though with α -toxin other results were different [34]. When injected into other cells (eggs [31]) or applied in patch-clamp experiments (mast cells [28,35]) GTP[S] stimulated exocytosis without secretagogue application. This discrepancy might be due to multiple effects of GTP or of its analogues [21].

Despite these uncertainties, we could demonstrate for the first time, by h.p.l.c., that GTP is hydrolysed in cells during synchronous exocytosis. The site where this occurs has yet to be determined. Assuming 10^3 exocytosis sites per cell [6], 10^7 GTP molecules would be consumed per event; considering the short time required for initiation of membrane fusion [6,36] and an acceptable catalytic-centre activity, the mass of enzyme protein required to explain the GTP turnover observed could not be accommodated exclusively at the fusogenic site proper. Therefore, as with some other systems, the precise site(s) of GTP consumption cannot yet be indicated. From our data we also have to assume the occurrence of guanine-nucleotide-binding proteins, possibly of different kinds, as postulated by others [37–39] for mast cells and neutrophils.

Conclusions

In the presence of 0.5 mM- Mg^{2+} , a pCa of 5 is required to trigger exocytosis, including membrane fusion, in cortices derived from *Paramecium* cells. ATP may prime sites relevant for exocytosis by an unknown mechanism; GTP increases Ca^{2+} sensitivity to concentrations occurring *in vivo*, but a local pCa increase also has to be considered. These effects might be more complex than previously assumed, since subcortical Ca^{2+} storage and release could also be ATP/GTP-dependent. Furthermore, some effects of nucleotide analogues are different *in vivo* and *in vitro*, possibly for one or more of the following reasons: (1) nucleotides have access to the intra- or extracellular side of the cell membrane; (2) GTP might act at the fusion site proper and possibly also directly or indirectly by mobilizing Ca^{2+} ; and (3) replacement of ATP by GTP at fusion sites or their sequential consumption.

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REFERENCES

- Rink, T. J. & Knight, D. E. (1988) *J. Exp. Biol.* **139**, 1–30
- Plattner, H. (1989) *Int. Rev. Cytol.* **119**, 197–286
- Baker, P. F. & Knight, D. E. (1981) *Philos. Trans. R. Soc. London B* **296**, 83–103
- O'Sullivan, A. J., Cheek, T. R., Moreton, R. B., Berridge, M. J. & Burgoyne, R. D. (1989) *EMBO J.* **8**, 401–411
- Whitaker, M. (1987) *Biosci. Rep.* **7**, 383–397
- Plattner, H. (1987) in *Cell Fusion* (Sowers, A. E., ed.), pp. 69–98, Plenum Press, New York
- Vilmart-Seuwen, J., Kersken, H., Stürzl, R. & Plattner, H. (1986) *J. Cell Biol.* **103**, 1279–1288
- Kersken, H., Vilmart-Seuwen, J., Momayez, M. & Plattner, H. (1986) *J. Histochem. Cytochem.* **34**, 443–454
- Jackson, R. C. & Crabb, J. H. (1988) *Curr. Top. Membr. Transp.* **32**, 45–85
- Detering, N. K., Decker, G. L., Schmell, E. D. & Lennarz, W. J. (1977) *J. Cell Biol.* **75**, 899–914
- Hansma, H. G. (1979) *J. Cell Biol.* **81**, 374–381
- Andrus, W. D. W. & Giese, A. C. (1963) *J. Cell. Comp. Physiol.* **61**, 17–30
- Ogura, A. & Machemer, H. (1980) *J. Comp. Physiol. A* **135**, 233–242
- Machemer, H. (1989) *J. Protozool.* **36**, 463–487
- DePeyer, J. E. & Deitmer, J. W. (1980) *J. Exp. Biol.* **88**, 73–89
- Naitoh, Y. & Kaneko, H. (1973) *J. Exp. Biol.* **58**, 657–676
- Matt, H., Bilinski, M. & Plattner, H. (1978) *J. Cell Sci.* **32**, 67–86
- Umbach, J. A. (1982) *Proc. R. Soc. London B* **216**, 209–224
- Schefer, U., Ammann, D., Pretsch, E., Oesch, U. & Simon, W. (1986) *Anal. Chem.* **58**, 2282–2285
- Tsien, R. Y. & Rink, T. J. (1980) *Biochim. Biophys. Acta* **599**, 623–638
- Knight, D. E., von Grafenstein, H. & Athayde, C. M. (1989) *Trends Neurosci.* **12**, 451–458

22. Blau, N. & Niederwieser, A. (1983) *Ann. Biochem.* **128**, 446–452
23. Howell, T. W., Cockcroft, S. & Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 191–197
24. Tatham, P. E. R. & Gomperts, B. D. (1989) *Biosci. Rep.* **9**, 99–109
25. Holz, R. W., Bittner, M. A., Peppers, S. C., Senter, R. A. & Eberhard, D. A. (1989) *J. Biol. Chem.* **264**, 5412–5419
26. Brooks, J. C. & Brooks, M. (1985) *Life Sci.* **37**, 1869–1875
27. Crossley, I., Whalley, T. & Whitaker, M. (1989) *J. Physiol. (London)* **415**, 90P
28. Fernandez, J. M., Lindau, M. & Eckstein, F. (1987) *FEBS Lett.* **216**, 89–93
29. Barrowman, M. M., Cockcroft, S. & Gomperts, B. D. (1986) *Nature (London)* **319**, 504–507
30. Haslam, R. J. & Davidson, M. M. L. (1984) *FEBS Lett.* **174**, 90–95
31. Turner, P. R., Jaffe, L. A. & Fein, A. (1986) *J. Cell Biol.* **102**, 70–76
32. Cran, D. G., Moor, R. M. & Irvine, R. F. (1988) *J. Cell Sci.* **91**, 139–144
33. Ahnert-Hilger, G., Bräutigam, M. & Gratzl, M. (1987) *Biochemistry* **26**, 7842–7848
34. Bader, M. F., Sontag, J. M., Thiersé, D. & Aunis, D. (1989) *J. Biol. Chem.* **264**, 16426–16434
35. Penner, R. & Neher, E. (1988) *FEBS Lett.* **226**, 307–313
36. Momayezi, M., Girwert, A., Wolf, C. & Plattner, H. (1987) *Eur. J. Cell. Biol.* **44**, 247–257
37. Burgoyne, R. D. (1989) *Trends Biochem. Sci.* **14**, 394–396
38. Gomperts, B. D., Barrowman, M. M. & Cockcroft, S. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 2156–2161
39. Cockcroft, S., Howell, T. W. & Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 2745–2750

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