Ca^{2+} Release from Subplasmalemmal Stores as a Primary Event during Exocytosis in *Paramecium* Cells

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Abstract. A correlated electrophysiological and light microscopic evaluation of trichocyst exocytosis was carried out with *Paramecium* cells which possess extensive cortical Ca stores with footlike links to the plasmalemma. We used not only intra- but also extracellular recordings to account for polar arrangement of ion channels (while trichocysts can be released from all over the cell surface). With three widely different secretagogues, aminoethyldextran (AED), veratridine and caffeine, similar anterior Na_{in} and posterior K_{out} currents (both known to be Ca^{2+}-dependent) were observed. Direct de- or hyperpolarization induced by current injection failed to trigger exocytosis. For both, exocytotic membrane fusion and secretagogue-induced membrane currents, sensitivity to or availability of Ca^{2+} appears to be different. Current responses to AED were blocked by W7 or trifluoperazine, while exocytosis remained unaffected. Reducing $[Ca^{2+}]_o$ to $\leqslant 0.16$ μM (i.e., resting $[Ca^{2+}]_i$) suppressed electrical membrane responses triggered with AED, while we had previously documented normal exocytotic membrane fusion. From this we conclude that the primary effect of AED (as of caffeine) is the mobilization of Ca^{2+} from the subplasmalemmal pools which not only activates exocytosis (abolished by iontophoretic EGTA injection) but secondarily also spatially segregated plasmalemmal Ca^{2+}-dependent ion channels (indicative of subplasmalemmal $[Ca^{2+}]_i$ increase, but irrelevant for Ca^{2+} mobilization). The $^{45}Ca^{2+}$ influx previously observed during AED triggering may serve to refill depleted stores. Apart from the insensitivity of our system to depolarization, the mode of direct Ca^{2+} mobilization from stores by mechanical coupling to the cell membrane (without previous Ca^{2+}-influx from outside) closely resembles the model currently discussed for skeletal muscle triads.

THERE is ample evidence that exocytosis regulation follows similar principles in all cells—from protozoan to mammalian—though with variations of the basic theme (for reviews see 6, 41). The ciliated protozoan *Paramecium* is a favorable model system because about 10^3 trichocysts are docked at the cell membrane (42). This allows their synchronous release within about 0.1 s (23) in response to different secretagogues, such as polyamines (40, 43, 45), veratridine (24), or caffeine (Klauke, N., and H. Plattner. 1993. *Eur. J. Cell Biol.* 60[suppl.]37:142). *Paramecium* cells possess extended subplasmalemmal Ca-storage compartments, the alveolar sacs (61). These underly almost all of the nonciliary (i.e., somatic) cell surface, except for sites occupied by cilia or trichocysts (1, 42).

Ca^{2+} plays a pivotal role in the regulation of exocytotic membrane fusion (for a review see reference 7) also in *Paramecium* (42) where the source of Ca^{2+} is still under debate. Based on theoretical considerations, polyamines were proposed by Cohen and Kerboeuf (11) to activate hyperpolarization-sensitive Ca channels (Ca_{hyp}[1]; c.f. [52, 53]). This was assumed to account for a rapid influx of $^{45}Ca^{2+}$ into the subplasmalemmal cytosol (22) accompanied by a Ca^{2+}-induced Ca^{2+} release (CICR) from alveolar sacs (11). The evidence presented here argues against this type of CICR.

Among the electrophysiological analyses with *Paramecium* (for reviews see 28, 47), none has dealt with secretion regulation. Only voltage-dependent Ca channels in ciliary membranes (for review see 49) are known not to play a role in exocytosis regulation (43), as we can confirm. Known somatic ion channels include a mechano-sensitive Ca (or Ba) channel at the anterior cell pole, a slow Na channel, different K channels including one in the posterior cell region (for reviews see 28, 47) as well as a newly described Ca_{hyp} (52, 53) and a Na channel which is also permeable to Ca (56). We show that several of these channels may be involved in the electrical response to secretagogues. We found that

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1. *Abbreviations used in this paper*: AED, aminoethyldextran; 4-AP, 4-aminopyridine; Ca_{hyp}, hyperpolarization-sensitive Ca channel; $Ca_{i,o}$, intra-(extra) cellular Ca^{2+}; $[Ca^{2+}]_{i,o}$, intra-(extra) cellular Ca^{2+} concentrations; CICR, Ca^{2+}-induced Ca^{2+} release; ICa_{hyp}, hyperpolarization-activated Ca current; SR, sarcoplasmic reticulum; TEA, tetraethylammonium; TFP, trifluoperazine.

Ca_{hyp} blockers neither inhibit exocytosis (monitored in the light microscope) nor fully block the electrophysiological responses by which it is accompanied.

Our work with *Paramecium* faced the following problems. (*a*) Whole cell patch-clamp recording, the method of choice for studying membrane fusion along with underlying currents in other systems (2), is not applicable to intact *Paramecium* cells (58) due to the rigid surface relief and the large size of the cell which prevents adequate control of the membrane potential by the whole cell method (see, e.g., 20). (*b*) Characterization and separation of the many ionic currents have been hampered by the fact that only a few channel inhibitors, such as tetraethylammonium (TEA), act as in higher eukaryotic cells (12, 49). (*c*) Some ciliary currents are superimposed on those of the somatic membrane. (*d*) Some somatic ion channels, like mechano-sensitive channels (28) display an anterior-posterior gradient. This would question the value of intracellular recordings in search of unknown channel functions during exocytosis. *Paramecium* cells are capable of performing exocytosis over the whole cell surface (43).

We tried here to overcome some of these drawbacks by also recording anterior and posterior extracellular currents and by including some mutant strains in our analyses. The data we obtained are compatible with activation of different currents by a primary release of $Ca^{2+}{}_i$ from subplasmalemmal stores and their simultaneous refilling. This may hold true for AED and caffeine, while the trigger mechanism of veratridine remains to be established. Thus, not only the numerous footlike links between alveolar sacs and the cell membrane (see Fig. 1 in reference 42) but also the signal transduction via a primary Ca^{2+} release from a mechanically coupled pool looks quite similar to skeletal muscle triads (54, 55, 63).

Materials and Methods

Cell Cultures

Paramecium tetraurelia cells were cultivated as previously described (44). Besides the wild type (7S), we also used the "pawn" mutant, d4-500 (kindly provided by Dr. C. Kung, Madison; c.f. [14, 57]), and the trichocyst-free "trichless" (tl) mutant (46).

Chemicals and Solutions

The composition of salines used for electrophysiology is shown in Table I. Experiments to analyze the secretagogue effects were conducted with cells in culture medium. For ion concentrations of the culture medium, see reference (44). For some controls, cells were washed in buffers indicated below. Compounds to be tested were dissolved in 5 mM TRIS-HCl or, for additional controls, in Pipes-HCl buffer, each pH 7.2. To test inhibitors of Ca_{hyp}, we additionally used the formula of Preston et al. (52, 53), i.e., 1 mM Hepes-buffer pH 7.2 with 4 mM KCl and 0.01 mM EGTA added. In some experiments we substituted Na-proprionate for chloride, or *N*-methylglucamine for sodium. For amiloride, a stock solution was prepared in dimethylsulfoxide or ethanol for immediate use by dilution to a final solvent concentration of $\leq$1 vol/vol %. Controls with solvents were without any effect.

Compounds used were from Sigma (Deisenhofen, FRG), with the exception of Hepes, Pipes (Serva, Heidelberg, FRG), TEA (Merck, Darmstadt, FRG), and trifluoroperazine (TFP) (Fluka, Buchs, Switzerland).

Trigger Experiments

Cells were mixed with equal parts of test solutions. Triggering of exocytosis was evaluated semiquantitatively by determining released trichocysts under the light microscope, as described previously (45). This proved most reliable, since any possible side effects can easily be monitored. Comparable ratings were achieved by light scatter evaluation of trigger experiments (data not shown) and by quantitative evaluation of freeze-fracture replicas (references 23–25). As a check test, residual, i.e., non-exocytosed, trichocysts were registered by adding picric acid (45).

Electrophysiology

Membrane potentials and currents were measured with a one electrode current/voltage clamp (Axoclamp 2, Axon Instruments, Foster City, CA) in the discontinuous mode (chopped clamp, switching rate 4-13 kHz). Electrodes were filled with a 1:1 mixture of 3 M KCl and K-citrate. Electrode resistance was between 15 and 30 MOhm.

Alternatively, extracellular currents were measured. For this purpose a patch electrode with a diameter of $\sim$20 μm was fabricated from 1.5 mm outer diameter soft glass (Hilgenberg, Malsfeld, Germany) and fire polished. The pipette was partially filled with saline (see below) and connected to a patch clamp amplifier (EPC 7, List Electronic, Darmstadt, Germany) via a conventional patch electrode holder. Under microscopic control either the anterior or the posterior part of a cell was sucked into the pipette, such that about 1/3 to 1/2 of the cell was in the interior of the pipette (Fig. 1).

In some experiments EGTA was iontophoretically injected into cells precisely as indicated in references (56, 57) using electrodes filled with 0.5 M K_2-EGTA pH 7.4 and with a resistance between 20 and 70 MOhm.

The secretagogues aminoethyldextran (AED, usually 0.01%), caffeine (50 mM) or veratridine (10 mM) were applied via another patch-type electrode (opening $\sim$1–2 μm) which was positioned about one cell length away from the exposed part of the cell (Fig. 1). Secretagogues were dissolved in saline of the same composition as the bath fluid and applied by a 0.5-s pressure pulse of 200 kPa (30 psi) to the electrode holder with a pneumatic pico pump (WPI). With each application, 2 nl of secretagogue solution were delivered to the cell, determined in mock experiments in which the volume of solution injected into an oil droplet was measured. Thus, the actual secretagogue concentration "seen" by the cell surface was below that in the pipette.

Extracellular Current Recordings

Simultaneous intra- and extracellular recordings show the validity of the latter method for registration of electrical events associated with exocytosis. The polarity of currents measured by the patch electrode amplifier corresponds to that recorded in the "cell-attached" configuration, i.e., an inward membrane current from the membrane within the electrode corresponds to a positive signal (upward deflection). Since trigger and recording pipettes face opposite parts of the cell (Fig. 1) and active membrane currents are presumably locally restricted to the part of the cell exposed to the trigger substance (see below), there will be a current flow within the cell comparable, e.g., to that of local synaptic currents at a synapse. The patch electrode will basically "see" a return current and, thus, record a current direction opposite to that of the stimulated part of the cell. While the amplitude of extracellularly recorded currents depends on the area of the cell in the pipette and "seal" resistance between cell surface and pipette, the time course mirrors

*Table I. Composition of Solutions (mM) Used For Electrophysiological Recording**

Solution	Pipes‡	Cl^-	Na^+	K^+	Ca^{2+}	EGTA	pH
Na-Pipes‡ 1 mM Ca^{2+}	5	3	8	1	1	0	7.0
Na-Pipes 10 μm Ca^{2+}	5	3	8	1	1	1.01	7.0
Na-Pipes 0.15 μM Ca^{2+}	5	3	8	1	1	2.5	7.0
Na-Pipes $\sim$10 nM Ca^{2+}	5	3	8	1	1	10	7.4
"8K-Pipes"	5	2	0	8	1	0	7.0
"20K-Pipes"	5	22	0	20	1	0	7.0
"40-K-Pipes"	5	42	0	40	1	0	7.0

* The pH of the solutions was adjusted with NaOH for solutions containing Na and with KOH for those with K^+ as the only monovalent cation. Other chemicals, listed in Table III and in the text, were added before adjusting the pH of the solutions.

‡ Referred to as standard Na-Pipes solution in the text.

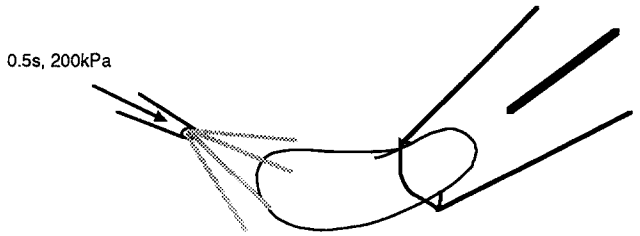

Figure 1. AED application. Schematic diagram showing the arrangement for extracellular recordings and secretagogue application. The cell is aspirated into the measuring pipette which is connected to the patch-clamp amplifier. The secretagogue is applied to the opposite end of the cell by pressure ejection from a patch-type electrode shown on the left. The orientation of the cell was determined visually by the cell shape and by the direction of normal forward rotation in the pipette. For intracellular current- or voltage-clamp, the cell was additionally impaled by a microelectrode (not shown).

that of the de- or hyperpolarizing currents triggered by the secretagogues. For the sake of clarity and to comply with the convention that a depolarization is caused by an inward current and a hyperpolarization by an outward current, extracellular currents are displayed inverted in the figures. An example of simultaneous intracellular recording of membrane potential and corresponding extracellular current signal for wild-type (7S) cells triggered by veratridine is shown in Fig. 2. Estimates from experiments with simultaneous intra- and extracellular recordings during which current was injected via the intracellular electrode show that the current amplitude measured through the patch pipette is roughly proportional to the area of the cell in the pipette.

While the extracellular recording technique is not suited for quantitative measurements and does not allow changing the membrane potential, it was found to be adequate to efficiently test the effect of blockers or of different ionic solutions on the secretagogue responses. In addition, since extracellular recording does not require penetration of the cell by an electrode, it minimizes unwanted discharge of trichocysts.

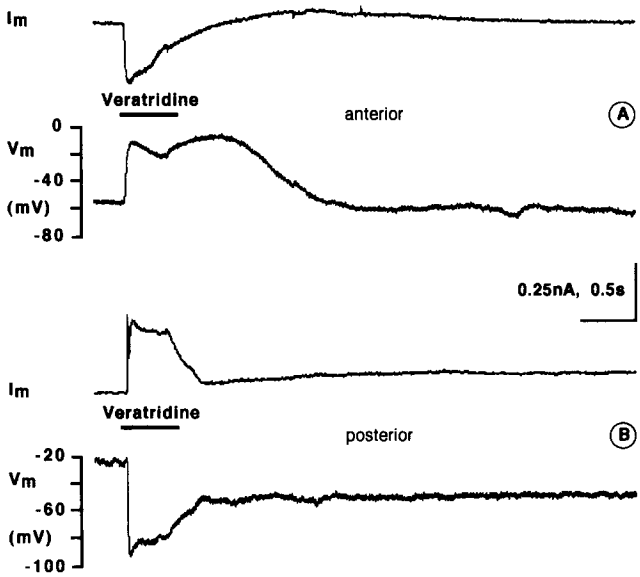

Figure 2. Intra-/extracellular recordings. Simultaneous intracellular voltage and extracellular current records from two 7S cells in standard Na-Pipes solution (as specified in Table I). The horizontal bar at the beginning of the traces marks beginning and duration of a 0.5-s pulse of veratridine. The upper trace of each set shows extracellular current recordings, while the lower trace shows the membrane potential. (*A*) Inward current and depolarization elicited by veratridine application to the anterior part of a cell. Cell hyperpolarized by constant current injection to the indicated potential (to increase inward current driving force). (*B*) Outward current and hyperpolarization elicited by veratridine application to the posterior part of a cell. Current calibration for both extracellular records.

Results

Data obtained by light microscopic evaluation of trigger experiments and by electrophysiology are summarized in Table II and III, respectively. For light microscopic evaluation three runs with ~50 cells each were analyzed. Electrophysiological recordings were obtained from >200 (AED), 20 (caffeine), or 17 cells (veratridine). The percentage of extracellular recordings was about three times higher than that of intracellular recordings, since we had to analyze effects of cell polarity (see Introduction) and those of potential inhibitors (preferably with all-or-none responses). About equal numbers of extra- and intracellular recordings are presented.

Secretagogue Effects on Trichocyst Exocytosis

The trigger effects of the secretagogues AED, caffeine, and veratridine are summarized in Table II, which also lists the effects of various potential agonists and antagonists. As indicated in Materials and Methods, the data are based on previous quantitative evaluation of some key experiments. The AED secretagogue effect was not inhibited by TEA, 4-aminopyridine (4-AP), Ba^{2+}, amiloride, or by W7 or TFP. Substi-

*Table II. Stimulation or Inhibition of Trichocyst Exocytosis by Different Agents**

Compounds	Effect on exocytosis‡
AED (0.005 % = 1.4 × 10^{-6} M)§	+++
TEA (10 nM)‖ → AED	+++
4-AP (10 mM)‖ → AED	+++
Ba^{2+} (10 mM)‖¶ → AED	+++
Amiloride (1 mM)‖¶ → AED	+++
N-Methylglucamine (Na-free medium, 10 mM, up to 4 h tested)‖ → AED	+++
Na-Proprionate (Cl-free medium, 10 mM, up to 4 h tested) ‖ → AED	+++
Caffeine (10, 20, 50 mM)	+/++/+++
Veratridine (2.5 mM)	+++
Veratridine (2.5 mM) + 10 mM Ca^{2+}	–
Veratridine + Ca^{2+} (as above) → AED	+++
EGTA (4.5 mM) → veratridine (2.5 mM)	–
W7 (10 μM)‖ → AED	+++
TFP (up to 100 μM tested)‖ → AED	+++
EGTA (5 mM, microinjected) → AED	–

* For mode of application, see Materials and Methods.
‡ Symbols: –, (no exocytosis); + or ++, (≤1/3 or 2/3, respectively, of trichocysts released); +++, (full exocytotic response [achieved with minimum concentration of trigger compounds indicated]). For reference data obtained by quantitative evaluation, see Materials and Methods.
§ Same concentration used throughout. Arrows indicate sequential application of another compound (usually tested in intervals of 5 s, occasionally up to 1 min or as indicated in Materials and Methods).
‖ No trigger effect without secretagogue.
¶ Also tested under conditions specified for Ca_{hyp} (see Materials and Methods).

*Table III. Summary of Electrophysiological Data Obtained with AED**

Blocker/treatment	Inward current	*n*	Outward current	*n*	In/outward current	*n*	Cell type
TFP 3–10 μM					blocked	10	7S
TFP 10 μM					blocked	9	tl
W7 10–100 μM	blocked	4	reduced	5			tl
Amiloride 1 mM	blocked	5	normal response	6			7S
$[Ca^{2+}]_o$ 10 μM	normal response	5	normal response	8			7S
$[Ca^{2+}]_o$ 0.15 μM			blocked	2	blocked	7	7S
$[Ca^{2+}]_o$ ~10 nM					blocked	2	7S
EGTA injection (~5 mM)			blocked	3			7S
Ba^{2+} 1 or 10 mM	normal response	3	normal response	3			7S
4-AP 1 or 5 mM			normal response	7			7S
TEA 1 mM			normal response	2			7S

* Summary of experiments where various channel blockers (added to standard Na-Pipes solution; see Table I) and $[Ca^{2+}]_o$ were tested for their effectiveness in blocking electrical responses triggered by the standard pulse of AED (0.01%, 0.5 s, 200 kPa). Whenever measurements before and after treatment were from the same cell, data are listed separately under inward current or outward current, respectively. Experiments in which a number of cells were sampled for their ability to respond before the application of the blocker are listed under in/outward current. *n* is the number of cells tested. (For experiments without blockers values for *n* are >200, 20, and 17 for AED, caffeine and veratridine, respectively.)

tution of *N*-methylglucamine for Na^+ or of Na-proprionate for Cl^- also remained without any effect on the AED response. Caffeine and veratridine were also effective secretagogues. Surprisingly the latter was inhibited not only by extracellular EGTA but also by an excess of exogenous Ca^{2+} and AED could overcome this inhibition.

Direction of the Elicited Current Depends on Site of Application

The direction of the electrical response was found to depend on the location of secretagogue application (Figs. 2–11). Application to the anterior end resulted in a depolarization of the cell with an inward current recorded extracellularly, and application to the posterior end elicited an outward current and hyperpolarization, respectively. This holds true for all of the secretagogues analyzed. Fig. 2 is a typical example using veratridine.

Both, de- and hyperpolarizing responses decreased upon repetitive application of a secretagogue to the same cell pole (Fig. 3).

When cells were impaled with an intracellular electrode, exocytosis of a few trichocysts could be seen. At the same time, small de- or hyperpolarizing currents were noticed, their frequency of occurrence decreasing in time (Fig. 4, *A*, *C*, and *D*). Whenever spontaneous discharge occurred with higher frequency (Fig. 4 *A*, *trace 5*), the resulting currents had the same appearance as responses during submaximal secretagogue stimulation, e.g., after repetitive application (Fig. 4 *B*).

For AED application in "standard Na-Pipes" (see Table I), peak currents measured under voltage-clamp conditions near the cell's normal resting membrane potential (−30 mV) averaged 1.8 ± 1 nA ($n = 4$) for inward currents and 3.5 ± 2 nA ($n = 5$) for outward currents.

Membrane responses began with a minimum delay of typically 59 ± 29 ms for inward and 89 ± 34 ms for outward currents (each determined for evidently fast rising responses) independent of the secretagogue used. But longer delays were also seen—in particular—for the inward current responses (Figs. 6 *C* and 11).

Several Secretagogues Are Capable of Eliciting Currents

Like the commonly used secretagogue AED (43, 45), veratridine, known as an agonist of voltage-dependent Na channels (see 16), was also found to trigger trichocyst exocytosis in *Paramecium* cells (24). Veratridine triggered similar electrical responses in 7S cells (Fig. 5 *C*) as did AED (Fig. 5 *A*) but the former could be blocked by raising $[Ca^{2+}]_o$ from the normal concentration of 1 mM to 10 mM ($n = 2$). Under the same conditions, induction of exocytosis could be prevented (Table II). Caffeine, known to readily permeate

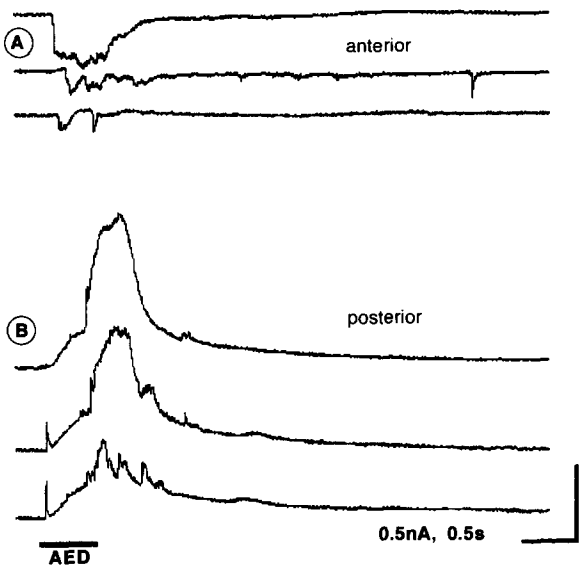

Figure 3. Repetitive secretagogue application. Consecutive extracellular current records showing inward (*A*) and outward (*B*) currents elicited by repetitive application of AED for 0.5 s to the anterior (*A*) and posterior (*B*) end of two 7S cells. Time between applications: 15 s. Cells in Na-Pipes solution, the beginning and duration of the application are indicated by the horizontal bar.

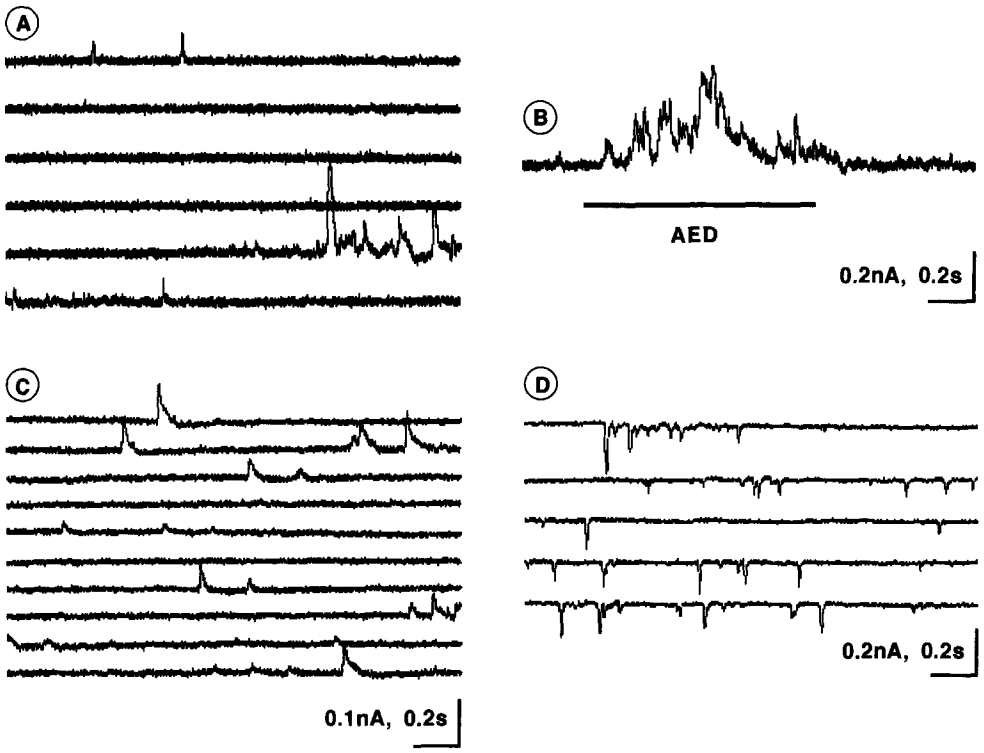

Figure 4. Spontaneous outward and inward currents. (*A*) Consecutive records of spontaneous outward currents recorded under voltage clamp (V_h = −35 mV, 7S cell, K-Pipes solution). (*B*) Currents triggered by 1-s AED application (*horizontal bar*). Same cell and same conditions as in *A*. Consecutive records of extracellularly recorded spontaneous outward (*C*) and inward (*D*) currents from 7S cells in Na-Pipes solution.

cells (64) and to release Ca^{2+} from internal pools, also elicits exocytosis (Table II) and electrical membrane responses similar to those seen with AED or with veratridine (Fig. 5 *B*).

Secretagogue-induced Currents in the Trichless Mutant

AED-triggered currents are not restricted to wild-type (7S) cells but could also be elicited in the tl mutant, with indistinguishable responses (Fig. 6, *A* and *B*). This excludes the contribution of any currents that might result from fusion of the trichocyst membranes to the secretagogue-induced currents. Also spontaneous currents occur in tl cells (data not shown).

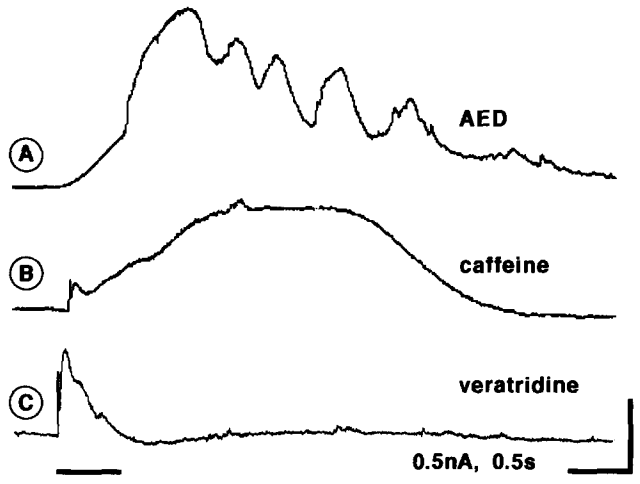

Figure 5. Comparison of AED, caffeine, and veratridine effects. Outward currents elicited by application of AED (*A*), caffeine (*B*), or veratridine (*C*) to the posterior end of different 7S cells in Na-Pipes solution. Extracellular recordings. The 0.5-s applications are marked by the horizontal bar.

Blockers of Ionic Currents in Paramecium

To determine which type of inward and outward currents contribute to the secretagogue response, various potential inhibitors were tested for their ability to block exocytosis (Table II) and the electrical response.

Compound W7 suppresses the inward currents and reduces outward currents elicited by AED (Fig. 6; Table III). Another calmodulin inhibitor, TFP, blocks both inward and outward currents (Table III).

Amiloride, which reversibly inhibits a somatic hyperpolarization-activated Ca current (ICa_{hyp}) in *Paramecium* with a dissociation constant of 0.4 mM (52, 53), suppresses secretagogue-elicited inward currents, but does not affect outward currents (Fig. 6; Table III). Another potent ICa_{hyp} blocker, Ba^{2+} ($K_d \leqslant 0.1$ mM; [52]), however, had no effect on inward or outward currents elicited by AED (Table III) even at 10 mM concentration.

Blockers of K conductances, TEA (12, 49), and 4-AP, had no effect on the AED-triggered responses.

Effects of External K^+ Concentration

The observation that the secretatogues tested elicit hyperpolarizing responses when applied to the posterior end of the cell suggested the activation of a K conductance. To substantiate this possibility, we tested the effect of increased extracellular K^+ on the secretagogue response and we found that the reversal potential of the hyperpolarizing response shifts to more positive potentials upon increase of external K^+ (Fig. 7). While the slope of 31 mV for a 10-fold change

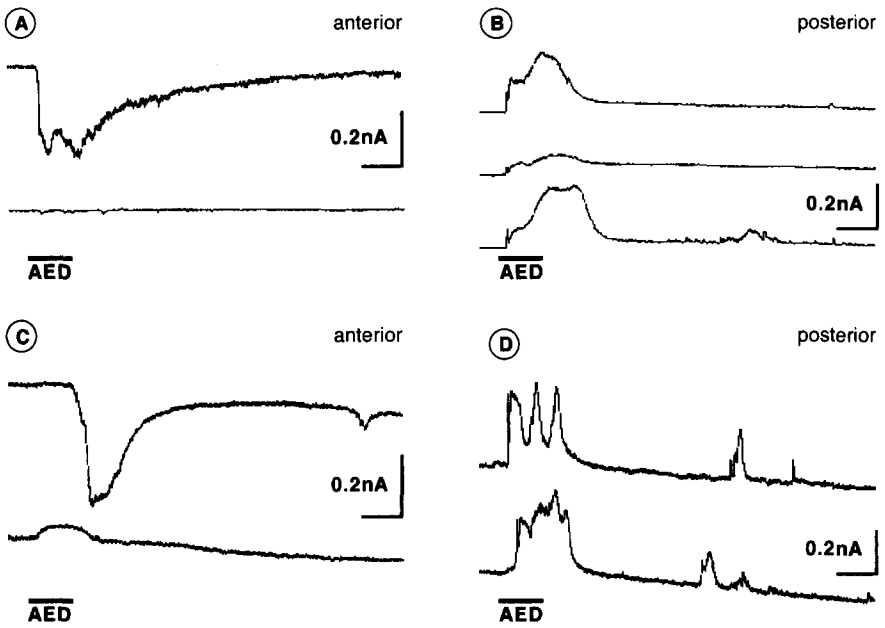

Figure 6. Effects of blockers on AED-induced currents, extracellular recordings. Effect of W7 and of amiloride on inward and outward currents elicited by AED applied to different strains, tl (*A* and *B*) or 7S (*C* and *D*). (*A*) Inward current recorded before (*upper trace*) and after (*lower trace*) block of current by addition of 10 μM W7 (*lower trace*). (*B*) Outward currents recorded before application of W7 (*upper trace*), reduced currents during the presence of 100 μM W7 (*middle trace*), and recovered currents after washout (*lower trace*). (*C*) Inward currents (*upper trace*) were blocked (*lower trace*) after addition of 1 mM amiloride (the remaining current signal is an application artifact). (*D*) Outward currents (*upper trace*) were unaffected by application of 1 mM amiloride (*lower trace*). AED application to the anterior (*A* and *C*) and posterior (*B* and *D*) end of two different cells in Na-Pipes solution. The 0.5-s applications are marked by the horizontal bars.

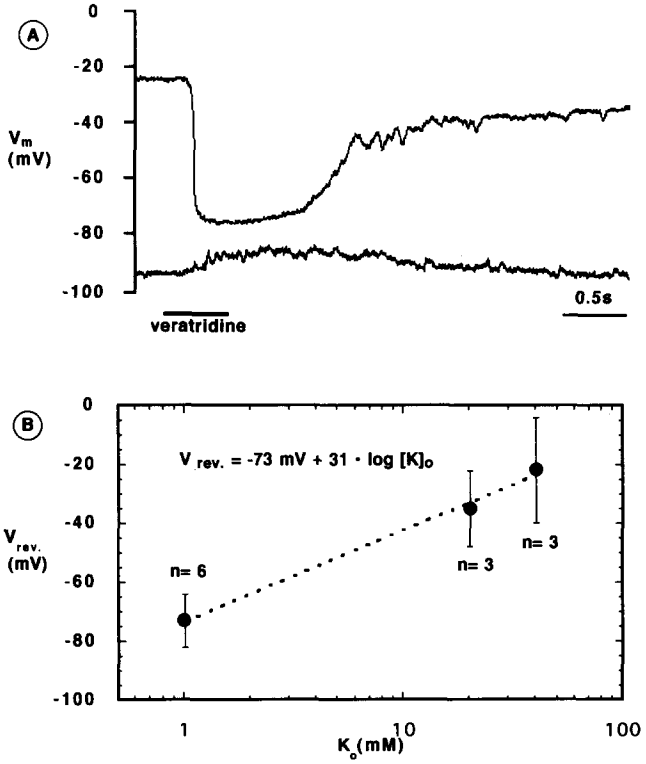

Figure 7. Reversal potential of the posterior current changes with external K^+. (*A*) Example of voltage responses elicited by application of veratridine in presence of 1 mM K^+ in Na-Pipes (see Table I) at the membrane resting potential (*upper trace*) and with the membrane hyperpolarized to −95 mV by current injection (−1 nA, *lower trace*). The 0.5-s application period is indicated by the horizontal bar. (*B*) Reversal potentials plotted as a function of external K^+. Reversal determined from voltage and current clamp experiments of responses triggered by the application of AED or veratridine to the posterior end of 7S cells in Na-Pipes. KCl added to give the concentrations indicated on the abscissa.

of K^+ deviates from the value of 58 mV expected for a pure K conductance, it is consistent with the notion that a major part of the current at the posterior cell pole is carried by K^+. Extracellular application of 1 mM amiloride did not significantly change reversal potential (data not shown, n = 5), thus indicating that the secretagogue-induced posterior conductance (Fig. 7) is not "contaminated" by anterior current components. It, thus, is mainly—though not exclusively—K^+ selective. Frequently the hyperpolarizing responses, in particular to veratridine, had the appearance of regenerative negative spikes with a plateau phase lasting several seconds

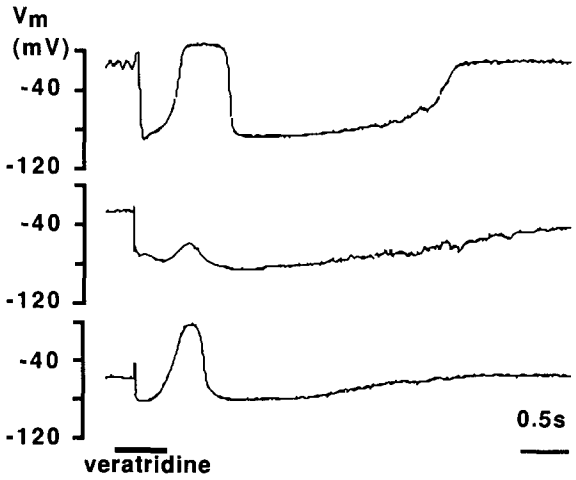

Figure 8. Negative spikes. Regenerative hyperpolarizing voltage responses elicited by application of veratridine to the posterior end of a 7S cell in Na-Pipes. Voltage responses elicited at the membrane resting potential (*middle trace*), and with the membrane polarized to −60 mV (*lower trace*) and −8 mV (*upper trace*) by current injection. The 0.5-s application periods are marked by the horizontal bar.

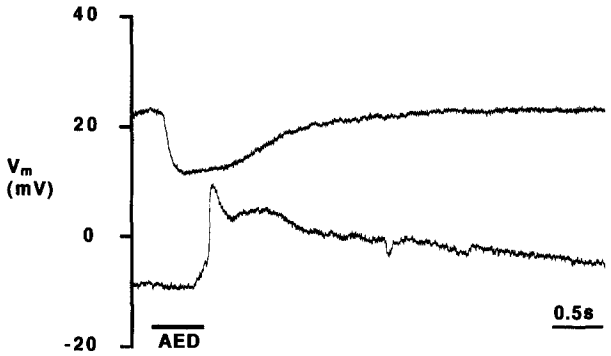

Figure 9. Reversal of the anterior depolarizing response. Example of voltage responses elicited in a 7S cell by application of AED in 20K-Pipes (see Table I) at the membrane resting potential (*lower trace*) and with the cell depolarized to +22 mV by current injection (+0.5 nA, *upper trace*). The 0.5-s application period is indicated by the horizontal bar.

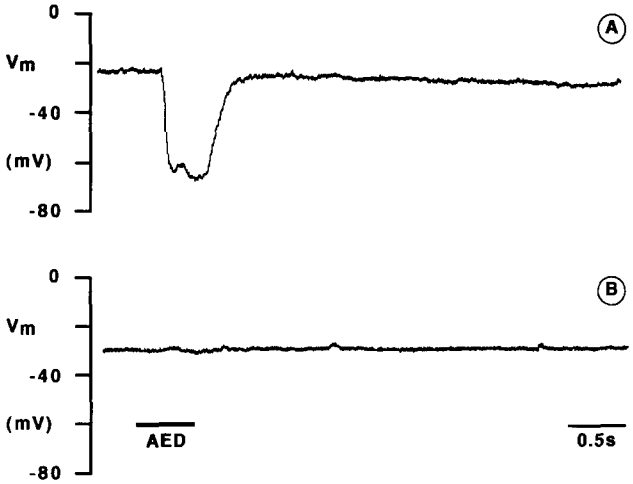

Figure 10. Effects of EGTA injection on AED-induced hyperpolarization. (*A*) Intracellular recording of a hyperpolarizing voltage response elicited by application of AED (*horizontal bar*) to the posterior end of a 7S cell in Na-Pipes. (*B*) Block of the response after iontophoresis of EGTA (60 s, −2nA) into the cell.

(Fig. 8); for more details, see "Shape of Current Curves," below.

In normal Na-Pipes, the anterior, depolarizing response reversed at an average near +8 mV (± 9 mV; $n = 5$), as shown in Fig. 9. While the currents were smaller in elevated K^+ solution, which is to be expected due to the depolarized membrane resting potential and, hence, a diminished driving force, the reversal potential was not significantly changed.

Effects of External Ca^{2+} Concentration

The requirement of the secretagogue-induced currents for Ca^{2+}_o was tested over a wide range of concentrations. Anterior inward as well as posterior outward currents elicited by AED were similar with elevated (10 mM) $[Ca^{2+}]_o$ and down to concentrations of 10 μM (Table III). With 0.15 μM $[Ca^{2+}]_o$ (corresponding to resting $[Ca^{2+}]_i$ levels [28]) or below, both currents were suppressed (Table III)—provided that the AED pipette also contained the same low Ca^{2+} as the medium—although exocytotic membrane fusion is normal (25). If the application pipette, however, contained $[Ca^{2+}]_o$, normally used (1 mM) currents could still be observed. Similarly, if the effects of lowering Ca^{2+} were studied on the same cell by merely exchanging the bath solution while keeping the solution in the "holding pipette" unchanged, an electrical response could still be elicited.

Buffering $[Ca^{2+}]_i$

To directly test for the involvement of a $[Ca^{2+}]_i$ increase in the AED-mediated secretory and current responses, we injected the Ca chelator EGTA. Iontophoretic injections were done under the same conditions as previously used to eliminate Ca^{2+}-dependent K^+ and Na^+ currents in references (56, 57). With ~5 mM intracellular EGTA, $[Ca^{2+}]_i$ levels are kept well below normal resting levels indicated by (29, 37). Fig. 10 shows the block of the AED-induced hyperpolarizing membrane response by a 1-min −2 nA iontophoretic EGTA injection. Similar results were obtained with several cells with injections of between 30 and 90 s with −2 nA.

Ciliary Ca^{2+} Channels Are Not Involved

AED was able to induce inward and outward currents in pawn cells, strain d4-500, a mutant devoid of functional ciliary Ca^{2+} channels (Fig. 11). These cells display normal exocytosis in response to AED (43). This suggests that ciliary Ca^{2+} channels are not involved in the secretagogue response.

Depolarization or Hyperpolarization of the Cell Does Not Trigger Exocytosis

As a corollary result of the intracellular current- and voltage-clamp experiments, we noticed that neither hyper- nor depolarization of the cell per se caused exocytosis. This held true at least as long as the membrane potential was changed within the "extended physiological range", i.e., about ±100 mV from the resting potential (−30 mV).

Shape of Current Curves

The general shape of the responses varied widely from fairly smooth rising waveforms (Fig. 3 *B*) to those with pronounced oscillations over several seconds (Figs. 5 *A*, 6 *D*,

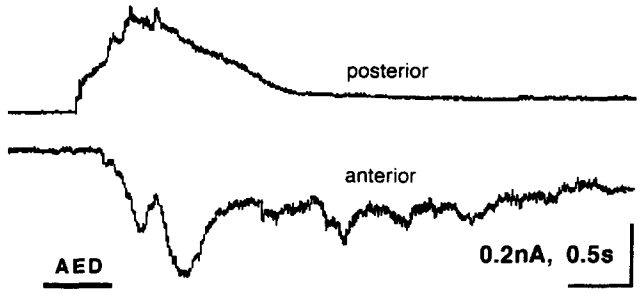

Figure 11. Response of pawn cells, strain d4-500, to AED. Extracellular outward (*upper trace*) and inward (*lower trace*) current recorded from the same cell in Na-Pipes. AED was applied posteriorly (*upper trace*) or anteriorly (*lower trace*) while the cell was held in the measuring pipette at the opposite end. The 0.5-s application periods are marked by the horizontal bar.

8, and 11). Outward currents often showed an initial fast spikelike component (Figs. 2 *B*, 3 *B*, 5, *B* and *C*, and 6 *D*), (the origin of which remains to be determined) which probably represents an initial fast outward current.

Currents elicited in 7S cells by AED and registered extracellularly may last from ~0.5 s to several seconds (Figs. 3, 5, and 6). Anterior and posterior recordings have different shapes. Occasionally oscillations occur, e.g., in Figs. 5 and 6 *D* (*upper trace*) or in Fig. 3 after the third application of AED (with decreasing current amplitude). The cause of the different shape of extracellular current curves observed in >150 extracellular recordings with AED remains to be analyzed. The same holds for veratridine and caffeine.

Although the primary currents are a Na^+ inward and a K^+ outward current (depending on the site to secretagogue application), we cannot exclude a secondary activation of other V-dependent currents. For instance, in Fig. 8 (*lowest trace*), where the membrane is already hyperpolarized by current injection, ICa_{hyp} may be activated in consequence of the additional hyperpolarization induced by posterior secretagogue application.

Discussion

Inward and Outward Currents Are Specific Effects of Secretagogues

The fact that the depolarizing and hyperpolarizing AED response coincides with the localization of the anterior and posterior mechanical sensitivity (28, 47) might suggest a simple mechanical activation. Several observations show, however, that anterior inward and posterior outward currents are secretagogue specific and not the result of mechanical stimulation due to the pressure application of the secretagogue solutions. First, control applications with solutions devoid of secretagogue hardly produced currents like those described in this paper. Fig. 12 vaguely shows a minimal upward deflection in the two lowest traces out of five controls.

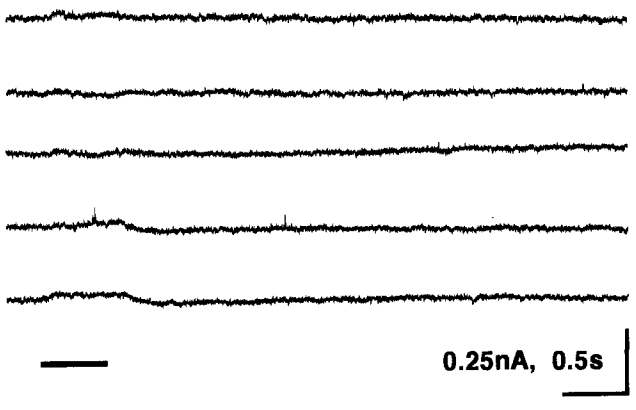

Figure 12. Control applications without secretagogue fail to elicit responses. Consecutive traces from repetitive pressure applications of saline (Na-Pipes) devoid of secretagogues do not cause mechanically triggered responses when 40 psi (more than usually) pressure was applied to cells (7S), even though the cell visibly bent during the application. The 0.5-s applications to the posterior cell pole are marked by the horizontal bar. Extracellular current recordings.

A similar upward deflection in experiments with secretagogues (as in Fig. 6 *C*, *bottom trace*) is quite exceptional. Microscopic observation showed that there was only slight cell movement by the "jet" from the pressure pipette but this movement was insufficient to trigger a mechano-electrical response. Second, repeated application of AED resulted in a decrease of both hyper- and depolarizing responses (Fig. 3). Even though it could not be analyzed quantitatively, the current response seemed proportional to the number of expelled trichocysts. Third, AED-induced currents were absent in cells that were pretriggered by incubation with AED or veratridine (data not shown).

In summary, all these observations support the conclusion that the electrical responses we observed are indeed specific for the application of the different secretatogues. Similar responses obtained with tl cells exclude any contribution caused by fusion of trichocyst membranes.

Anterior Depolarizing Response

An obvious question is, which currents are activated by AED? A number of inward currents have been described in *Paramecium* (see 28, 47) besides the mechanically activated anterior receptor current discussed above.

The ciliary Ca current carried by voltage-dependent Ca^{2+} channels, located exclusively in the ciliary membrane, is responsible for the "ciliary reversal" reaction (for reviews see 28, 49). This current can be ruled out as a primary current elicited by the secretagogue for the following reasons. First, the ciliary Ca current shows pronounced inactivation and subsides in the order of 100 ms (28), whereas secretagogue triggered inward currents last up to several seconds. Because of the Ca^{2+}-dependence of inactivation, this process can indeed be expected to be particularly pronounced for the large inward currents observed during the secretagogue response (c.f. Figs. 2 *A*, 3 *A*, and 6, *A* and *C*). Second, the AED response is unaffected in the pawn mutant, d4-500 (Fig. 11), which lacks ciliary Ca current. It should also be noted that deciliated wild-type cells as well as pawn cells show normal exocytotic response to AED (43).

Another type of Ca inward current which has been characterized recently, is ICa_{hyp} (52, 53). ICa_{hyp} is effectively blocked by extracellularly applied amiloride or Ba^{2+}. The attractive idea that this current would be activated during exocytosis in *Paramecium* has been put forward by Cohen and Kerboeuf (11). They proposed that polycationic secretagogues, such as AED, would act by hyperpolarizing the actual transmembrane potential via surface charge effects. There are, however, several arguments against the relevance of ICa_{hyp} for AED-triggered exocytosis. First, although the AED-triggered inward currents were indeed found to be sensitive to 1 mM amiloride, they were not at all sensitive to Ba^{2+} (Table III). Outward currents are insensitive not only to Ba^{2+}, but also to 1 mM amiloride. Second, neither amiloride nor Ba^{2+} reduced AED-triggered exocytosis. This was repeatedly tested, also under conditions indicated by Preston et al. (52, 53), with different solutions and incubation times before AED application (see Materials and Methods and Table II). Third, direct electrical hyperpolarization of the cells, which activates ICa_{hyp}, did not trigger trichocyst release.

Posterior Hyperpolarizing Response

The hyperpolarization induced by veratridine (Fig. 8) is reminiscent of the regenerative hyperpolarization in *Paramecium* (38, 50, 59). The veratridine response has the same shape and a comparable duration and it is of regenerative nature.

Unlike the regenerative hyperpolarization, which is only observed in low (<1 mM) external K^+ (38, 59), the secretagogue responses can be observed at K^+ concentrations well above 1 mM. Furthermore, the regenerative hyperpolarization is blocked by 1 mM external TEA (38), while the electrical secretagoue response is not (Table III). These data are compatible with the lack of effect of K channel blockers on exocytosis (Table II).

Possible Involvement of Ca^{2+}-dependent Conductances

Several ion channels able to mediate inward currents in *Paramecium* are activated by an increase in $[Ca^{2+}]_i$ (47). Two examples are a Ca^{2+}-dependent Mg current (48) and a Ca^{2+}-activated Na current (56, 57, 58). The Ca^{2+}-dependent Mg current can be ruled out as a carrier of the secretagogue-produced inward current since our solutions contained no added Mg^{2+} (Table II). Our main arguments in favor of Ca^{2+}-activated somatic conductances as mediators of not only the inward current but also of the outward current (Ca^{2+}-dependent K current; [51]), both of which are associated with exocytosis, stem from the following observations. First, both depolarizing and hyperpolarizing responses are blocked by W7 and by TFP (the block of ciliary voltage-dependent Ca channels by W7 [13, 15] being irrelevant in this context, as discussed above). Second, reducing $[Ca^{2+}]_o$ to sub-micromolar concentrations also blocks AED-triggered currents (Table III), as it reversibly inhibits, e.g., somatic Ca^{2+}-activated Na channels (58).

The independence of the electrical response to AED over a wide range of $[Ca^{2+}]_o$ (Table III) is not surprising if one assumes that the main source of Ca^{2+} is the extensive subplasmalemmal stores (see below). Reducing $[Ca^{2+}]_o$ does not block exocytotic membrane fusion (23). Release of internal Ca^{2+} might suffice to induce membrane fusion without activating currents if one considers the high Ca^{2+} sensitivity of fusion sites (close to resting levels, mediated by guanosine triphosphate; [27]) and/or possibly a selective release of Ca^{2+} at trichocyst docking sites (Klauke, N., and H. Plattner, unpublished results), similarly as in terminal cisterns of sarcoplasmic reticulum (SR) (54, 55). When stores cannot be refilled during Ca^{2+} release, the subplasmalemmal $[Ca^{2+}]_i$ increase must be restricted indeed. Concomitantly, AED application repeated in 15-s intervals caused decreasing current amplitudes (Fig. 3).

If in "standard Na-Pipes" (see Table I) the inward current is mainly carried by a Na^+ influx through the Ca^{2+}-dependent Na channel (58), how can we explain that there is still an inward current in nominally Na-free solution (see "Effects of External K^+ Concentrations" in Results)? It is possible that the Ca^{2+}-dependent Na channel is not very Na-selective, but also permeable to Ca^{2+}, particularly in the absence of extracellular Na^+, as described by Saimi (51).

Effects of Caffeine and Veratridine

Both agents trigger trichocyst exocytosis (Table II) and elicit similar electrical currents as AED, as we have shown. Caffeine readily enters the cells (64) and mobilizes Ca^{2+} from SR (see 34, 39) or from stores in various secretory cells (see reference 8). The Na channel agonist, veratridine (see 16), also triggers trichocyst exocytosis (24). Although veratridine was assumed to activate ciliary V-dependent Ca channels in *Paramecium* (60), it triggers exocytosis with EC_{50} ~1 mM (24) independent of these channels (see above). In the present study it also triggered similar electrical responses as AED or caffeine. Surprisingly, veratridine-triggered exocytosis and accompanying currents can be suppressed not only with extracellular EGTA but also with increased $[Ca^{2+}]_o$ (Tables II and III) and AED overcomes or bypasses the Ca^{2+}-mediated inhibition of veratridine-triggered exocytosis (Table II). The precise mechanism of veratridine stimulation remains to be determined, since it might be different from that of AED and caffeine.

Possible Role of Ca Stores

Ca^{2+} release in our system apparently occurs in packages (Fig. 4), i.e., in a quasi "quantal" fashion, as reported for other secretory systems (lacrimal [31, 65], pancreatic acinar [36], chromaffin [9] cells) and smooth muscle cells (4, 17, 67). Though correlation with individual, morphologically defined packages of intracellularly released Ca^{2+} is still missing (5), activation, e.g., of an "alveolar sac" may give rise to a spontaneous Ca-activated current (Fig. 4), while massive release from many sacs may result in the macroscopic currents. Increase in Ca^{2+} should be highest in the confined space between the subplasmalemmal alveoli and the surface membrane, and this we could in fact observe (25). Because the Ca-activated conductances seem spatially segregated on the *Paramecium* surface (47), this would lead to inward Na and outward K currents at the anterior and posterior pole of the cell, respectively, as we have observed.

There is increasing evidence of a primary Ca^{2+} release from stores, paralleled by a Ca^{2+} influx from the medium. This is true, e.g., of mast cells (18, 33), platelets (3), glioblastoma cells (62), and sea urchin eggs (35). However, structural coupling of stores to the plasmalemma by "footlike" connections (as occurring all over the alveolar sac-cell membrane contact area) have not yet been visualized in other secretory systems (though they might exist in a less distinct fashion). Interestingly, footlike connections, as in *Paramecium*, are widely distributed also in invertebrate muscles (26). As from alveolar sacs in *Paramecium*, caffeine can release Ca^{2+} from the so strikingly similar SR (30, 39, 54, 55), as can various polyamines (10, 19, 21, 39, 66). Also in mast cells or pancreatic acinar cells exogenous polyamines liberate internal Ca^{2+} (18, 32).

Conclusions

Three chemically different secretagogues, a polyamine (AED), veratridine, and the general Ca^{2+} mobilizing agent, caffeine, produced very similar electrical signals in *Paramecium*. Since we showed by widely different methods (25 and this study) that subplasmalemmal $[Ca^{2+}]_i$ increases upon AED stimulation and since this causes membrane fusion (23, 25, 27, 42), Ca^{2+} represents an essential second messenger. Concomitantly we here showed that $Ca^{2+}{}_i$ chelation abolished exocytosis and currents. The pivotal question arose in

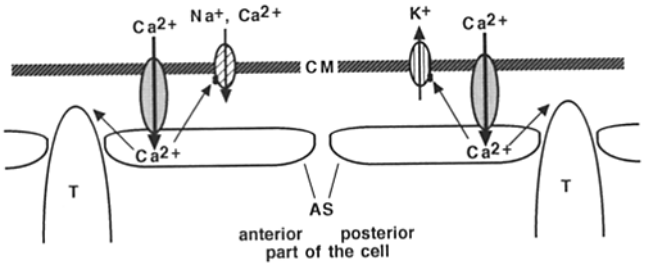

Figure 13. Summary of steps proposed to occur in *Paramecium* in response to the secretagogue, AED, according to the arguments presented in the text (also see Conclusions). Release of Ca^{2+} from alveolar sacs (*AS*) is an early direct response. The resulting increase of subplasmalemmal pCa_i causes activation of Na^+ and Ca^{2+} influx at the anterior part and of K^+ efflux at the posterior part of the cell (*CM*, cell membrane). Ca^{2+} release may be site-directed and/or Ca^{2+} sensitivity of currents and of membrane fusion, respectively, may be different. Mobilization of Ca^{2+} from the stores also causes an influx of $Ca^{2+}{}_o$ directly into the store (if $Ca^{2+}{}_o$ is available, as it normally is) which might reflect the slightly delayed $^{45}Ca^{2+}$ influx accompanying secretion (24). The store is thus refilled during depletion. The model accounts for the fact that Ca^{2+} mobilization from stores suffices to induce exocytotic membrane fusion at sites of trichocyst (*T*) attachment at the cell membrane, even when $Ca^{2+}{}_o$ has been removed (23, 25) and when in- and outward currents are suppressed (this paper).

the current analysis, whether (*a*) a Ca^{2+} influx, as suggested by Cohen and Kerboeuf (11, 22), or (*b*) mobilization from an internal pool without a previous Ca^{2+} influx, as proposed by Knoll et al. (25), is relevant for inducing exocytotic membrane fusion with AED.

Our hypothesis (*b*) is supported by the following arguments. First, although all currents are blocked or reduced by low $[Ca^{2+}]_o$ or by TFP and W7, exocytosis is not. The different responses of currents and of membrane fusion might be due to different Ca^{2+} sensitivity and/or site-directed release from stores which tightly surround every exocytosis site. Second, a primary Ca^{2+} influx could activate posterior currents only when it comes from inside the cell, since the Ca^{2+} inward current is restricted to the anterior part of the cell. Third, according to our time resolved measurements, influx of $^{45}Ca^{2+}$ during AED stimulation occurs only slightly after the occurrence of most membrane fusion events (24) and, thus, might serve to refill the stores (Fig. 13). Fourth, by analyzing isolated alveolar sacs and by microinjection studies we have excluded the involvement of any of the known second messenger compounds in activating $^{45}Ca^{2+}$ release and exocytosis, respectively (Länge, S., N. Klauke, and H. Plattner, manuscript submitted for publication).

Based on all these arguments, the currents we observed with AED most probably represent a secondary, rather than a primary step in signal transduction in *Paramecium* cells. However, they can be considered as indicative of a subplasmalemmal $[Ca^{2+}]_i$ increase (as with the other secretagogues tested). Apart from our lack of knowledge how the cell surface senses a trigger, like AED, our hypothesis (summarized in Fig. 13) assumes a Ca^{2+} release mechanism analogous to that currently discussed for skeletal muscle triads (54, 55, 63). These also release stored Ca^{2+} in response to caffeine, but without a primary $[Ca^{2+}]_i$ increase in the subplasmalemmal space, and physical connections exist for mechanical coupling and for site-directed Ca^{2+} release. Remarkably alveolar sacs, like SR, contain a calsequestrin-like protein (Plattner, H., A. Habermann, N. Klauke, I. Majoul, and H.-D. Söling, manuscript in preparation). All this makes alveolar sacs appear quite similar to SR. It remains to be seen whether our model would be applicable also to other secretory systems.

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