Volume 304, number 2,3, 265-268 FEBS 11166 June 1992

A rapid calcium influx during exocytosis in *Paramecium* cells is followed by a rise in cyclic GMP within 1 s

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Received 28 April 1992

The synchrony of trichocyst exocytosis in *Paramecium* allows temporal correlation of associated events. Using quenched flow we observed a Ca^{2+} influx concurrent with exocytosis within 80 ms after stimulation with the secretagogue aminoethyldextran. Cyclic AMP did not change in depency of stimulation. Cyclic GMP transiently increased after 500 ms, culminating at 2 s, and thus considerably lags behind exocytosis induction and influx of Ca^{2+}. Both Ca^{2+} influx and rise in cGMP are known to be induceable also by Ba^{2+} or veratridine, allegedly via the opening of ciliary Ca^{2+} channels. However, only veratridine stimulated exocytosis. We conclude that both aminoethyldextran and veratridine induce an exocytosis-associated Ca^{2+} influx, which is responsible for the rise in cGMP, through an as yet unknown pathway.

Calcium; Exocytosis; Cyclic AMP; Cyclic GMP; *Paramecium*; Veratridine

1. INTRODUCTION

Ca^{2+} and cyclic nucleotides are involved in the regulation of ciliary beat of *Paramecium* cells [1,2]. Less is known concerning their roles in trichocyst exocytosis, a well established experimental system for the study of secretory mechanisms [3,4]. Whereas a crucial role for Ca^{2+} has been derived from effects of ionophores [5], a Ca^{2+} influx associated with exocytosis has been demonstrated only recently [6]. However, the temporal correlation of this influx (2s after stimulation) with exocytosis (within 80 ms after stimulation [7,8]) could not be strictly established due to experimental limitations. Therefore, we now applied quenched flow [7] measurements to resolve subsecond time periods and observed a Ca^{2+} influx already after 80 ms.

In *Paramecium*, cAMP levels are increased by hyperpolarization [9], and cGMP levels by a Ca^{2+} influx after depolarization [10,11], but no information is available yet concerning the role of these nucleotides during trichocyst exocytosis. In other cell types, cAMP production is affected by Ca^{2+} [12], and cGMP mediates Ca^{2+} influx in stimulated pancreatic acinar cells [13]. In order to follow the fate of these cyclic nucleotides during exocytosis in *Paramecium*, we determined their respective levels from 30 ms up to 10 s after stimulation. Whereas no consistent changes of cAMP were found, a rise of cGMP was determined after 500 ms, thus secondary to exocytosis and to the accompanying Ca^{2+} influx. Since a similar increase in cGMP was shown earlier to be induced by Ba^{2+} or veratridine, supposed to provoke long lasting opening of the voltage-dependent Ca^{2+} channels in the ciliary membrane [11,14], we tested the effects of these substances on exocytosis. Ba^{2+} had no effect but veratridine stimulated trichocyst exocytosis. Therefore, we assume that different targets are concerned by the action of Ba^{2+}, veratridine and aminoethyldextran (AED), the two latter ones only being relevant for exocytosis stimulation. In addition, we suspect that the cell response to veratridine, previously attributed to ciliary voltage-dependent channels [14], could be in fact related to exocytosis-associated events.

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2. MATERIALS AND METHODS

2.1. *Cells and stimulus*

Paramecium tetraurelia 7S (wild type) was grown axenically until early stationary phase, washed in buffer (5 mM PIPES, 1 mM $CaCl_2$, 1 mM KCl, pH 7) and starved in this buffer overnight as described [15]. See the same reference for specification of the secretagogue aminoethyldextran and [7] for short-time stimulation using quenched flow.

2.2. *Ca^{2+} influx*

Measurements were performed in principal according to [6], but the quenched flow equipment described in [7] was used to resolve subsecond times. Cells were adjusted to 30,000 cells/ml buffer (5 mM PIPES, 40 μM $CaCl_2$, 1 mM KCl, pH 7) and mixed with the same buffer containing ^{45}Ca (final specific activity 1 μCi/ml)±AED (0.01% final concentration). Samples were chased by spraying into 10-fold volume of ice-cold buffer (5 mM PIPES, 5 mM $CaCl_2$, 1 mM KCl, pH 7), vacuum-filtered and washed 3 times in the same buffer. Filters were counted by liquid scintillation.

2.3. *Cyclic nucleotides*

Cells were mixed with AED or Ba^{2+} (10 mM in 5 mM PIPES, 1 mM

$CaCl_2$, 1 mM KCl, pH 7) for defined times and stopped in a second mixing chamber by ice-cold perchloric acid (1 M final concentration). Cyclic nucleotides were determined with radioimmuno-assay kits obtained from NEN, Dreieich, FRG.

2.4. *Effects of Ba^{2+} and veratridine on exocytosis*

Exocytosis was monitored by light microscopy after 1 to 1 mixture of cells (in 5 mM PIPES, 1 mM $CaCl_2$, 1 mM KCl, pH 7) and the same buffer containing the appropriate concentration of Ba^{2+} or veratridine. Quantification of exocytosis was performed by rough estimation of the number of trichocysts excreted.

3. RESULTS

3.1. *Ca^{2+} influx*

We determined the Ca^{2+} influx in a time range from 30 ms up to 1 s after stimulation (for longer times, see [6]). Fig. 1 shows a significant AED-dependent Ca^{2+} influx 80 ms after stimulation. At the shortest time available, 30 ms, no stimulated influx could be detected. The rate of the uptake showed a progressive decrease from the initial maximal rate at 80 ms. The maximal rate would support a rise of intracellular [Ca^{2+}] of more than 100 μM/s (calculated for total cell volume).

3.2. *Cyclic nucleotides*

We determined the level of cyclic nucleotides in a time range from 30 ms up to 10 s after stimulation. For comparison, and as a standard, we also determined the well characterized [11] effect of Ba^{2+}, which we now extended to short-time incubations. Fig. 2 shows the time course of changes in cAMP levels. We see considerable variations between different time points and also between experiments, as indicated by the error bars. Possibly the fluctuations were induced by occasional hyperpolarization [9] due to turbulences during flow through the mixing chamber and tubing, and by vigorous pipetting. However, no effect of AED-stimulation as compared to controls can be recognized. This is in contrast to cGMP levels, which showed a pronounced transient rise 1 s after stimulation by AED (Fig. 3). This rise culminated after 2 s and then declined again, whereas the rise induced by Ba^{2+} reached the maximum only after 5 s and remained elevated for 10 s (after this time the level falls slowly, see [2]). Also the rate of the increase was higher after AED stimulation (up to 27

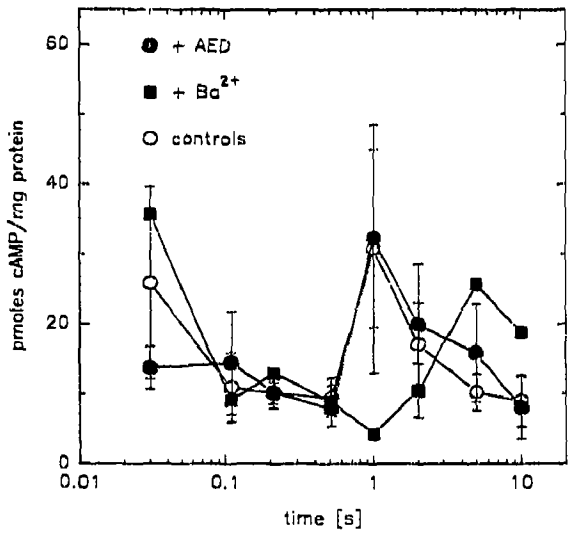

Fig. 2. Levels of cAMP within 10 s after stimulation. Data represent means ±S.E.M. (n = 3 for controls and AED-stimulation, n = 2 for Ba^{2+}-treatment). No consistent differences after AED-stimulation as compared to controls become significant.

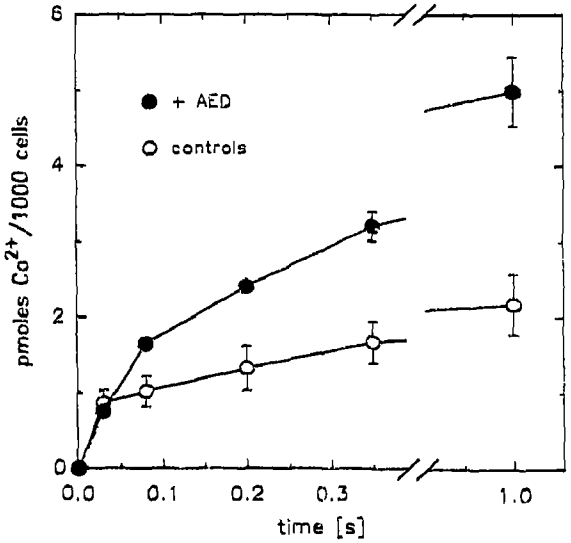

Fig. 1. Ca^{2+} influx within 1s after stimulation by AED. Data represent means±S.E.M. (n = 8). 30 ms after stimulation no difference as compared with controls could be found. After 80 ms and longer times the increase is significant (Students t-test, P<0.05).

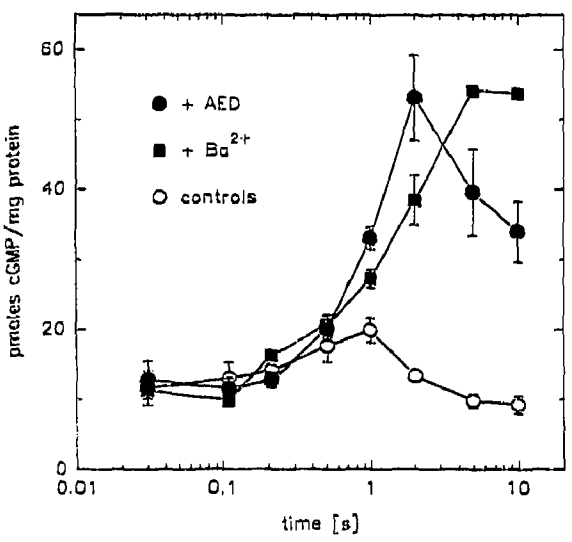

Fig. 3. Levels of cGMP within 10 s after stimulation. Data represent means ±S.E.M. (n = 3 for controls and AED-stimulation, n = 2 for Ba^{2+}-treatment). Differences as compared to controls become significant at 1 s.

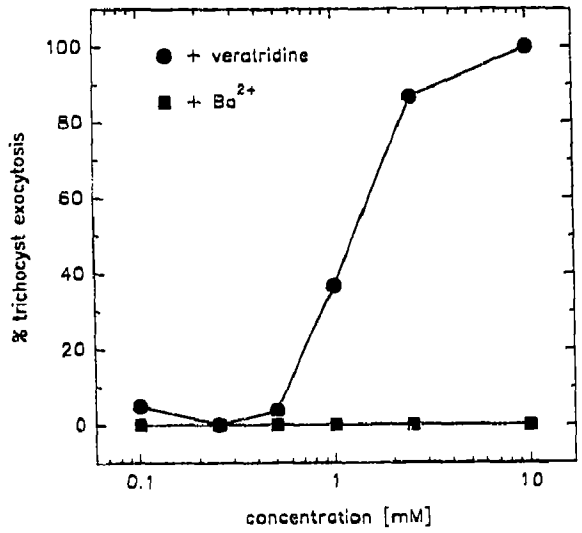

Fig. 4. Effects of Ba^{2+} and veratridine on trichocyst exocytosis. Data represent means of semiquantitative determinations (n = 2–4).

pmol·s·mg protein) as compared to Ba^{2+} treatment (up to 17 pmol·mg protein). This high cGMP increase observed with AED, since the mode of action of this drug is still unknown, might reflect the superposition of two effects, the one on exocytosis-associated Ca^{2+} influx, the other one on voltage-dependent ciliary channels.

3.3. *Effects of veratridine and Ba^{2+} on exocytosis*

Both Ba^{2+} as well as AED lead to a rise in cGMP level, which is assumed to be caused by Ca^{2+} influx [2]. Recently veratridine has been shown to cause an increase of cGMP in a similar manner to Ba^{2+} [14]. Therefore, we tested the effects of Ba^{2+} and veratridine on trichocyst exocytosis. Figure 4 shows the inefficiency of Ba^{2+}, whereas veratridine triggered exocytosis in a dose-dependent fashion with a half-maximal efficiency of about 1 mM.

4. DISCUSSION

Global trichocyst exocytosis stimulated by AED is accompanied by an influx of Ca^{2+} already detectable 80 ms after stimulation. At this time the trichocyst contents are extruded [7]. Before extrusion plasma and trichocyst membranes have to fuse, a process which can be observed already 30 ms after stimulation [7]. However, no stimulated Ca^{2+} influx could be detected at this time point. This result may indicate either that the influx is delayed with respect to exocytosis and thus secondary to faster events stimulated by AED (as e.g. an efflux from the cortical Ca-stores described in [4]) or that the influx is early but too small to be detected, as compared to the binding background seen at 30 ms in both stimulated and not stimulated cells. Within this range of time, asynchrony and heterogeneity between cells could be a problem and this question should be addressed at the single cell level, by electrophysiological methods, for instance.

The rise of cGMP after the rapid Ca^{2+} influx calls to mind the situation during the depolarization-induced ciliary reversal, where Ca^{2+} enters via voltage-dependent Ca^{2+} channels and activates guanylate cyclase [2]. However, ciliary Ca^{2+} channels have already been shown to be irrelevant for exocytosis. (i) Exocytosis can be induced in deciliated cells devoid of the Ca^{2+} channel [16]. (ii) The exocytotic response to local stimulation does not propagate [8,16]. (iii) Ca^{2+} influx and exocytosis can be induced in *pawn* mutants (defective in the ciliary channel) by stimulation with AED [6]. A Ca^{2+} influx and rise in cGMP in *pawns* has also been observed after treatment with veratridine [14], which is shown in the present study to also induce exocytosis. Therefore, we conclude that veratridine does not (at least not exclusively) act on voltage-dependent ciliary Ca^{2+} channels, but instead on some unknown target related to exocytosis induction, similarly to AED. The conclusion that veratridine activates voltage-dependent Ca^{2+} channels in *pawn* mutants [14] needs to be re-examined in view of our present results.

Since a rise in $[Ca^{2+}]_i$ (produced by ionophores) is sufficient in competent cells for induction of exocytosis [5], efficient intracellular compartmentation has to be postulated which prevents Ca^{2+} from reaching exocytosis sites after influx through the ciliary Ca^{2+} channels. The induction of ciliary reversal by a mechanical stimulus, depolarization by K^+ or even Ba^{2+} (leading to an exaggerated response) is never accompanied by trichocyst exocytosis. On the other hand, if an influx were responsible for induction of exocytosis [6], clearly the pathway has to be different from the ciliary channels [16]. This holds for both AED and veratridine stimulation, although no Ca^{2+} channel relevant for exocytosis has been identified yet.

Acknowledgments: We are grateful for expert technical assistance by Claudia Braun, help with the gamma counter (in the laboratory of Dr. V. Ullrich) by Xiao Binh, and critical reading of the manuscript by Jean Cohen. Supported by the Deutsche Forschungsgemeinschaft (SFB 156) and the Institut National de la Santé et de la Recherche Médicale (Grant 910503).

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