Oscillating response to a purine nucleotide disrupted by mutation in *Paramecium tetraurelia*

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The purine nucleotide GTP, when added extracellularly, induces oscillations in the swimming behaviour of the protist *Paramecium tetraurelia*. For periods as long as 10 min the cell swims backwards and forwards repetitively. The oscillations in swimming behaviour are driven by changes in membrane potential of the cell, which in turn are caused by periodic activation of inward Mg^{2+}- and Na^{+}-specific currents. We screened for and isolated mutants that are defective in this response, exploiting the fact that the net result of GTP on a population of cells is repulsion. One mutant, GTP-insensitive (*gin A*), is not repelled by GTP. In addition, GTP fails to induce repetitive backwards swimming in *gin A* mutants, although they swim backwards normally in response to other stimuli. GTP fails to evoke oscillations in membrane potential or Mg^{2+} and Na^{+} currents in the mutant, although the Mg^{2+} and Na^{+} conductances are not themselves measurably affected. A small, oscillating Ca^{2+} current induced by GTP in the wild type, which might be part of the mechanism that generates oscillations, is also missing from *gin A* cells. To our knowledge, *gin A* is the first example of a mutant defective in a purinergic response. We discuss the possibility that the *gin A* lesion affects the oscillator itself.

INTRODUCTION

In addition to having roles in metabolism, purine nucleotides also act as extracellular messengers for many types of cell. Extracellular ATP at micromolar concentrations can induce diverse responses, including the modulation of ciliary beat frequency in epithelial cells from frog oesophagus [1], and the carrying of fast synaptic neurotransmission in the central nervous system of vertebrates [2–4]. Mediating these varied responses to nucleotides are purinergic receptors (purinoceptors), which lie in the plasma membrane. ATP receptors fall into at least two classes: metabotropic receptors known as P2Y and ionotropic receptors known as P2X [5,6]. Extracellular GTP, which has been found to produce relaxation in rat mesenteric artery and smooth muscle, might activate a distinct purinoceptor [7].

The first observation of a purinergic effect in a microbe was that of GTP on the ciliate *Paramecium tetraurelia* [8]. However, whereas purinergic receptors in vertebrate tissue respond primarily to ATP and UTP, *Paramecium* responds preferentially to GTP and its close structural analogues, such as guanosine 5′-[γ-thio]triphosphate and guanosine 5′-[β,γ-imido]triphosphate; ATP is 1/1000 as potent, whereas other nucleotides such as CTP, XTP, UTP and ITP produce no apparent response [8]. Given the specificity of this response, it is likely that a receptor mediates nucleotide signal transduction in *Paramecium*, although this receptor has yet to be identified at the molecular level.

P. tetraurelia normally swims through its freshwater environment with its anterior end forward. GTP induces oscillatory changes in the cell's swimming direction, causing repetitive episodes of backwards swimming. As illustrated in Scheme 1, the GTP response pathway is presumably initiated by GTP binding to its receptor on the plasma membrane of the cell. Receptor binding is then transduced by an unknown mechanism into an oscillating intracellular signal that periodically activates Mg^{2+} and Na^{+} conductances [9]. Because these conductances are known to be operated by intracellular Ca^{2+} [10,11], it is likely that this ion provides the oscillating signal that mediates the GTP response. Oscillating Ca^{2+} triggers oscillating Mg^{2+} and Na^{+} currents, which in turn produce oscillations in membrane potential. On each depolarization, voltage-sensitive Ca^{2+} channels in the ciliary membrane open and admit Ca^{2+} into the cilia. The increase in Ca^{2+} concentration in the cilia leads to a reversal in the direction of the ciliary power stroke, causing the cell to swim backwards [12]. The net effect of these events on a population of cells is repulsion from GTP.

Purinergic receptors employ a variety of second messengers inside the cell. Often, however, they initiate signalling pathways that result in oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [13–15]. Oscillating $[Ca^{2+}]_i$ is a common response to stimulation by many different agents in tissues as diverse as muscle cells, hepatocytes, lymphocytes and fibroblasts [16–19]. Little is known, however, about the mechanism by which Ca^{2+} oscillations are established or how the purinergic signalling system might be coupled to a calcium oscillator. Although $[Ca^{2+}]_i$ has an important role in several physiological processes in *P. tetraurelia* [20–24], no stimulus other than GTP is known to induce oscillations in $[Ca^{2+}]_i$. The GTP response might thus provide a unique opportunity to study the biology of Ca^{2+} oscillations and possibly to identify genes that are important in both Ca^{2+} and purinergic signalling mechanisms. To dissect the GTP response pathway genetically, we screened for mutants that were defective only in their behavioural response to GTP. We present here a behavioural and electrophysiological characterization of a new mutant of *P. tetraurelia*, called *GTP-insensitive A* (*gin A*), which is defective in its response to GTP. Other stimuli elicit normal responses from the mutant. Furthermore no ab-

Abbreviations used: BST, backward-swimming time, expressed as a percentage; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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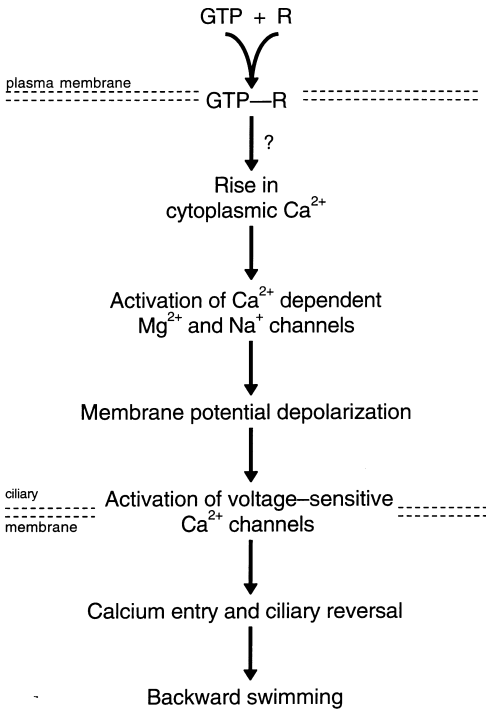

Scheme 1 Sequence of events triggered by extracellular GTP

The GTP response pathway is initiated when GTP binds to its putative receptor on the plasma membrane. The GTP signal is transduced and converted into an oscillating signal by mechanisms that are currently unknown, but probably involve intracellular Ca^{2+}. With each rise in intracellular Ca^{2+} concentration, Ca^{2+}- dependent Na^+ and Mg^{2+} channels in the plasma membrane become activated, allowing those ions to flow into the cell, depolarizing the membrane. This depolarization opens voltage-operated Ca^+ channels located in the ciliary membrane and allows Ca^{2+} to enter the cilia, which reverses the direction of the ciliary power stroke and induces backwards swimming.

normalities in the mutant could be found in the membrane ion conductances known to participate in the wild-type response to GTP. Our results suggest that *gin A* is a new mutation that blocks an early event specific to the GTP response pathway, perhaps by disrupting the oscillating mechanism itself.

EXPERIMENTAL

Cell stocks and culture conditions

The present studies were conducted with *P. tetraurelia*, stocks 51s (wild type) and GTP-insensitive A. The mutant is an F6 descendant of the original isolate, obtained through three successive backcrosses to the wild type. Both the wild-type and *gin A* stocks also contained the trichocyst non-discharge mutation *nd6* [25], which was used as a marker during genetic crosses and to facilitate the insertion of microelectrodes during electrophysiological experiments. Cells were grown at room temperature (22–25 °C) in wheat grass medium as described [9,26].

Mutagenesis and screening

Wild-type cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), as described by Kung [27]. Briefly, approx. 10^6 cells were incubated in 150 μg/ml MNNG for 1 h. After MNNG had been washed out, cells were separated into 12 groups, starved to induce homozygosity (by autogamy), and allowed to undergo six to eight fissions before screening. Exautogamous survival was approx. 40%. GTP-insensitive mutants were selected by using the galvanotaxis trough method described by Hinrichsen et al. [28] with the following modifications: mutagenized cells were concentrated in their growth medium by centrifugation. The growth medium was then diluted 1:1 with distilled water and loaded into the holding compartment of a galvanotactic trough. The outer chambers of the trough contained growth medium diluted 1:1, to which 10 μM GTP had been added. The voltage induced across the length of the trough was 40 V, in 50 ms pulses at 4 Hz. The first 10–20 cells to reach the cathode were isolated, single-cell cloned, and retested with the GTP behavioural assay described below. To enrich for cells specifically defective in their response to GTP, those individuals that could not respond to 30 mM K^+ (as described below) were discarded. These screens yielded three individuals that were unable to respond to GTP: one that produced a cell line that consistently failed to respond to GTP and was named *gin A*, one that produced a cell line that showed variable responsiveness to GTP and was named *gin B*, and one that produced an unnamed line that consistently responded to GTP, although more weakly than the wild type. Because the genetics of *gin B* has proved to be more troublesome than the wild type or *gin A*, its relationship to *gin A* has not been unequivocally determined.

Solutions

Several membrane ion conductances are known to be involved in the behavioural response of *P. tetraurelia* to GTP. 'GTP-testing solution' contained all the ions necessary for a strong behavioural response to GTP: 4 mM KCl, 1 mM Ca^{2+} [$CaCl_2$ and $Ca(OH)_2$], 1 mM Hepes buffer, 0.5 mM $MgCl_2$ 5 mM NaCl and 10 μM EDTA, pH 7.2. This solution was used in measuring the swimming response to GTP and to lysozyme, another stimulant found to cause cells to swim backwards [29]. In measuring the membrane potential response to GTP, we used GTP-testing solution modified by the addition of 10 mM tetraethylammonium chloride.

We measured the swimming response of *P. tetraurelia* to other stimuli by using solutions designed to depolarize the membrane potential of the cell: all solutions contained 1 mM Ca^{2+} [$CaCl_2$ and $Ca(OH)_2$], 1 mM Hepes buffer and 10 μM EDTA, pH 7.2; 'resting solution' additionally contained 4 mM KCl; 'Mg^{2+} solution' additionally contained 5 mM $MgCl_2$ and 10 mM tetraethylammonium chloride; 'Na^+ solution' additionally contained 10 mM NaCl; 'K^+ solution' contained 30 mM KCl; 'Ba^{2+} solution' contained 6 mM $BaCl_2$.

We obtained electrophysiological measurements of ion-specific conductances under voltage clamp with solutions designed to isolate single membrane ion conductances: 'Ca^{2+} solution', which contained 1 mM Ca^{2+} [$CaCl_2$ and $Ca(OH)_2$], 1 mM Hepes buffer and 10 μM EDTA, pH 7.2. We also used Mg^{2+} solution and Na^+ solution as described above, except that in these electrophysiological experiments, Na^+ solution was modified by the addition of 10 mM tetraethylammonium chloride.

Behavioural assays

Geotaxis assay

Wild-type and GTP-insensitive cells were washed, concentrated and preincubated for 30 min in GTP-testing solution. Approx. 200 μl of this cell suspension was then placed into 6 mm × 50 mm borosilicate culture tubes (VWR Scientific). We gently layered approx. 200 μl of GTP-testing solution with and without 10 μM GTP on top of the cell suspensions. To help prevent mixing, Protoslo (Carolina Biological Supply) was added [to approx.

10% (v/v)] to all solutions. The tubes were photographed 10–15 min after the upper solutions were layered on the cell suspensions.

GTP response

All cells were preincubated for at least 30 min in GTP-testing solution before being tested in GTP. Individual cells were then selected with a micropipette and ejected forcibly into GTP-testing solution containing concentrations of GTP stated in the text. Repeated episodes of swimming backwards were recorded on a computer in real time, as described by Clark et al. [8].

Ionic solution tests

Cells were preincubated in resting solution for approx. 30 min, then selected with a micropipette and ejected forcibly into the lysozyme-containing or ionic test solutions stated in the text, after which their behaviour was monitored for up to 5 min. Single episodes of swimming backwards were timed with a stopwatch.

Electrophysiological assays

Membrane potential measurements

To measure the resting membrane potential, two capillary microelectrodes (containing 0.5 M KCl with tip resistances of approx. 120 MΩ) were inserted briefly into and then removed from a cell bathed in resting solution. The difference between the voltage measured outside and inside the cell was taken to be the resting membrane potential. GTP-induced oscillations in membrane potential were measured with techniques described by Clark et al. [8]. Cells were bathed in GTP-testing solution while GTP was perfused into the experimental chamber (capacity approx. 1 ml) at a rate of approx. 20 ml/min. Membrane potential was recorded on a chart recorder.

Membrane current measurements

GTP-induced currents were measured with established techniques [9]. Capillary microelectrodes used to establish a voltage clamp contained 1 M CsCl and had tip resistances of approx. 40 MΩ. Cell membranes were clamped at −25 mV in Ca^{2+} solution, −20 mV in Na^+ solution, and −15 mV in Mg^{2+} solution. Currents were filtered at 10 Hz and recorded on a chart recorder. Depolarization- or hyperpolarization-induced membrane currents were measured by the method of Preston et al. [30]. The capillary microelectrodes used to establish a voltage clamp contained either 3 M KCl (for K^+ currents) or 3.5 M CsCl (for Mg^{2+}, Na^+ or Ca^{2+} currents) and had tip resistances of 20–25 MΩ. Cell membranes were clamped at −40 mV in K^+, Na^+ and Ca^{2+} solutions or at −30 mV in Mg^{2+} solution. Currents were filtered at 1–2 kHz and were recorded and analysed with pCLAMP software (Axon Instruments). All recordings were made at room temperature (22–25 °C).

RESULTS

Screening for mutants

Although several components of the GTP signalling pathway have been identified, we have little understanding of the genes involved. To dissect the GTP-signal transduction pathway genetically, we screened for mutants that were no longer repelled by GTP. A general strategy for isolating *Paramecium* mutants is to set up two stimuli in opposition to one another [27]. In wild-type cells, for example, repulsion by GTP prevents cells from responding to a second stimulus such as gravity or electric current. Mutants that are unable to respond to GTP, however, remain responsive to the second stimulus and can be enticed away from the bulk of the mutagenized cell population. In our screen we employed the strong tendency of paramecia to migrate in an electric field toward the cathode, a phenomenon known as galvanotaxis ([31]; also see the Experimental section). Mutagenized cells unable to respond to GTP migrated freely toward the cathode, whereas those that remained responsive to this chemical were unable to respond to the electric field.

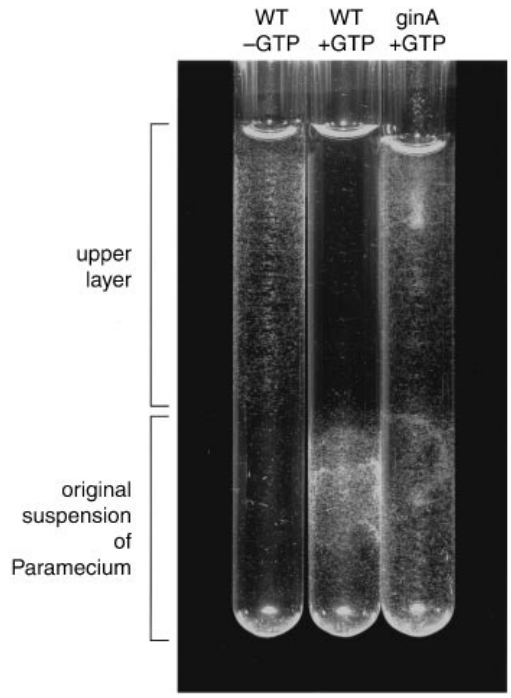

Figure 1 Effect of 10 μM GTP on the negative geotactic response of wild-type and *gin A* mutant cells

Paramecium tends to swim upwards against gravity. Within a few minutes after a fresh layer of medium was gently placed on top of a suspension of *Paramecium*, wild-type (WT) cells had swum through the new layer and reached the top of the tube (left). When the upper layer contained 10 μM GTP, however, wild-type cells were repelled by this nucleotide and prevented from swimming to the top of the tube (middle). As *gin A* mutants came into contact with the GTP-containing layer, they paused at the interface for a few minutes before continuing their migration upwards (right).

gin A mutants are defective in behavioural response to GTP

The screen described above yielded three individuals that were unable to respond to GTP and were thus named GTP-insensitives. Here we present the characterization of *gin A*, which is an F6 descendent of the original isolate. Genetic analysis (results not shown) suggests that the gin A phenotype results from a recessive single-site mutation. Insensitivity of the mutant to GTP was readily apparent in a simple geotaxis assay (Figure 1). When GTP-free buffer was gently layered on top of a suspension of wild-type cells, they migrated freely through the new layer to the top of the tube, in a characteristic response to gravity called negative geotaxis (left tube). When 10 μM GTP was included in the new layer of buffer, however, the bulk of the wild-type cells were prevented from migrating upwards for at least 30 min (middle tube). *gin A* mutants under the same conditions migrated

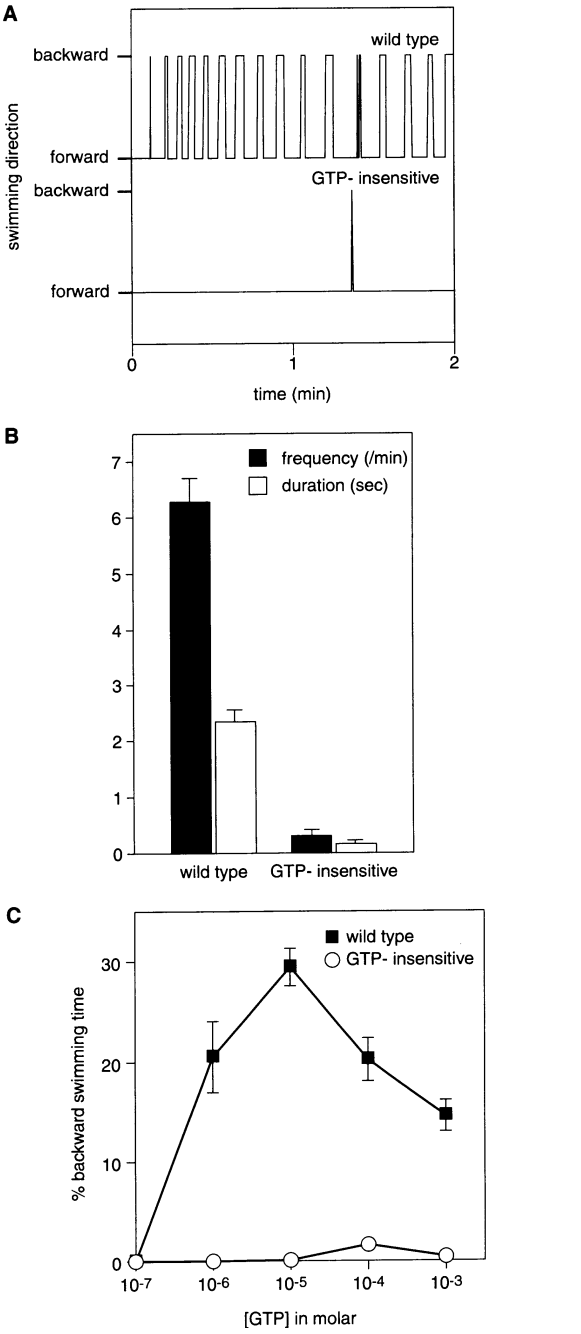

Figure 2 Swimming behaviour of wild-type and *gin A* cells in GTP

(**A**) The swimming direction of individual cells. The traces illustrate the periodic transitions between swimming forwards (baseline) and swimming backwards (plateaus) in a wild-type cell (upper trace) and a *gin A* mutant (lower trace) bathed in a solution of 10 μM GTP. (**B**) The average frequency of transitions from forwards to backwards swimming and the average duration of episodes of swimming backwards in wild-type and *gin A* cells bathed in 10 μM GTP. Each bar represents the average of three separate experiments ($n = 16$, error bars represent S.E.M.). (**C**) The effect of GTP concentration on swimming behaviour. Increasing concentrations of GTP cause wild-type cells (■) to spend a greater percentage of their time swimming backwards (larger BST), but have little effect on *gin A* cells (○). Each point represents the mean for three separate experiments ($n = 12$, error bars represent S.E.M.).

through the GTP-containing layer to the top of the tube, although they paused briefly (3–5 min) at the interface between the two layers (right tube).

The repellent effect of GTP on a population of cells is the sum of its effect on individuals. We therefore evaluated the swimming behaviour of individual cells in GTP. Unstimulated wild-type cells normally swim forwards and occasionally jerk briefly backwards in what is called an avoiding reaction [31]. GTP induces a strikingly different behaviour pattern: wild-type cells initially whirl for approx. 5 s, gyrating about a pivot point near their posterior. They then begin repeated and prolonged episodes of alternately swimming forwards and backwards that are often punctuated with additional whirling. In the studies reported here we individually tested several wild-type and *gin A* cells in 10 μM GTP. The upper trace in Figure 2(A) shows changes in the swimming direction of a typical wild-type cell in 10 μM GTP. Episodes of swimming backwards occurred in wild-type cells with an average frequency (mean $\pm$ S.E.M.) of 6.3 ± 0.4 times/min, whereas the average duration of each episode was 2.3 ± 0.2 s (Figure 2B; $n = 16$). In contrast, *gin A* mutants under the same conditions typically whirled for approx. 30 s (results not shown in Figure 2), then resumed forwards swimming with few interruptions by either whirling or swimming backwards (the response of a typical *gin A* mutant is shown in the lower trace in Figure 2A). The mean frequency of backwards-swimming events among *gin A* cells was 0.3 ± 0.1 min^{-1} ($n = 16$), with an average duration of 0.2 ± 0.0 s ($n = 16$; Figure 2B). After recovery from whirling, *gin A* cells swam forwards with occasional avoiding reactions. Thus *gin A* mutants do not display the repeated backwards swimming in GTP characteristic of the wild type.

We wanted to know whether the failure of GTP to elicit repetitive backwards swimming in *gin A* was due to a decrease in the sensitivity of these mutants to GTP. We therefore quantified the swimming responses of wild-type and *gin A* cells to GTP over a range of nucleotide concentrations. As a measure of the intensity of the behavioural response to GTP, we used the percentage of assay time spent by the cell in swimming backwards (BST) in GTP solutions [8]. Wild-type cells displayed increasing values for BST as the concentration of GTP increased from 0.1 to 10 μM GTP, with an apparent EC_{50} of approx. 0.3 μM (Figure 2C). In both Figure 2(C) and in previously published studies [8], concentrations of GTP greater than 10 μM yielded less than maximal values for BST, although the reasons for this remain unclear. In contrast with the wild type, *gin A* mutants yielded consistently low values for BST (Figure 2C), even at concentrations of GTP 50-fold that eliciting maximal BST values in the wild type. This suggests the phenotype of *gin A* cannot be explained by a simple shift in sensitivity to GTP.

Mutants remain responsive to other stimuli

Next we studied whether the *gin A* mutation interferes with the ability of the mutant to swim backwards. We compared the swimming responses of wild-type and *gin A* cells to a battery of depolarizing solutions that induce backwards swimming [28,32–34]. Table 1 summarizes our findings that *gin A* mutants do not differ significantly from wild-type cells in their behaviour in solutions containing 5 mM Mg^{2+}, 10 mM Na^{+}, 30 mM K^{+}, 6 mM Ba^{2+} or 1 mM lysozyme. Thus the *gin A* mutation does not impair the ability of cells to swim backwards.

Mutants fail to show GTP-dependent oscillations in electrophysiological responses to GTP

The observations reported above suggest that the cellular mechanisms responsible for ciliary reversal are not grossly affected by the *gin A* mutation. To determine where the lesion that renders this mutant insensitive to GTP might be located in the putative

Table 1 Behaviour of wild-type and *gin A* cell lines in various depolarizing solutions

Cells tested with ionic solutions were transferred individually from resting solution to a test solution containing 5 mM Mg^{2+}, 10 mM Na^+, 30 mM K^+ or 6 mM Ba^{2+}. Cells tested in lysozyme were transferred from GTP-testing solution into 1 μM lysozyme. The values obtained for wild-type cells and *gin A* mutants are not significantly different, as determined by Student's *t* test. Abbreviations: BS, continuous backwards swimming for the time indicated in parentheses (mean duration $\pm$ S.E.M. in seconds; $n = 17$); AR, repeated avoiding reactions, or brief backward jerks ($n = 17$ for Ba^{2+} tests; $n = 7$ for lysozyme tests).

		Response (duration in seconds)				
Cell line	Test solution ...	Mg^{2+}	Na^+	K^+	Ba^{2+}	Lysozyme
Wild type		BS (10.6 $\pm$ 1.1)	BS (4.0 $\pm$ 0.9)	BS (20.9 $\pm$ 1.4)	AR	AR
gin A		BS (9.6 $\pm$ 0.9)	BS (2.9 $\pm$ 0.8)	BS (18.5 $\pm$ 0.8)	AR	AR

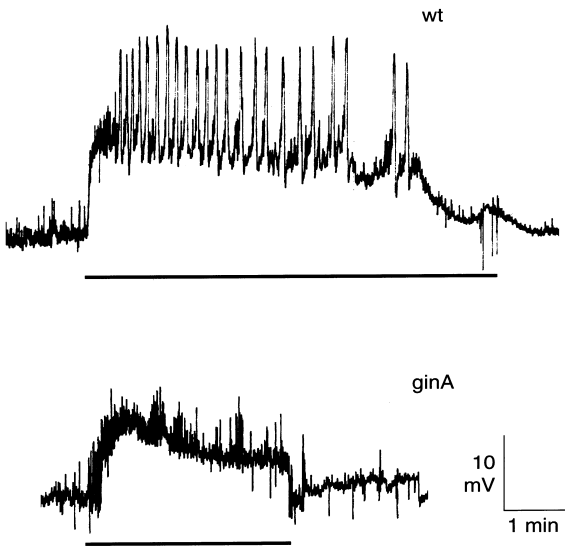

Figure 3 Membrane potential response of wild-type and *gin A* cells to GTP

Traces show changes in membrane potential during addition and removal of 10 μM GTP (bar). The upper trace was recorded from a typical wild-type (wt) cell (similar recordings were obtained from 24 out of 24 wild-type cells). The lower trace was recorded from a single *gin A* cell (eight out of 12 *gin A* cells yielded similar traces with no oscillations, whereas four out of 12 yielded traces with only one or two oscillations).

signal transduction pathway, we next examined the ability of GTP to induce changes in the membrane potential and in specific ion conductances of *gin A*. Figure 3 illustrates the changes in membrane potential of a typical wild-type cell and a *gin A* cell perfused with 10 μM GTP. The wild-type response was a complex depolarization consisting of two components: oscillating depolarizations of approx. 20 mV in amplitude that appeared about five times per minute and were superimposed on a sustained depolarization of approx. 25 mV in amplitude that was sustained over a period of several minutes (Figure 3, upper trace). All wild-type cells tested (24 out of 24) produced both the sustained and oscillating depolarizations on the application of GTP. In contrast, whereas all *gin A* mutants tested (12 out of 12) under the same conditions displayed the sustained depolarization, only four displayed oscillations in the presence of GTP (Figure 3, lower trace). Furthermore these oscillations were relatively rare, occurring at a frequency far lower (less than 0.7 min^{-1}) than that of the wild type. Thus *gin A* mutants typically lack the GTP-induced oscillating depolarizations observed in the wild type.

Next we examined the effects of GTP on cells under voltage clamp. When GTP is applied to wild-type cells under these conditions, it activates a Mg^{2+} current, a Na^+ current and a Ca^{2+} current, all of which oscillate simultaneously [9]. All three of these currents might contribute to the GTP-induced depolarizations observed in wild-type cells. We therefore compared the effects of GTP on wild-type and *gin A* cells in each of three ion solutions designed to isolate the Mg^{2+}, Na^+ and Ca^{2+} currents (Figure 4). GTP-induced currents from wild-type cells were similar in form to depolarizations induced by GTP, as described above, in that they were complex and seemed to be the sum of two components: an oscillating current superimposed on a smaller, sustained current (Figure 4A). We observed this pattern in eight out of nine wild-type cells bathed in Ca^{2+} solution, eight out of eight in Na^+ solution, and ten out of ten in Mg^{2+} solution. In contrast, *gin A* mutants displayed only the slow, sustained current when tested under similar conditions (Figure 4B). There was no evidence of an oscillatory current in seven out of eight mutants tested in Ca^{2+} solution, ten out of ten mutants tested in Na^+ solution, and nine out of nine mutants tested in Mg^{2+} solution. Thus all three oscillating currents typically fail to activate *gin A* mutants on the application of GTP.

Previous studies have shown that mildly hyperpolarizing (by approx. 5 mV) wild-type cells under voltage clamp completely and reversibly inhibits the oscillatory currents induced by GTP [9]. If the *gin A* mutation were to shift the voltage dependence of this inhibition, it could prevent cells from responding to GTP at resting membrane potential. In this case, depolarizing the membrane might be expected to relieve this inhibition and enable *gin A* cells to respond normally to GTP. To test this possibility we depolarized, by 20 mV, voltage-clamped cells that were actively responding to GTP. Depolarization had no effect on oscillating currents in wild-type cells and failed to restore GTP-induced oscillating currents in 26 out of 26 *gin A* cells tested (results not shown). Thus it is unlikely that a shift in voltage sensitivity of the voltage-dependent inhibition accounts for the phenotype of *gin A*.

Major ion conductances are unaffected by *gin A* mutation

Because the application of GTP to mutant cells failed to elicit the oscillating currents observed in the wild type, we next studied whether one or more of the conductances responsible for those currents might be missing in *gin A* mutants. The oscillating Mg^{2+} and Na^+ currents that are induced by GTP reflect the periodic activation of Mg^{2+}- and Na^+-specific ion conductances, both of which have been described in detail previously and whose

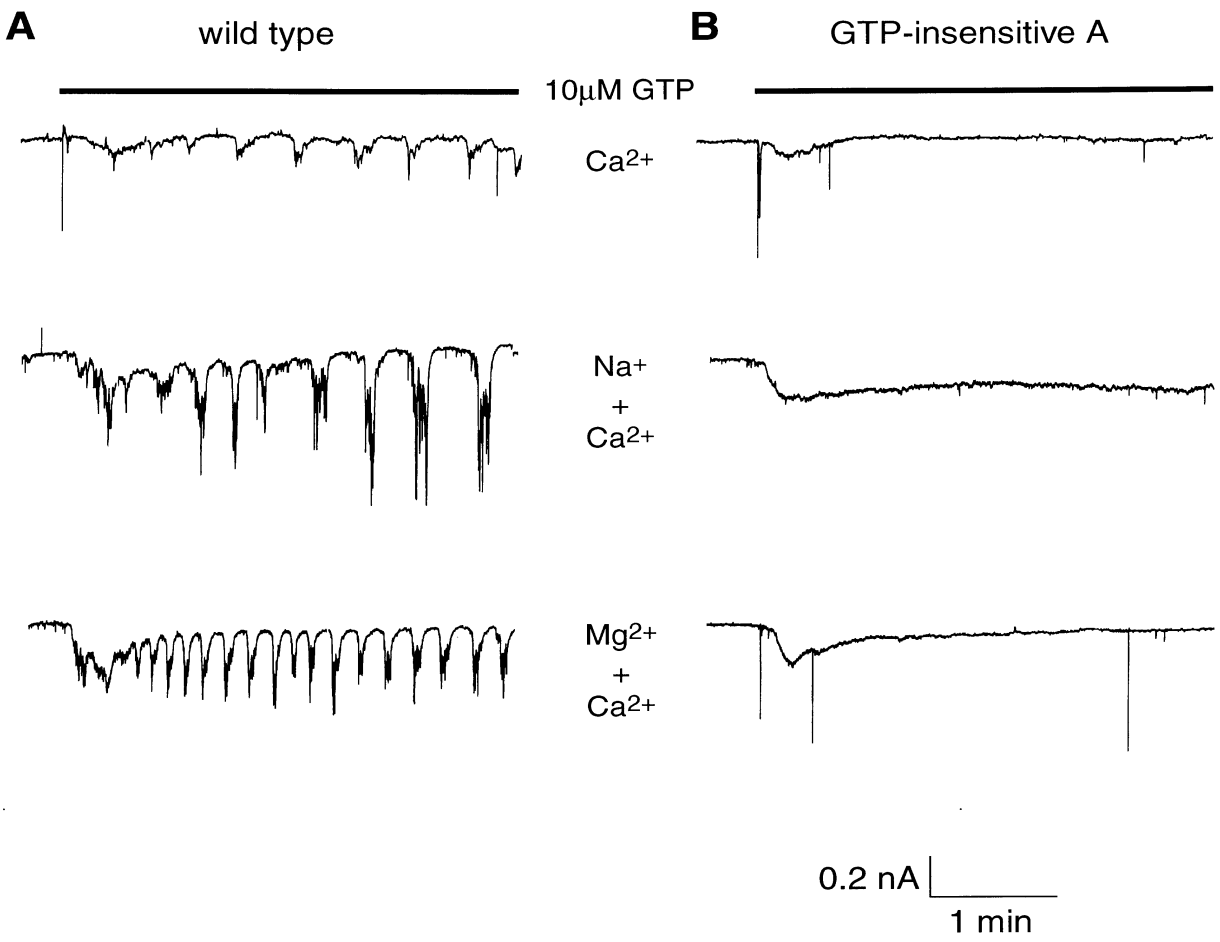

Figure 4 GTP-induced membrane currents in wild-type and *gin A* cells

Current traces recorded under two-electrode voltage clamp from an individual wild-type (**A**) or *gin A* (**B**) cell. Each trace represents the currents activated on the addition of 10 μM GTP to cells bathed in a solution containing 1 mM Ca^{2+} (top traces), 10 mM Na^+ plus 1 mM Ca^{2+} (middle traces) and 5 mM Mg^{2+} plus 1 mM Ca^{2+} (bottom traces).

Table 2 Electrophysiological properties of wild-type and *gin A* cell lines

Values are current amplitudes, presented as means ± S.E.M. for *n* determinations. The numerals in the (*n*) column represent the number of wild-type and *gin A* cells tested respectively. Amplitudes of tail currents were obtained by fitting traces of tails to one- or two-exponential equations, and extrapolating to the end of the voltage step. I_{Na} tail currents were recorded at −40 mV after a 500 ms step to +10 or −110 mV, as indicated. I_{Mg} tail currents were recorded at −30 mV after a 500 ms step to +20 or −100 mV, as indicated. K^+ currents ($I_{K(h)}$ and $I_{K(Ca,h)}$) were elicited by a 500 ms step to −120 mV: the peak K^+ influx (I_{peak}) and the fast component of the tail current (I_{tail}) were due to $I_{K(h)}$; the current at 500 ms (I_{t-500}) and the slow component of the tail current were due to $I_{K(Ca,h)}$. $I_{K(d)}$ tail currents were recorded at −40 mV after a 1.5 s step to −5 mV. Membrane potentials (V_m) were measured as described in the Experimental section.

Current or potential	Parameter	Wild-type cells	*gin A* cells	(*n*)
I_{Na} (nA)	I_{tail}, +10 mV	−0.98 ± 0.11	−1.24 ± 0.09	(6, 4)
	I_{tail}, −110 mV	−3.24 ± 0.22	−3.60 ± 0.16	(6, 4)
I_{Mg} (nA)	I_{tail}, +20 mV	−0.85 ± 0.19	−0.92 ± 0.11	(4, 7)
	I_{tail}, −100 mV	−1.56 ± 0.27	−1.82 ± 0.29	(5, 7)
$I_{K(h)}$ (nA)	I_{peak}, −120 mV	−20.7 ± 1.9	−21.5 ± 2.3	(11, 10)
	I_{tail}, −120 mV	7.0 ± 0.8	10.0 ± 1.2	(11, 10)
$I_{K(Ca,h)}$ (nA)	I_{t-500}, −120 mV	−14.4 ± 1.7	−19.2 ± 1.8	(11, 10)
	I_{tail}, −120 mV	1.33 ± 0.42	1.65 ± 0.44	(11, 10)
$I_{K(d)}$ (nA)	I_{tail}, −5 mV	0.81 ± 0.24	0.69 ± 0.13	(5, 5)
V_m (mV)		25.4 ± 1.4	25.75 ± 3.0	(5, 6)

properties are well known [10,11]. Mg^{2+} and Na^+ currents were elicited from wild-type and *gin A* cells by using 500 ms step depolarizations or hyperpolarizations under voltage clamp. There were no significant differences between the amplitudes of either current in the wild-type and *gin A* cell lines (Table 2).

Although the Mg^{2+}- and Na^+-specific ion conductances described above are known to be operated by an increase in $[Ca^{2+}]_i$ [10,11], the membrane conductance that might be the source of this Ca^{2+} has not yet been identified. Two voltage-sensitive Ca^{2+} conductances have been described previously in *P. tetraurelia*: one activated on depolarization and one on hyperpolarization. Depolarization-activated Ca^{2+} currents were elicited in cells by using 20 ms step depolarizations from −40 mV (Figures 5A and 5B). These currents inactivate quickly, but a comparison of the currents at their peak suggested that there are no significant differences between the wild-type and *gin A* cell lines in terms of amplitude or voltage dependence. Hyperpolarization-activated currents were elicited by using 300 ms steps from −40 mV (Figures 5C and 5D). This Ca^{2+} current also inactivated quickly, and again a comparison of peak values failed to show significant differences in amplitudes or voltage dependences between the wild-type and *gin A* cell lines.

We also examined the resting membrane potential and the four known K^+ conductances of *P. tetraurelia*. K^+ currents are

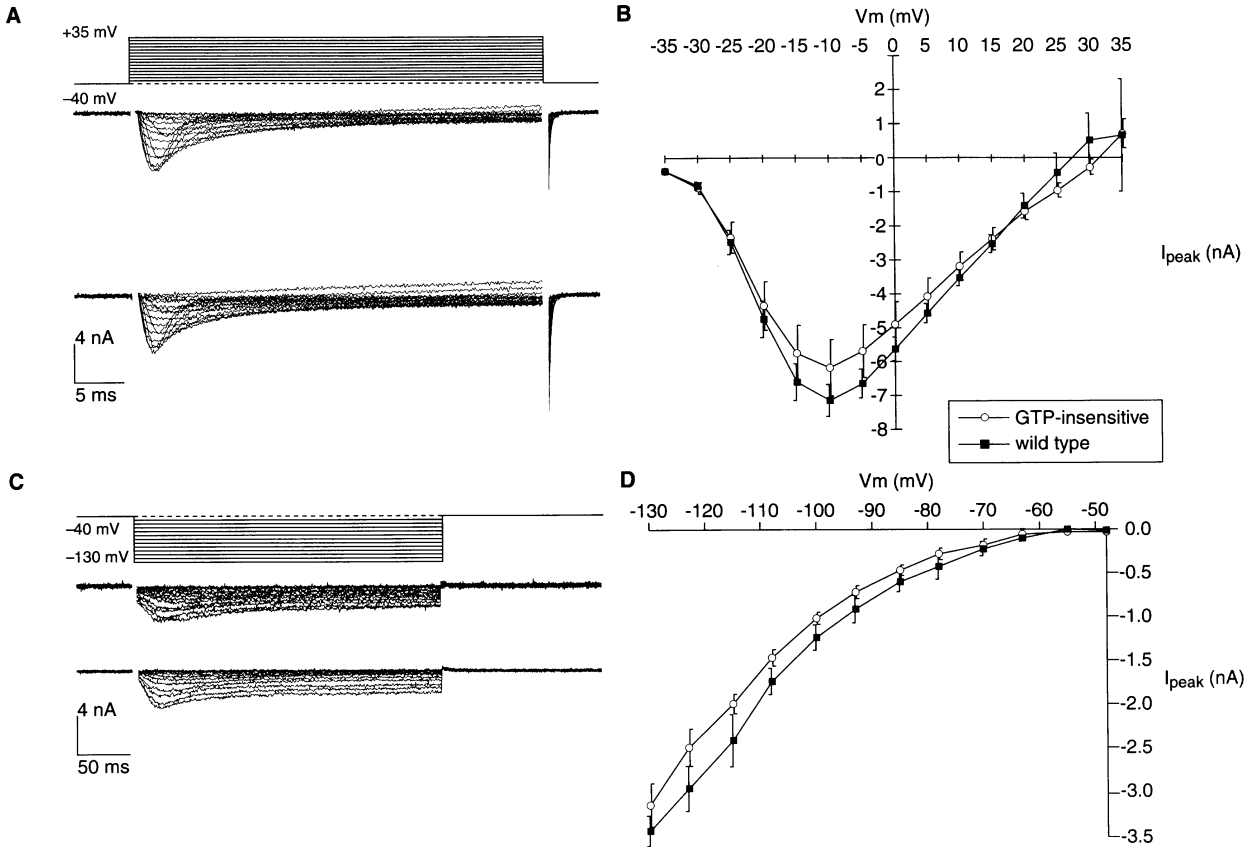

Figure 5 Voltage dependence of $I_{Ca(d)}$ and $I_{Ca(h)}$ in wild-type and *gin A* cells

(**A**) Families of Ca^{2+} currents elicited on depolarization of a typical wild-type cell (upper trace) and *gin A* mutant (lower trace) in Ca^{2+} solution. Traces have been corrected for linear leak current. (**B**) Peak amplitude of $I_{Ca(d)}$ (I_{peak}) plotted as a function of membrane potential (V_m). Points are means ± S.E.M. from seven wild-type cells (■) and ten *gin A* cells (○). (**C**) Families of Ca^{2+} currents elicited on hyperpolarization of a typical wild-type cell (upper trace) and *gin A* mutant (lower trace) in Ca^{2+} solution. (**D**) Peak amplitude of $I_{Ca(h)}$ (I_{peak}) plotted as a function of membrane potential (V_m). Points are means ± S.E.M. from eight wild-type cells (■) and eight *gin A* cells (○).

important in setting resting membrane potential and in repolarizing the membrane after periods of excitation. Thus it is conceivable that a defect in one of these conductances might account for the phenotype of the *gin A* mutant. The voltage-dependent and Ca^{2+}-dependent K^+ currents activated on depolarization were elicited by using 1 s steps from −40 mV, whereas the voltage-dependent and Ca^{2+}-dependent K^+ currents activated on hyperpolarization were elicited by using 500 ms steps. We found that the resting membrane potentials and the amplitudes and kinetics of all of the K^+ currents (Table 2) in both wild-type and *gin A* cell lines were similar.

DISCUSSION

GTP-insensitives are a new class of mutant of *P. tetraurelia* that fail to respond normally to extracellular GTP. Wild-type paramecia are repelled by micromolar concentrations of GTP, a behaviour that results from lengthy episodes of swimming backwards that can be repeated several times per minute for up to 10 min in the continued presence of nucleotide. In contrast, *GTP-insensitive A* (*gin A*) mutant cells respond to GTP with whirling that causes them to pause briefly but that seldom results in swimming backwards or repulsion from the stimulus.

The sensory transduction pathway that mediates repulsion from GTP in the wild type (Scheme 1) involves many steps, several of which have been characterized previously. The specificity with which GTP produces repulsion in the wild type suggests that a receptor in the cell membrane allows *P. tetraurelia* to recognize the presence of this nucleotide [8]. Receptor binding is transduced by unknown means to yield an oscillating intracellular signal, which then causes oscillations in $[Ca^{2+}]_i$, perhaps through activation of a Ca^{2+} conductance in the plasma membrane. Each rise in $[Ca^{2+}]_i$ activates separate Ca^{2+}-dependent Mg^{2+}-specific and Na^+-specific conductances, causing Mg^{2+} and Na^+ influx. This depolarizes the membrane enough to activate a voltage-sensitive Ca^{2+} conductance in the ciliary membrane. The resultant increase in intraciliary Ca^{2+} level causes a reversal in the ciliary power stroke and the cell swims backwards. Recovery after each intracellular signal oscillation is presumed to involve renormalization of membrane potential and of cytosolic and intraciliary Ca^{2+} concentration, allowing the cell to resume swimming forwards. This sequence of events is repeated several times per minute, causing the repetitive backwards swimming that is characteristic of *P. tetraurelia*'s response to GTP.

Although we do not know precisely where the *gin A* mutation disrupts this sequence of events, our results allow us to begin to narrow the possibilities, as follows.

The *gin A* phenotype is not caused by the disruption of processes that regulate ciliary reversal or the depolarization-

activated Ca^{2+} channels in the ciliary membrane. *Atalanta* is a mutant with a defect in the ciliary reversal mechanism that prevents it from swimming backwards in response to any external stimuli [35]. The *gin A* phenotype is clearly distinct from that of *atalanta*, for although it does not respond to GTP, it does swim backwards in various depolarizing test solutions (Table 1). It is therefore unlikely that the *gin A* mutation disrupts ciliary motor function. Similarly, *pawn* mutants fail to swim backwards because they lack a functional ciliary Ca^{2+} influx pathway [36]. Not only have we shown that *gin A* mutant cells are capable of swimming backwards normally in response to various stimuli (Table 1), we have also shown this Ca^{2+} conductance to be both present in the mutant and to be of comparable amplitude to that of the wild type (Figure 5A).

The *gin A* mutation does not seem to disrupt the mechanisms that regulate membrane potential. Because swimming backwards in *P. tetraurelia* requires membrane depolarization to activate the voltage-sensitive Ca^{2+} channels in the cilia, a defect in the mechanisms that regulate membrane potential could account for the loss of the GTP response in *gin A* mutant cells. However, our evidence suggests that this is not so. Behavioural studies show that *gin A* cells swim backwards in various test solutions for times that approximate those of the wild type (Table 1). Durations of swimming backwards are a direct reflection of membrane potential [37,38], suggesting that the mechanisms for controlling membrane excitation and repolarization are normal in *gin A*. Furthermore an examination of the K^+ currents in *gin A* cells under voltage clamp failed to show any abnormality that might lead to a shift in resting membrane potential in this mutant (Table 2). The K^+ currents are largely responsible for determining membrane potential: a K^+ current that activates unusually fast is thought to be responsible for the behavioural insensitivity of the *TEA-insensitive* (where TEA stands for tetraethylammonium chloride) and *restless* mutants of *P. tetraurelia*. A comparison of the resting membrane potential of *gin A* and wild-type cells confirmed that no significant difference exists between these two cell lines (Table 2).

The *gin A* mutation does not disrupt Mg^{2+}-specific and Na^+-specific conductances. In the wild type, GTP induces the periodic activation of a Mg^{2+}-specific current and a Na^+-specific current. The oscillation of these currents ultimately drives the repeated episodes of swimming backwards which cells typically display in the presence of GTP. Although both currents are activated in the GTP response pathway, either alone is sufficient to produce the behavioural response. *Eccentric* and *fast-2* mutations specifically inhibit the Mg^{2+} current and the Na^+ current respectively [11,39] and thus prevent GTP responses in one ionic solution but not the other [9]. In contrast, *gin A* prevents the cells from responding to GTP by swimming backwards in solutions containing both Mg^{2+} and Na^+ (Figure 2). This suggests that both currents might be suppressed in this mutant, a notion supported by the voltage clamp records showing that GTP fails to elicit oscillating currents in Mg^{2+} or Na^+ solutions (Figure 4). The *gin A* mutation does not seem to affect the Mg^{2+} or Na^+ conductances themselves, however, for both can be activated normally in response to step changes in membrane potential under voltage clamp (Table 2). The mutation therefore seems to disrupt the activation of these currents by GTP specifically, and not their activity in general.

Having eliminated several possibilities for the location of the *gin A* defect, several possible targets remain: the Ca^{2+} oscillator, the plasma membrane receptor for GTP, or any protein that might mediate a signal in between. Our results suggest that the *gin A* mutation might disturb the regulation of intracellular Ca^{2+} or perhaps even the oscillator itself. The Mg^{2+} and Na^+ conductances that mediate the GTP response both require that $[Ca^{2+}]_i$ rise above resting levels to activate. If the *gin A* mutation were to prevent a rise in $[Ca^{2+}]_i$, it would explain how a single-site mutation could suppress two independent ion currents. Previous work [9] has suggested that GTP-induced oscillations in $[Ca^{2+}]_i$ precede the activation of Mg^{2+} and Na^+ currents in the wild type. Ligand-induced Ca^{2+} oscillations are relatively common in biology, including purinoceptor-activated oscillations [13,15]. In these cases the source of Ca^{2+} is thought to be either internal stores and/or the external medium by means of a plasma membrane Ca^{2+} conductance. In *P. tetraurelia* the source of Ca^{2+} for the proposed intracellular Ca^{2+} oscillations is still uncertain. This cell contains massive intracellular Ca^{2+} stores, the alveolar sacs [40], which could well be mobilized in response to GTP. However, a periodic GTP-induced Ca^{2+} conductance in the somatic membrane that would be more than sufficient to allow the activation of the Mg^{2+} and Na^+ currents has also been identified [9]. At present it is still unclear whether this Ca^{2+} current is a third Ca^{2+}-dependent current also activated by an intracellular Ca^{2+} oscillator or whether this current represents the oscillator itself. Future experiments are likely to resolve this issue. Note that the GTP-induced oscillating Ca^{2+} conductance is missing in *gin A* mutant cells, which, although being consistent with either hypothesis, might explain why GTP fails to activate the Mg^{2+} and Na^+ currents in the mutant.

If the *gin* A^+ gene product were the GTP receptor, it would also readily explain how the *gin A* mutation disrupts the responses to GTP. A mutation in the GTP-binding site would probably be apparent in studies of receptor affinity and/or specificity and is an avenue for further research. If, however, the mutation affected receptor transduction, the isolation and molecular characterization of the *gin A* gene and its products might be necessary before its effects could be understood fully. Note, however, that we have presented data suggesting that *gin A* cells are still able to recognize GTP, even though this recognition fails to produce repulsion. Applying GTP to mutant cells causes whirling, a weak backwards swimming response reflecting sustained (non-oscillating) membrane depolarization (Figure 3). That *gin A* mutants whirl in GTP probably accounts for their pausing at the GTP/buffer interface in the negative geotaxis assay (Figure 1). *gin A* mutant cells also respond to GTP with sustained inward currents in Ca^{2+}, Mg^{2+} and Na^+ (Figure 4). There are two plausible explanations for these observations: the first is that there are actually two distinct classes of GTP receptor, one of which gives a sustained (whirling) response, whereas occupancy of the other causes $[Ca^{2+}]_i$ oscillations. In this scenario, the *gin A* mutation would disrupt the oscillation pathway. The second possibility is that both sustained and periodic responses are mediated by the same receptor and transduction pathway, but that the *gin A* gene product is a vital component only of the mechanism that initiates and maintains $[Ca^{2+}]_i$ oscillations. At present we have no evidence indicating which of these two explanations is the more likely cause of the *gin A* phenotype.

Although there are many examples of extracellular purine nucleotide signalling in the literature, including those that trigger changes in $[Ca^{2+}]_i$, little is known about the mechanisms and importance of the effects of extracellular GTP. Furthermore the fundamental mechanisms underlying $[Ca^{2+}]_i$ oscillations, such as positive feedback, co-operativity, deactivation and reactivation, have yet to be elucidated at the molecular level [41]. Whether the *gin A* mutation lies in a GTP-specific purinergic receptor or in mechanisms responsible for Ca^{2+} oscillations, it represents the first disruption of this complex signalling pathway by mutation, and an opportunity to identify the molecular components involved.

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