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Ca^{2+} oscillations mediated by exogenous GTP in *Paramecium* cells: assessment of possible Ca^{2+} sources

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Abstract

We applied exogenous guanosine trisphosphate, GTP, to *Paramecium tetraurelia* cells injected with Fura Red for analysing changes of free intracellular Ca^{2+} concentrations, $[Ca^{2+}]_i$, during periodic back-/forward swimming thus induced. Strain *ginA* (non-responsive to GTP) shows no Ca^{2+} signal upon GTP application. In strain *nd6* (normal Ca^{2+} signalling) an oscillating $[Ca^{2+}]_i$ response with a prominent first peak occurs upon GTP stimulation, but none after mock-stimulation or after 15 min adaptation to GTP. While this is in agreement with previous electrophysiological analyses, we now try to identify more clearly the source(s) of Ca^{2+}. Stimulation of *nd6* cells, after depletion of Ca^{2+} from their cortical stores (alveolar sacs), shows the same Ca^{2+} oscillation pattern but with reduced amplitudes, and a normal behavioural response is observed. Stimulation with GTP, supplemented with the Ca^{2+} chelator BAPTA, results in loss of the first prominent Ca^{2+} peak, in reduction of the following Ca^{2+} amplitudes, and in the absence of any behavioural response. Both these observations strongly suggest that for the initiation of GTP-mediated back-/forward swimming Ca^{2+} from the extracellular medium is needed. For the maintenance of the Ca^{2+} oscillations a considerable fraction must come from internal stores, probably other than alveolar sacs, rather likely from the endoplasmic reticulum.

Keywords: Ca^{2+}; Calcium; Cilia; Oscillations; *Paramecium*

1. Introduction

Ca^{2+} regulates many cellular processes, like stimulated secretion by exocytosis, gene transcription, cell division [1–4], and ciliary activity [5–9]. The origin of a Ca^{2+} signal can be manyfold, e.g., influx from the outside medium and/or release from internal stores by widely different signalling mechanisms [1,3,4,10].

As in mammalian cells, these aspects also occur in ciliated protozoa including *Paramecium* [11]. In these cells, the normal intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, mediates normal ciliary beat during forward swimming, while increase of $[Ca^{2+}]_i$ in cilia causes beat reversal and backward swimming [12–14]. A reversal reaction is easily observed during depolarization-induced activation of voltage-dependent Ca^{2+} channels in the ciliary membrane [15,16]. But spill-over from cortical regions during exocytosis stimulation can also produce ciliary reversal [17,18].

In many cell types $[Ca^{2+}]_i$ can oscillate with widely different periodicity (from the sub-second range to hours), either spontaneously, or after different exogenous triggers, with the involvement of widely different signal transduction pathways [19–21].

In *Paramecium* the discovery has been made that exogenous guanosine trisphosphate, GTP, causes periodic back- and forward swimming, paralleled by oscillating Ca^{2+} currents [22]. This observation has been extended to the related species, *Tetrahymena* [23]. Evidence has been found that during GTP stimulation Ca^{2+} may in part come from the outside and in part from internal stores. Among them the cortical Ca^{2+} stores (alveolar sacs) have been envisaged [24].

From this, the occurrence of surface receptors for GTP has been inferred in *Paramecium* [25–27] and *Tetrahymena* [23,28,29]. The typical receptor property, i.e., adaptation [30,31], has also been observed with GTP in *Paramecium* [25] and *Tetrahymena* [23]. By definition, receptors would have to be of the purinergic type. For GTP this is remarkable, as up to now from other cells such receptors are known only for ATP, ADP, UTP, and UDP [32,33]. In contrast, in *Paramecium* GTP is ∼1000 times more potent than ATP [26]. Only the non-hydrolysable β-γ-methylene

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ATP analogue can produce a similar effect in *Paramecium* [28].

If one considers a purinergic receptor for GTP in *Paramecium*, one would also have to consider the existence of different subtypes. This would include those represented (i) by multimers formed from units with two membrane-spanning domains, and those (ii) with seven membrane-spanning domains, as known from higher eukaryotes [33–35]. The first type is reported to form ion channels with low specificity [36]. This may account for the well known permeabilising effect of exogenously added nucleoside trisphosphates [36]. This can induce Ca^{2+}-dependent processes, such as exocytosis [37], provided Ca^{2+} is in the medium. The second type is involved in signal transduction by coupling to trimeric G-proteins, followed by activation of either adenylate cyclase or phospholipase C (PL-C) and formation of the Ca^{2+}-mobilising agent, inositol 1,4,5-trisphosphate ($InsP_3$), [35].

None of these molecules and pathways are established in *Paramecium* on a molecular level. Therefore, analysis of GTP-mediated effects on Ca^{2+}-based swimming behaviour necessarily remains, though functionally stringent, on a merely descriptive level at this stage. However, by a more detailed analysis of the phenomena and by inclusion of Ca^{2+} imaging technology, we here try to set a new baseline, considering the rapidly expanding molecular biology work with *Paramecium*. Essentially we use fluorochrome imaging to localise Ca^{2+} signals under conditions of varying extracellular Ca^{2+} concentrations, $[Ca^{2+}]_o$, and we also deplete alveolar sacs of their Ca^{2+} before subsequent GTP stimulation. We aim at dissecting more clearly the different components of the GTP-mediated Ca^{2+} signals.

2. Materials and methods

2.1. Cell materials

Paramecium tetraurelia cells, mutant *nd6* (without trichocyst discharge [38]), were cultured in dried lettuce medium, monoxenically inoculated with *Enterobacter aerogenes* as feeding bacteria, at $[Ca^{2+}]_o = 50\,\mu M$. In addition we used the mutant *pawnB* [39] and *ginA* [40]. All cell lines used additionally contained the *nd6* mutation. Both, non-discharge (*nd*) and *pawn* properties (lack of ciliary reversal reaction upon depolarization) were tested before use: (i) by adding the secretagogue aminoethyldextran, AED (40 kDa, 1-NH_3^+/kDa [17]), and (ii) by chemical depolarization by adding 20 mM KCl, respectively.

2.2. Stimulation conditions

For $[Ca^{2+}]_i$ analysis, cells where washed in 1 mM HEPES buffer adjusted to pH 7.2 with Tris. The solution was supplemented with 1 mM KCl and 1 mM Ca^{2+} (modified after Clark et al. [41]). Eventually lower values of $[Ca^{2+}]_o$ where achieved by adding the ultrafast Ca^{2+}-chelator, BAPTA (time constant = 0.5 μs [42]), to the trigger solution to produce $[Ca^{2+}]_o$ of ∼30 nM, i.e., slightly below $[Ca^{2+}]_i$ at rest (∼65 nM [43]). Aliquots were stimulated by AED to deplete cortical Ca^{2+} stores [44] before GTP was added within ≤3 min.

Cells were stimulated by adding, through a pipette, GTP (in 10 mM Tris buffer, pH 7.2) to the medium at the anterior pole of individual cells. The actual concentration of GTP reaching the cell surface was estimated from the dilution after release from the microcapillary as 15 μM, according to the approach previously described [43]. Although this is slightly above the 10 μM generally used in the *Paramecium* literature, in fact, slightly higher concentrations are required for maximal stimulation [40].

Mock applications were performed with buffer, without GTP added to the trigger solution. $[Ca^{2+}]_o$ was eventually reduced to a calculated value of ∼30 nM by adding BAPTA to the stimulation medium—not before. Simultaneous application of stimulant and the Ca^{2+} chelator, BAPTA free acid, is an established method that avoids cell damage [45].

2.3. Ca^{2+} fluorochrome analysis

Cells loaded by microinjection with the Ca^{2+} fluorochrome Fura Red were stimulated and evaluated in a conventional light microscope by 2λ analysis, as previously described [43,45]. Fura Red was used at a concentration to yield an intracellular concentration of ∼50 μM. In the f/f_0 ratio analysis, any fluorescence readings during stimulation (f) are expressed as a ratio referred to the reading at rest, time t_0 (f_0), just before stimulation. The ratio method allows measurements of $[Ca^{2+}]_i$ independently from fluorochrome concentration in the cell. The maximum emission of Fura Red upon excitation is 650 nm. For excitation, wavelengths of 440 and 490 nm are used and the ratio of emission at both wavelengths is calculated. Thus, interference from autofluorescence and effects from shape change are eliminated [46], although the time required for filter changes (1.5 s per final data point) restricts time resolution. We recorded f/f_0 ratios over a time period of up to 1 min.

$[Ca^{2+}]_i$ analysis was performed at the cortical site (below cilia) where the stimulus had been applied. A frame of ∼3 × 10 μM was adjusted to such an area, just as in our previous work [43,45]. The f/f_0 ratios thus obtained were evaluated by the ANOVA test which allows statistical comparison of several data sets. Usually, per data set shown, the time course of five cells (unless indicated otherwise) has been analysed by this point-per-point digital analysis.

3. Results

3.1. Compatibility with previous electrophysiological and behavioural analyses

Before analysing new aspects by Ca^{2+}-fluorochrome imaging we tried to reproduce important aspects of

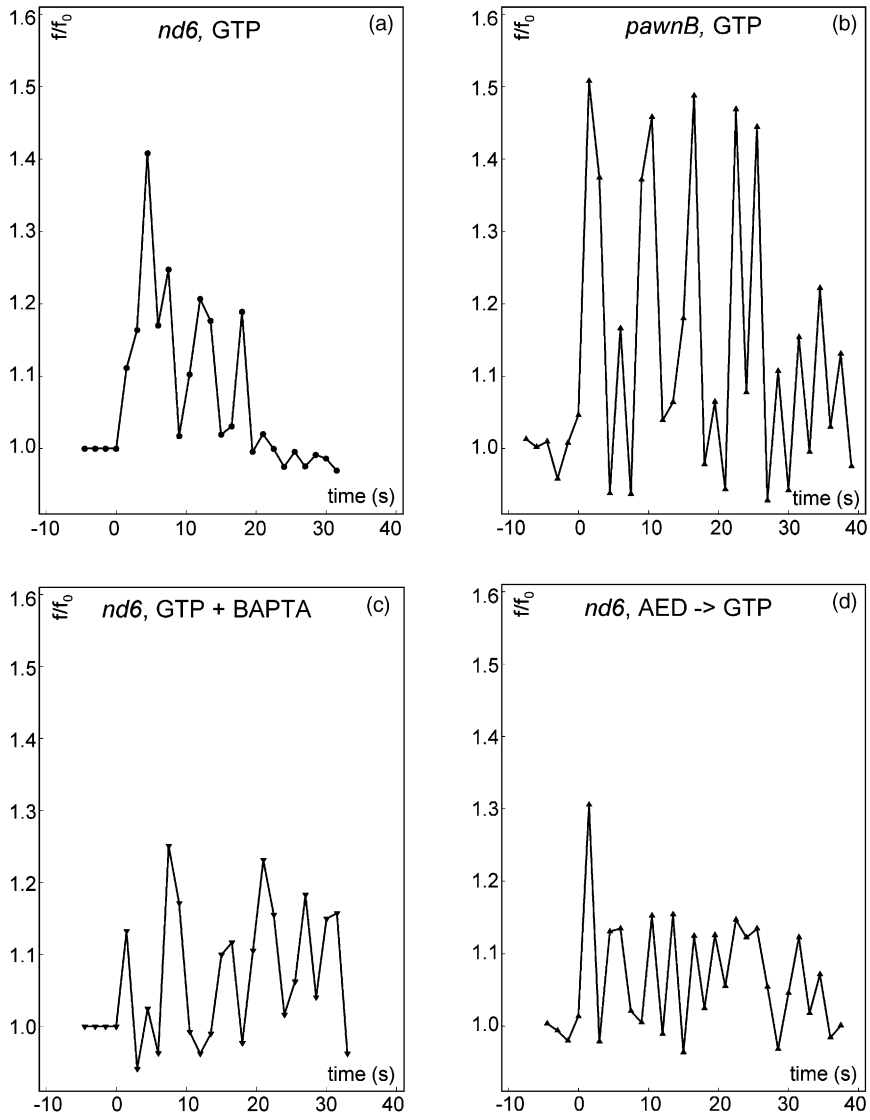

Fig. 1. Examples of Fura Red f/f_0 recordings during GTP stimulation in *nd6* (a, c, d) and *pawnB* (b) cells under the conditions indicated. GTP was added to the cells at $t = 0$ s, usually in presence of Ca^{2+} (a, b, d), while in (c) BAPTA was added to the GTP stimulant, as specified in Section 2. In (d) AED was applied shortly before GTP stimulation (but after decay of the AED-mediated Fura Red f/f_0 signal shown in Fig. 5d) to probe the response to GTP when alveolar sacs were depleted of most of their Ca^{2+}. Note the eventual occurrence of a predominating first peak (a, d) which becomes more clear in the statistical evaluations shown in Fig. 3.

GTP-mediated Ca^{2+} signalling in *Paramecium*, as reported from electrophysiological work. These analyses have been conducted in presence of extracellular Ca^{2+}, $[Ca^{2+}]_o = 1$ mM. We measured f/f_0 ratios in a cortical region adjacent to the stimulation site, as specified in Section 2.

In all strains we analysed (except *ginA*), GTP-induced $[Ca^{2+}]_i$ oscillations with a period of about 7 s (Figs. 1 and 2). This is within the frame of previous work, with periods of about 5–10 s [24,26,40,41], always depending on whether smaller peaks are also taken into consideration. Occurrence and absence of oscillating Ca^{2+} signals strictly correlates with the occurrence and absence of periodic ciliary reversal, respectively, interrupted by forward swimming (Table 1), also as reported in previous work on GTP stimulation. In *nd6* cells, for unknown reasons, adaptation to GTP reduces the oscillation frequency (an aspect not pursued here). Any further variation to this reaction pattern is specified in Table 1. Our findings include the occurrence of periodic $[Ca^{2+}]_i$ fluctuations in *nd6* cells as well as in both *pawn* strains analysed, *pawnA* (not shown) and *pawnB*. The intensity (f/f_0) of GTP-induced $[Ca^{2+}]_i$ signals is evaluated in Fig. 3, either the first (abundant) peak only or the average of the following peaks, respectively. This appeared feasible to us since we frequently see that the first f/f_0 peak induced by GTP is larger than the subsequent ones (Fig. 3 columns a versus columns b). For unknown reasons *pawn* cells produce rather variable $[Ca^{2+}]_i$ amplitudes (see large S.E.M. for *pawnB* in Fig. 3) and their behavioural response also

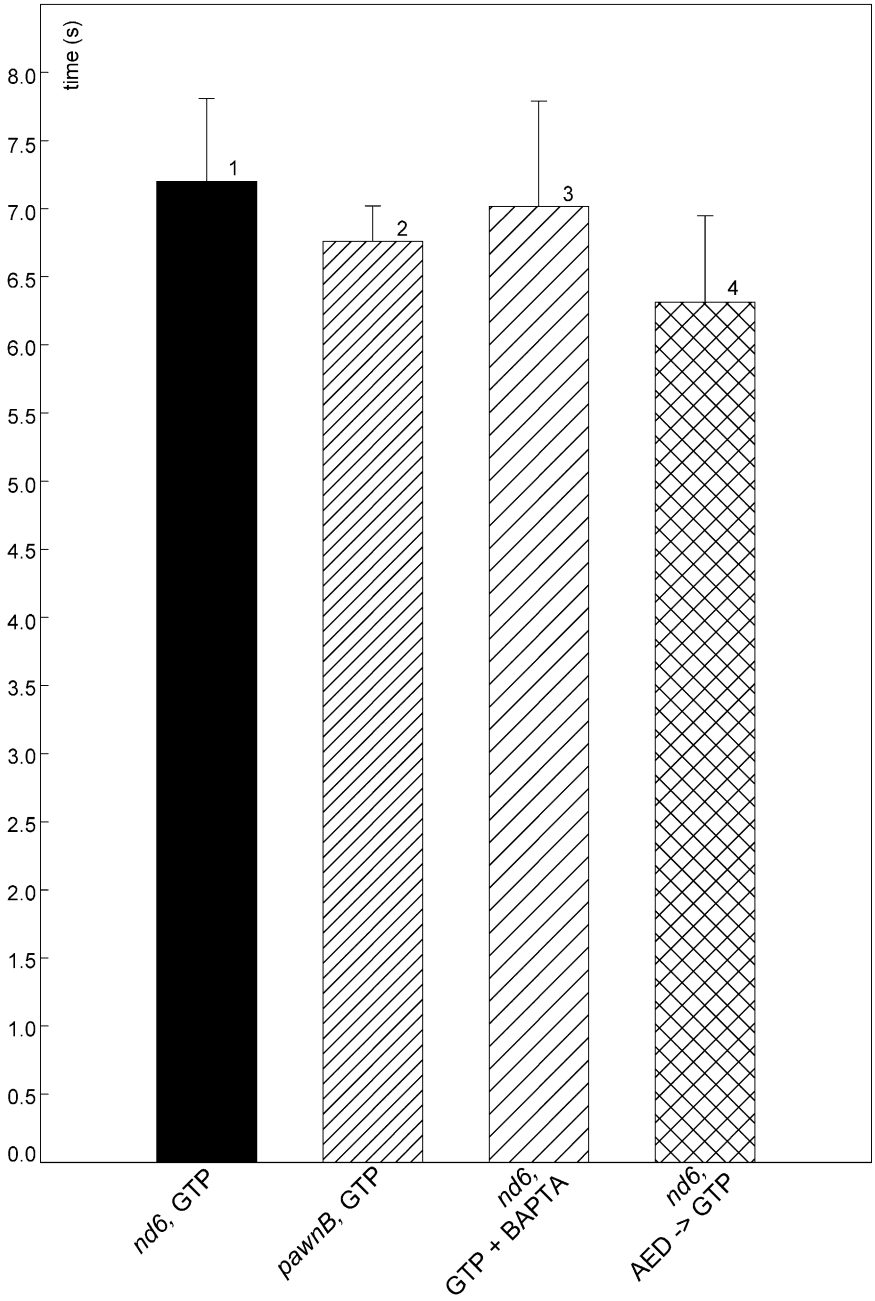

Fig. 2. Periodicity of Fura Red f/f_0 signals recorded after GTP stimulation in *nd6* and *pawnB* cells under the conditions indicated. Note that in both strains the response shows similar periodicity of ∼7 s, regardless whether Ca^{2+} is present in, or absent (+BAPTA) from the GTP stimulation solution, or whether alveolar sacs have been depleted of their Ca^{2+}. Number of cells analysed in columns 1–4, $N = 5, 5, 5, 4$. Error bars = S.E.M. According to the ANOVA test applied, the data contained in columns 1–4 are not significantly different from each other.

varies (Table 1). We can well reproduce reports on adaptation of *Paramecium* to GTP [25] showing that *nd6* cells become adapted to GTP over ∼15 min (Figs. 4 and 5a). While the predominance of the first peak is a new aspect, all data are well compatible with the electrophysiological work cited above. This, together with the subsequent controls, made us confident about the imaging methodology we used for the first time on this subject.

Before extending our studies to new aspects we performed the following controls. Application of buffer to *nd6* cells does not evoke any Ca^{2+} signal (Fig. 3, 5th column pair, and Fig. 5b), thus excluding mechanical stimulation by the flush. This is important considering ongoing discussions on the interacting effects of mechanical stress and of nucleoside trisphosphates [47]. Similarly we see neither any $[Ca^{2+}]_i$, nor any behavioural response in the non-responder, strain *ginA*, when exposed to GTP (Fig. 3, 6th column pair, and Fig. 5c). All GTP-mediated $[Ca^{2+}]_i$ responses are clearly different from those obtained during AED-stimulated exocytosis (Fig. 5d), which are

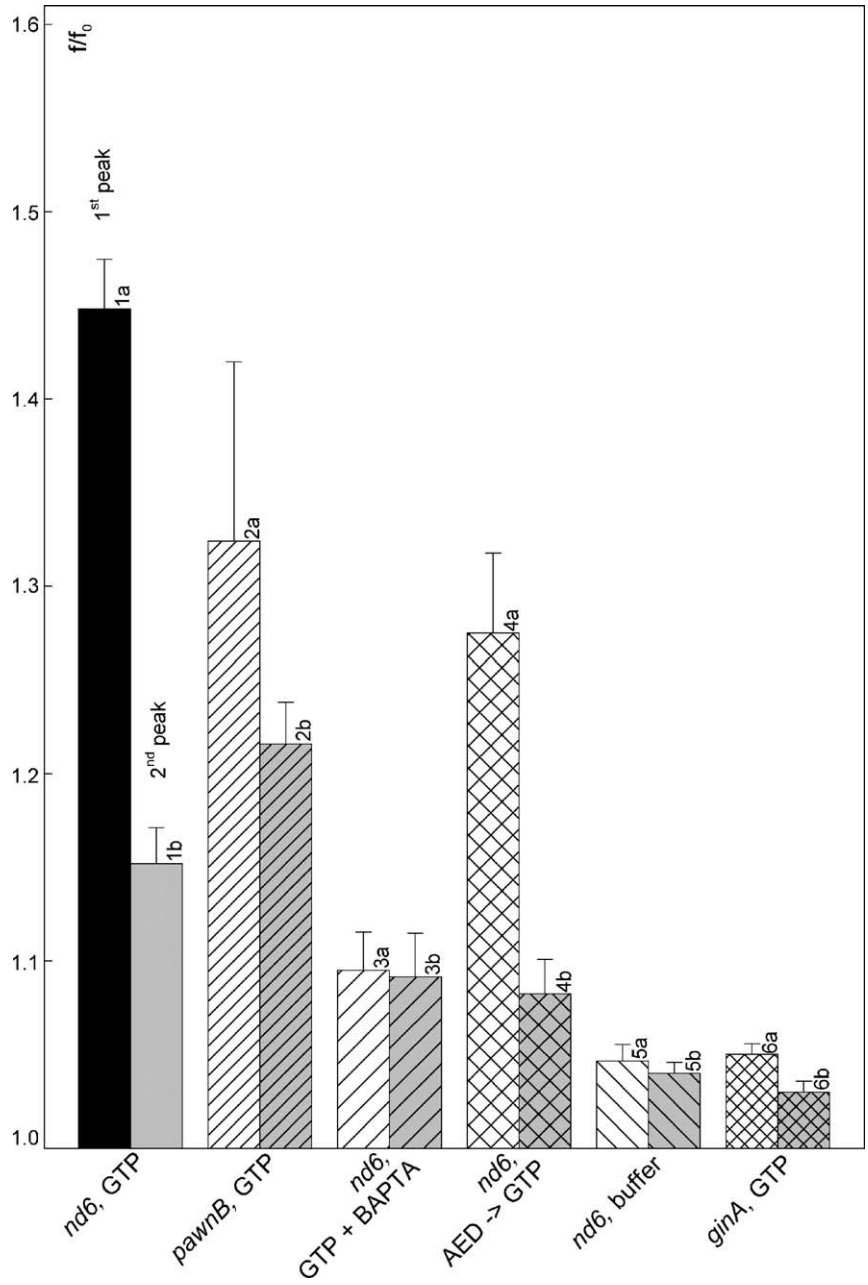

Fig. 3. Fura Red f/f_0 signal increase upon GTP stimulation in different strains and under conditions indicated for the respective column pairs, 1–6. The 1st column ("a") of each pair shows the size of the first peak recorded immediately after stimulation, column "b" designates the average of all following peaks recorded. The first peak frequently predominates, e.g., in the columns 1a, 2a and 4a, but not in the column pair 3 (+BAPTA added), 5 (mock stimulation) and 6 (non-responding *ginA* cells). $N = 5$ (for each of the individual columns, 1a through 3b), 4 (columns 4a, 4b), 3 (columns 5a through 6b). $P < 0.05$ for comparison of columns 1a and 1b, 2a/2b, 4a/4b. No significant difference was found between columns 1a/3a, 1a/4a, 3a/3b, 3a/5a, 5a/5b, 6a/6b, 5a/6a, 3b/5b, 5b/6b. For the column pair 1a/2a significance is only $P = 0.127$, for 1b/3b $P = 0.087$. Error bars = S.E.M. For abbreviations, see Fig. 2.

of the monophasic type reported in our previous work [43].

3.2. Additional new aspects of GTP-mediated Ca^{2+} signalling

This includes the observation that, in all GTP-sensitive strains analysed, the first f/f_0 peak generated by GTP frequently, although not always, exceeds any further peaks. These remain of fairly constant height, at least over several oscillations to follow (Figs. 1a, d, and 2). We analysed GTP effects in *nd6* cells not only in presence of $[Ca^{2+}]_o = 1$ mM, but also with GTP with BAPTA added (Figs. 1c and 3). This is a very fast Ca^{2+} chelator, with a time constant of 0.5 μs [42]. Such methodology—avoiding preincubation with the chelator—has been reliably applied to *Paramecium* in another context, without any deleterious side-effects [45]. Our findings with stimulation in presence of BAPTA are as fol-

Table 1
$[Ca^{2+}]_i$ oscillations[a] and behavioural response

Strain	Stimulation	$[Ca^{2+}]_i$ oscillations occurrence/1st peak	Behavioural response
nd6	GTP[b]	Oscillation/abundant	Periodic reversal
	GTP, 5 min	Oscillation/abundant	Periodic reversal
	GTP, 15 min	No oscillation/missing	None
	GTP + BAPTA[c]	No oscillation/missing	None
	Buffer	No oscillation/missing	None
	AED[d] → GTP	Weak oscillation/occurring (but weaker)	Less pronounced periodic response
pawnB	GTP	Weak oscillation/occurring (but weaker)	Less pronounced periodic response
ginA	GTP	No oscillation/missing	None

[a] Data are based on the respective figures. For number of cells analysed, see figure legends.
[b] This indicates the immediate response to ∼15 μM GTP, unless indicated otherwise.
[c] BAPTA added to GTP, not before.
[d] AED: aminoethyldextran secretagogue used at ∼2 μM.

lows (Fig. 3, 3rd column pair). (i) The abundant first peak is reduced to the size of the following peaks. Concomitantly, in Fig. 3, column 3a is significantly smaller than column 1a ($P < 0.05$). (ii) All peaks are smaller with BAPTA present, when compared with stimulation by GTP in presence of $[Ca^{2+}]_o$. In Fig. 3, the difference between columns 1b and 3b is $P = 0.087$. This suggests two sources of Ca^{2+} during GTP-mediated signalling: (i) influx from the medium (GTP + $[Ca^{2+}]_o$), and (ii) mobilisation from internal stores (GTP + BAPTA). It also implies (iii) that the latter component must be independent of the influx component, thus excluding a mechanism of the type Ca^{2+}-induced Ca^{2+}-release.

In sum, our data indicate that in *Paramecium*, during GTP stimulation, Ca^{2+} from influx and from store mobilisation are independent processes which are superimposed to each other. There is no significant delay in the onset of the first Ca^{2+} oscillation peak (Fig. 1), regardless of whether $[Ca^{2+}]_o$ is high or low. Since a Ca^{2+}-induced Ca^{2+}-release mechanism seems to be excluded, one would have to expect some other intracellular signal, as discussed below.

Similar signalling occurs with *nd6* cells and with strain *pawnB* (Figs. 1–3). This excludes ciliary voltage-dependent Ca^{2+}-channels as mediators of Ca^{2+}-influx. It suggests involvement of somatic channels or of ciliary channels of another type which is not known as yet. While this has been known from *pawn* cells already [22], the involvement of any Ca^{2+} channels has been questioned altogether [41]. To us, involvement of some somatic Ca^{2+} channels appears appealing, not only because we see a Ca^{2+} influx component (Fig. 3 column 1a versus column 3a, and column 1b versus column 3b), but also because in another context we have found that spill-over of Ca^{2+} from somatic domains into cilia can easily take place, but not in the opposite direction [18]. One has to recall that we record f/f_0 changes not directly in cilia, but in the nearby cell cortex, as frequently done in work with cilia [5,9,48]. Our findings favour as a second component the involvement of intracellular Ca^{2+} stores in GTP-mediated Ca^{2+} signalling. However, for the following reasons we envisage other stores than previous work [24].

We tried to figure out which intracellular calcium pool, i.e., particularly the cortical stores (alveolar sacs) or the endoplasmic reticulum (ER), may contribute to GTP-mediated Ca^{2+}-oscillations. The corollaries and the rationale of such analyses are as follows. From energy-dispersive X-ray microanalysis (EDX) we know that alveolar sacs (which line most part of the cell membrane) have a total calcium concentration, [Ca], of ∼43 mM [44,49], most of it presumably bound to high capacity/low affinity Ca-binding proteins [50]. During stimulation of synchronous exocytosis by AED, [Ca] in alveolar sacs is reduced to ∼20% of its original value within 1 s [44]. We also know that refilling is unexpectedly slow, with a half-time of ∼60 min [51]. Although [Ca] may be less high in the ER, it deserves interest in the context of GTP-mediated Ca^{2+} signalling because it approaches ciliary bases where no alveolar sacs occur [52]. To differentiate between the two candidates for stores possibly involved in GTP-mediated signalling, i.e., the alveolar sacs and the ER, we proceeded as follows.

In a series of experiments with *nd6* cells we have depleted alveolar sacs of their Ca^{2+} by massive stimulation with AED. Following this, these cells have been stimulated with GTP. The time elapsed between the two stimuli was only ≤3 min, which is much too short to allow for any significant refilling [51]. Under these conditions, during GTP stimulation, we observe oscillating f/f_0 signals with a large first peak (Figs. 1d and 3, column 4a); subsequent peaks are quite similar to those occurring during stimulation by GTP + BAPTA, without previous depletion of Ca^{2+} from alveolar sacs (Fig. 1c versus d). Notably the first peak is very high, irrespective of AED pretreatment (Fig. 3, column 4a). Taken together this suggests the occurrence not only of a Ca^{2+} influx, but also the release from a store other than the alveolar sacs. The behavioural response under these conditions is variable, as summarised in Table 1, but periodic back- and forward swimming is observed.

Behavioural responses parallel rather strictly the periodic Ca^{2+} signals, notably the occurrence of a dominant first peak (Table 1). GTP + BAPTA causes no periodic back- and forward swimming in *nd6* cells. A normal, though

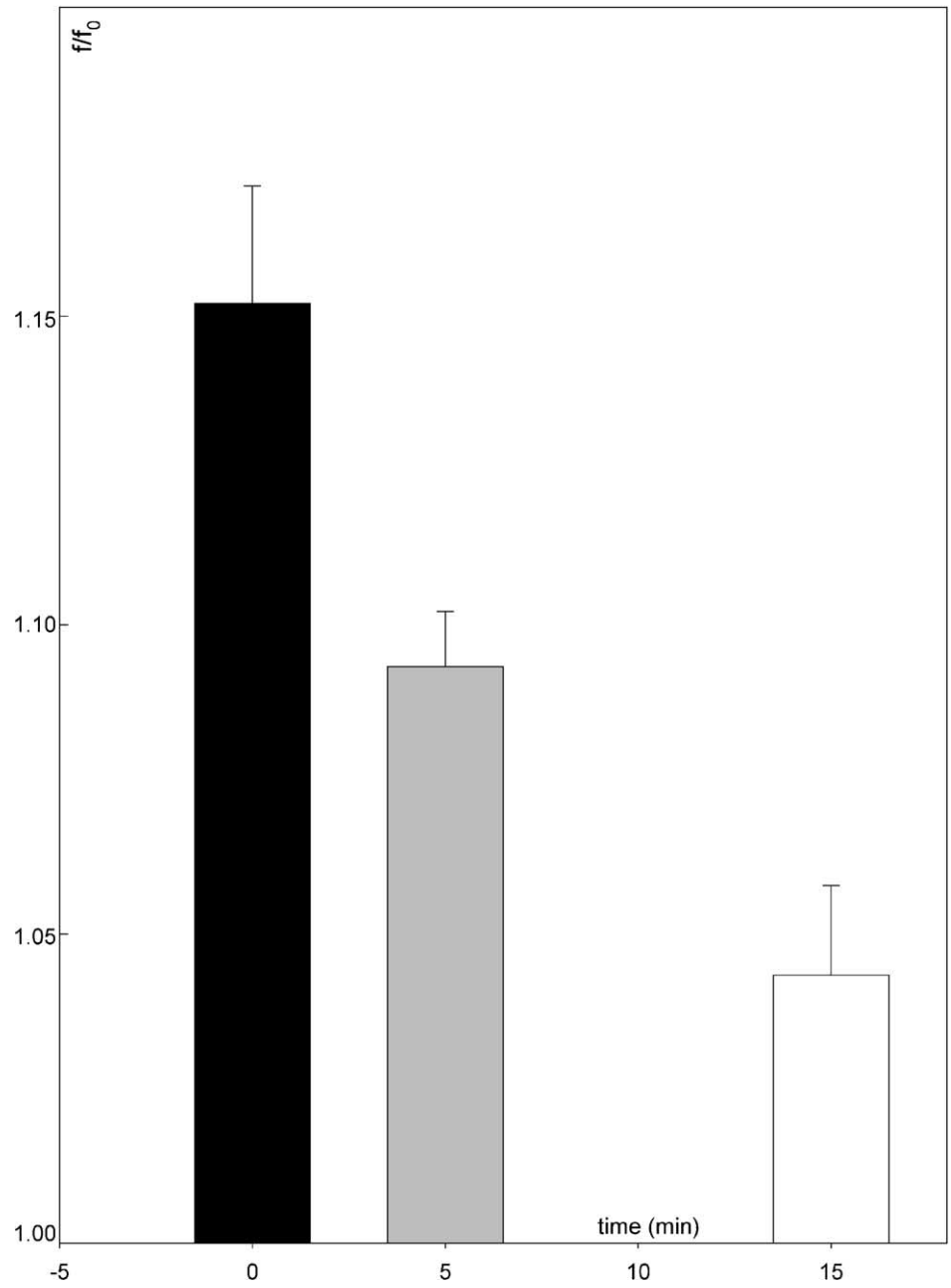

Fig. 4. Adaptation to GTP. The Fura Red f/f_0 response, averaged from all peaks, has been determined immediately after GTP addition (column 1, black) and 5 (column 2, grey) or 15 min later (column 3, white). Column 3 is not significantly different from controls (*nd6* mock stimulation, *ginA*) shown in Fig. 3. $N = 5, 3, 3$ (columns 1–3). Error bars = S.E.M. Abbreviations as in Fig. 3.

weaker response occurs after liberating Ca^{2+} from alveolar sacs by AED pretreatment. The extent of the behavioural reaction to GTP is weaker in *pawnB* cells. The reason of this minor inconsistency between *nd6* and *pawnB* cells may be as follows. Absolute $[Ca^{2+}]_i$ levels (not determined by the method used here) at rest or after stimulation may vary from strain to strain [43]. This discrepancy can suffice to yield quantitatively different responses, as also shown in previous work [53], considering that we measure f/f_0 ratios irrespective of $[Ca^{2+}]_i^{rest}$. Beyond this variability it is important to note that all strains, but *ginA*, produce Ca^{2+} oscillations under the conditions indicated. The requirement of a first strong peak for induction of a reversal reaction and the absence of both these phenomena in presence of BAPTA indicates the relevance of a Ca^{2+} influx signal, while ongoing $[Ca^{2+}]_i$ oscillations can be driven by internally released Ca^{2+}. The response after pretreatment with AED indicates that the internal store contributing to f/f_0 oscillations during GTP stimulation is most likely the ER.

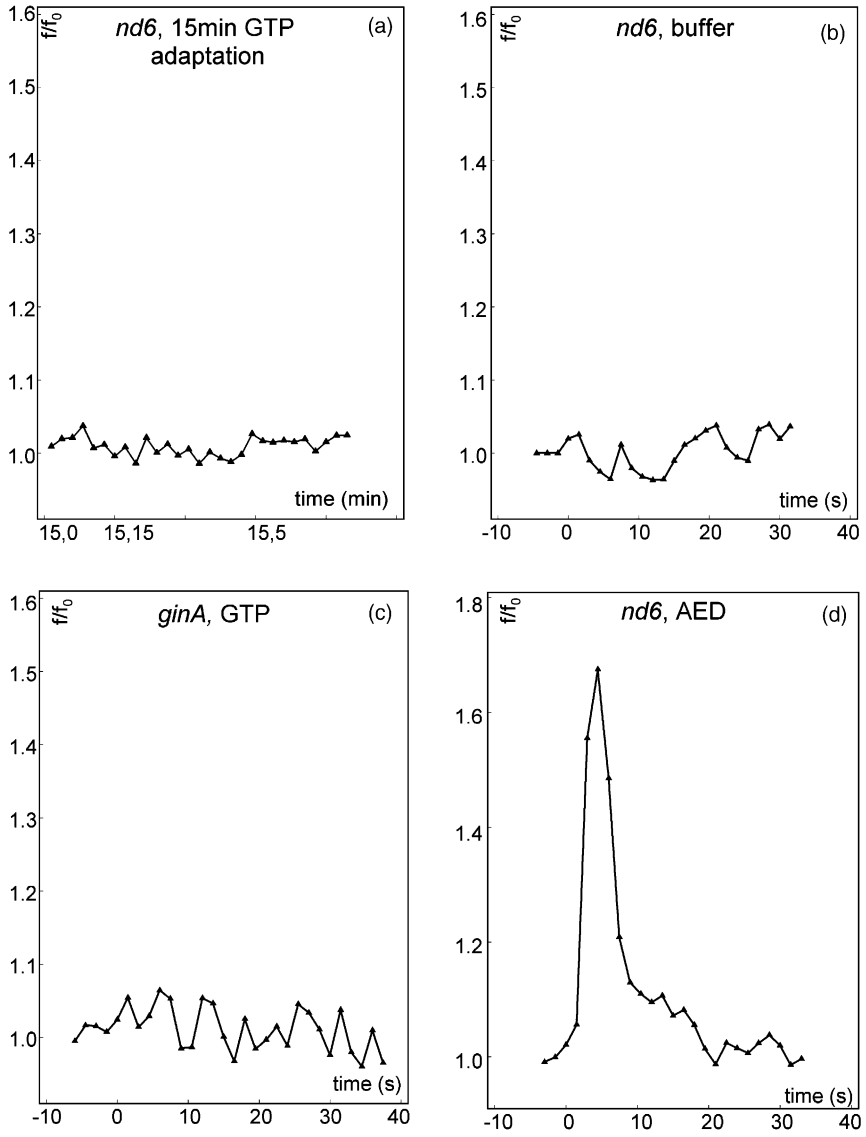

Fig. 5. Fura Red f/f_0 recordings representative of different controls. (a) Recording from a *nd6* cell 15 min after GTP stimulation (time shown: 15.0, 15.15, 15.5 min, etc.) shows adaptation when compared with Fig. 1a. (b) *nd6* cell, mock-stimulation, (c) *ginA* cell, GTP stimulation (no response). For comparison with GTP-mediated responses, we show in (d) the response of a *nd6* cell to AED (no GTP), as occurring during induction of synchronous exocytosis—in agreement with the literature cited in the text. This is to be compared with GTP stimulation *after* AED application (Fig. 1d).

4. Discussion

Using fluorochrome analysis, we first confirm that GTP-mediated periodic back- and forward swimming in *Paramecium* is paralleled by Ca^{2+} oscillations, as previously found by electrophysiology (see Sections 1 and 3). The new information we obtained includes the following aspects. (i) The occurrence of a dominant first peak (with higher amplitude than the following ones) is important for the induction of the behavioural response described. (ii) The occurrence of lower $[Ca^{2+}]_i$ amplitudes, though with unchanged period, at low $[Ca^{2+}]_o$, implies that these signals are driven from internal Ca^{2+} stores. (iii) The occurrence of identical oscillations after depleting alveolar sacs of their Ca^{2+} disqualifies these stores as relevant source of Ca^{2+} during GTP stimulation. From this we imply (i) the occurrence of GTP-activated Ca^{2+}-influx channels, most likely outside cilia, and (ii) the activation of Ca^{2+} release independent of Ca^{2+} influx. Our data allow us to re-interpret origin, mechanism and potential functional aspects of GTP-mediated Ca^{2+} signalling in *Paramecium*.

4.1. Are there purinergic surface receptors in Paramecium and what may be their function?

To our knowledge there are no GTP receptors reported to occur on the cell surface of any other eukaryote [33]. Is it

feasible to assume such an aberrant situation for *Paramecium*? In fact, as far as the signal transduction machinery in *Paramecium* is concerned, we already know several striking deviations from the rule. An example relevant for a re-interpretation of previous data is as follows. For instance, the SERCA-type Ca^{2+}-pump is strictly insensitive [54] to the classical inhibitor, thapsigargin [55], probably because of an aberrant binding site [56], while its activity is inhibited by caffeine [57]. No Ca^{2+} release from alveolar sacs is achieved, neither with ryanodine, nor with $InsP_3$ [54], but with the polyamine secretagogue AED [44,53]. These data, together with the unexpectedly slow refilling [51], shed some doubt on conclusions derived from experiments with SERCA inhibitors, assuming a role for alveolar sacs in Ca^{2+} release and uptake cycles during GTP stimulation [24]. Considering the endogenous Ca^{2+} signalling component which we assume for GTP activation, the ER is a more likely candidate than alveolar sacs (see below), unless one would concede to alveolar sacs an unexpected hybrid character. However, these aspects also require more detailed analysis, since one store may be susceptible to two different activators, at least in mammalian cells [58].

The fact that GTP causes the phenomena described, while ATP or UTP do not [26], remains undisputed, although non-hydrolysable ATP analogues work equally well at very low concentrations [27]. Binding studies strongly suggest the occurrence of purinergic receptors in *Paramecium* [27] and *Tetrahymena* [29]. In the future, molecular cloning and modelling may specify such receptors in more detail.

In metazoans, two subgroups of purinergic receptors exist [33]: (i) one family with Ca^{2+}-channel properties [34,36], and (ii) another one involved in signalling by second messengers, e.g., $InsP_3$ [35]. According to our findings with *Paramecium*, both receptor types would be required to account for the two components observed, one depending on $[Ca^{2+}]_o$ and another one operating by internal store mobilisation, respectively. The first type is of unknown cation specificity and its relation to channels described in *Paramecium* electrophysiologically [12–14,59] remains open. This is in contrast to previous studies with *Paramecium* which did not consider the involvement of Ca^{2+} influx [41]. (On the other hand, our data are not appropriate to exclude the involvement of any other specific ion channel.) The second receptor type may use trimeric G-proteins with $G\alpha_{0/i}$ subunits of the type suggested to occur in *Paramecium* by DeOndarza et al. [60] on the basis of Western blots with heterologous antibodies and of pertussis toxin-mediated ADP-ribosylation. Clearly, molecular identification is required also for these aspects. The large sequences of bona fide $InsP_3$ receptors meanwhile cloned in our lab (E.-M. Ladenburger, I. Korn, R. Kissmehl and H. Plattner, in preparation) may be another detail of the emerging puzzle. Channels with hybrid properties, sharing characteristics of $InsP_3$- and ryanodine-receptors, have been described on a pharmacological basis in *Toxoplasma gondii* [61], a closely related member of the phylum Alveolata. Furthermore, a crosstalk between both types of stores in producing Ca^{2+} oscillations has been discussed in some mammalian systems [19].

4.2. How would Ca^{2+} oscillations be produced?

A feedback between Ca^{2+} mobilisation and Ca^{2+}-activated and Ca^{2+}-inhibited processes can cause $[Ca^{2+}]_i$ oscillations in widely different cells [62–69]. This could encompass, e.g., phospholipase C and Ca^{2+}-inhibited Ca^{2+} release via $InsP_3$ receptors. To explain oscillations at low $[Ca^{2+}]_o$ in *Paramecium*, this is just one theoretical option to be pursued. $InsP_3$ receptors may also occur in the cell membrane, at least in mammals [70]. If this turns out to be the case, it could be one among several possible explanations for our observation that, in *Paramecium*, oscillations are quite similar, though of different amplitudes, with and without Ca^{2+} in the medium. However, other activators of Ca^{2+} release channels can also produce $[Ca^{2+}]_i$ oscillations [71,72]. Examples are the interference of Ca^{2+} pumps and ion exchangers [73] or other ion pumps with $InsP_3$ receptors [74]. Specifically, purinergic receptor activation in mammalian cells can produce Ca^{2+} mobilising second messengers other than $InsP_3$ [75]. Finally, feed-back with, as well as adequate positioning of intracellular Ca^{2+} binding proteins has to be considered [72,76]. In *Paramecium*, the periodic response may also be influenced by the ecto-ATPase which hydrolyses GTP equally well [77].

At this time, for *Paramecium* one can only speculate about the molecular machinery potentially involved in GTP-mediated $[Ca^{2+}]_i$ oscillations. Clearly more work is required to elucidate the chain of events leading to the ciliary response to be discussed below.

4.3. Effect on ciliary beat

It is established that $[Ca^{2+}]_i$ in the ciliary basis is critical for beat regulation [6,78]. Depending on the $[Ca^{2+}]_i$ actually "seen" by the basal portion of cilia, the actual Ca^{2+} level serves to drive either regular beating (forward swimming), or a beat reversal. The latter occurs in *Paramecium* after a $[Ca^{2+}]_i$ increase beyond a certain threshold [18].

Could the behaviour observed be accounted for by store mobilisation? We have seen in *Paramecium* cells by EDX analysis that, during stimulated exocytosis, Ca^{2+} spill-over occurs from the somatic cytosol into cilia, whereas Ca^{2+} fluxes in the opposite direction have never been observed [18]. Since depletion of Ca^{2+} from alveolar sacs still allows Ca^{2+} oscillations to occur in our experiments with GTP, we presume the ER as the relevant store, particularly since it reaches quite far out to the cell periphery [52], where alveolar sacs spare the basis of cilia.

$[Ca^{2+}]_i$ oscillations could contain a code for back- and forward swimming. Such oscillations have been shown to activate in mammalian cells a Ca^{2+}/calmodulin-dependent

protein kinase by superposition of waves [79]. A related kinase with calmodulin motifs [80] has recently been discussed in the context of ciliary beat regulation in *Paramecium* [81]. This is another track to be followed up.

4.4. Why should a cell release GTP?

Release of ATP from the cytosol of undamaged cells is established with mammalian cells [47] which may thus achieve an auto- and/or paracrine effect via purinergic surface receptors. Like in mammals, in *Paramecium* cells ATP and GTP occur in concentrations of 1 and 0.3 mM/l, respectively [82]. Considering the small cell volume of 0.7×10^{-10} l [83], even a dense population (e.g., 10^3 cells/ml) could hardly account for the release of any sufficient GTP into the medium to deliver a concentration sufficiently high for a long range signalling effect ("blood in the water" effect after cell damage). Since purinergic receptors are activated by micromolar ligand concentrations [84], an auto-/paracrine effect appears much more plausible, i.e., for near-range signalling by GTP in *Paramecium*. Along these lines, Iwamoto and Nakaoka [85] have shown that GTP at a concentration of 10 μM stimulates cell division in *Tetrahymena*. Moreover they found that shearing forces induced by slight centrifugation have the same effect. This is known from mammalian cells to cause nucleotide leakage [47]. In another study with *Paramecium*, Prajer et al. [86] observed $[Ca^{2+}]_i$ oscillations accompanying cell division. More concise ideas on signalling into the nucleus by superposition of $[Ca^{2+}]_i$ oscillations are available from lymphocytes [72]. In a *P. caudatum* strain we had previously observed a spontaneous 8-s period (similar to our current observations) whose generation and functional effect has not been persued [11]. Taken together, all this would be compatible with an auto- and/or paracrine effect of GTP in viable cells via purinergic receptors. In fact, exogenous nucleoside trisphosphates are also known to stimulate ciliary beat activity, at least in higher eukaryotes [9,34] which, in contrast to lower eukaryotes, do not have the capacity to reverse their ciliary beat.

Could all this, in functional terms, imply that cells would be made susceptible by an auto-/paracrine pulse of GTP to ongoing divisions? Would such cells be at the same time liable to periodic back- and forward movement, thus to remain in a favourable environment? This is another distinct question raised by our observations. Altogether, the function we envisage is widely different from what has been discussed up to now for GTP-mediated $[Ca^{2+}]_i$ oscillations.

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