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Extracellular GTP Causes Membrane-Potential Oscillations through the Parallel Activation of Mg^{2+} and Na^+ Currents in *Paramecium tetraurelia**

K.D. Clark[1], T.M. Hennessey[2], D.L. Nelson[1], R.R. Preston[3]

[1]Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin–Madison, Madison, WI 53706, USA
[2]Department of Biological Sciences, State University of New York, Buffalo, NY 14260, USA
[3]Department of Physiology, Allegheny University of Health Sciences, Philadelphia, PA 19129, USA

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Abstract. *Paramecium tetraurelia* responds to extracellular GTP ($\geqslant$ 10 nM) with repeated episodes of prolonged backward swimming. These backward swimming events cause repulsion from the stimulus and are the behavioral consequence of an oscillating membrane depolarization. Ion substitution experiments showed that either Mg^{2+} or Na^+ could support these responses in wild-type cells, with increasing concentrations of either cation increasing the extent of backward swimming. Applying GTP to cells under voltage clamp elicited oscillating inward currents with a periodicity similar to that of the membrane-potential and behavioral responses. These currents were also Mg^{2+}- and Na^+-dependent, suggesting that GTP acts through Mg^{2+}-specific (I_{Mg}) and Na^+-specific (I_{Na}) conductances that have been described previously in *Paramecium.* This suggestion is strengthened by the finding that Mg^{2+} failed to support normal behavioral or electrophysiological responses to GTP in a mutant that specifically lacks I_{Mg} (''*eccentric*''), while Na^+ failed to support GTP responses in ''*fast-2,*'' a mutant that specifically lacks I_{Na}. Both mutants responded normally to GTP if the alternative cation was provided. As I_{Mg} and I_{Na} are both Ca^{2+}-dependent currents, the characteristic GTP behavior could result from oscillations in intracellular Ca^{2+} concentration. Indeed, applying GTP to cells in the absence of either Mg^{2+} or Na^+ revealed a minor inward current with a periodicity similar to that of the depolarizations. This current persisted when known voltage-dependent Ca^{2+} currents were blocked pharmacologically or genetically, which implies that it may represent the activation of a novel purinergic-receptor–coupled Ca^{2+} conductance.

Key words: GTP — *Paramecium* — Electrophysiology — Purinergic — Mg^{2+} — conductance — Na^+ conductance — Ca^{2+} conductance

Introduction

Purinergic responses (responses elicited by purine nucleosides and nucleotides, but not by cyclic nucleotides) are common among metazoa (Gordon, 1986), but little is known about their existence in lower organisms. To date, the only purinergic responses described in microbes are those exhibited by the ciliated protists *Paramecium tetraurelia* (Clark, Hennessey & Nelson, 1993) and *Tetrahymena thermophila* (Francis & Hennessey, 1995) to extracellular GTP. At a concentration as low as 10 nM, GTP causes *Paramecium* to switch from its unstimulated pattern of predominantly forward swimming to an unusual pattern of alternating forward and backward swimming with transition periods of whirling (randomly directed motion). This behavioral response translates into avoidance of GTP in a chemoresponse assay (Clark et al., 1993; Francis & Hennessey, 1995). Pharmacologically, the behavioral response is specific to GTP or its closely related analogue GTP-γ-S. GDP, for example, is about 10-fold less active than GTP, ATP is 1,000-fold less active, and other nucleotides such as CTP, UTP, ITP, and XTP show no activity (Clark et al., 1993). Currently, no other characterized purinergic

* Dedicated to Julius Adler on his 65th birthday.

Correspondence to: D.L. Nelson

Abbreviations: BST, backward swimming time; $[Ca^{2+}]_{cyto}$, concentration of Ca^{2+} in the cell body; $[Ca^{2+}]_{cil}$, concentration of Ca^{2+} in the ciliary compartment; I_{Mg}, Mg^{2+}-specific current; I_{Na}, Na^+-specific current; TEA, tetraethylammonium chloride

responses are guanine-nucleotide-specific, making *Paramecium's* response to GTP a new pharmacological class of purine reception.

Paramecium's swimming behavior is governed by changes in its membrane potential (Naitoh & Eckert, 1969). Membrane hyperpolarization results in increased forward swimming speed, whereas depolarization causes the cell to slow down. If a depolarization is sufficiently strong, voltage-dependent Ca^{2+} channels present in the ciliary membrane open, resulting in the firing of a graded, Ca^{2+}-based action potential (Naitoh & Kaneko, 1972). The consequent rise in intraciliary Ca^{2+} concentration $[Ca^{2+}]_{cil}$ causes reversal of the ciliary power stroke, so that the cell whirls or swims backward. *Paramecia* ultimately adapt to a depolarizing stimulus by a return to forward swimming. Consistent with this electrophysiological control of swimming behavior, each GTP-induced backward swimming event is associated with a membrane depolarization whose magnitude is dependent on GTP concentration (Clark et al., 1993).

Initial investigation into the GTP signal-transduction pathway revealed that the oscillating depolarizations are not dependent on the voltage-dependent Ca^{2+} current that yields the upstroke of the previously characterized, Ca^{2+}-based action potential; while a mutant lacking this current fails to show GTP-induced backward swimming episodes, it retains the oscillating depolarizations (Clark et al., 1993). This suggests that the depolarizations are an upstream event in the GTP signal-transduction pathway and that they are most likely used to directly open the voltage-sensitive Ca^{2+} channels, which results in backward swimming. Here we show that the oscillating depolarizations actually result from the parallel activation of two separate depolarizing cation currents, either of which is capable of producing the behavioral response. One of these is a Ca^{2+}-dependent and Mg^{2+}-specific current (Preston, 1990) and the other is a Ca^{2+}-dependent and Na^{+}-specific current (Saimi & Kung, 1980). Not only do these observations reveal a novel chemoeffector pathway in *Paramecium*, they also define an important and previously unsuspected physiological role for these two cation currents.

Materials and Methods

Cell Stocks and Culture

Paramecium tetraurelia, stock 51S, was cultured in either a wheat grass medium or in a semi-defined artificial medium, as described (Clark et al., 1993; Preston, Saimi & Kung, 1990). Both were inoculated with a food organism, *Enterobacter aerogenes*. In addition to this wild-type stock, the following mutants derived from the wild type were used: d4-95 *pawn B* (Kung, 1971), d4-623 *Dancer* (Hinrichsen & Saimi, 1984), d4-700 *eccentric A* (Preston & Kung, 1994), d4-652 *fast-2* (*cam*^{13}) (Kink et al., 1990). All cell lines additionally contained a mutation, *nd6*, that prevents trichocyst discharge (Lefort-Tran et al., 1981), to minimize cell trauma during microelectrode insertion.

Behavioral Assay

One hour prior to experimentation, cells were washed from culture fluid into 10 mM HEPES buffer adjusted to pH 7.2 with Tris. For studies of the ion dependence of the GTP response, this solution was supplemented with stated concentrations of (a) $CaCl_2$, (b) KCl plus 1 mM $CaCl_2$, (c) $MgCl_2$ plus 1 mM $CaCl_2$ and 4 mM KCl, or (d) NaCl plus 1 mM $CaCl_2$ and 4 mM KCl. $CaCl_2$ (1 mM) was included under conditions (b), (c), and (d) to allow for the activation of Ca^{2+}-dependent K^{+}, Mg^{2+}, and Na^{+} currents. Cells were allowed to adapt to the experimental solutions for 1 hr before adding GTP. The effects of GTP on *Paramecium* behavior were assessed for a period of 90 sec, beginning approximately 2 sec after adding nucleotide to the cells. Backward swimming episodes were registered using a computer, which then calculated percentage backward swimming time (%BST) as a function of the total observation period (Clark et al., 1993) (program available upon request).

Electrophysiology

Conditions used to record membrane potential have been described (Clark et al., 1993). Free-running membrane potentials were recorded using a capillary intracellular microelectrode containing 0.5 M KCl, tip resistance *ca.* 150 MΩ. The cells were placed in ''resting solution'' (4 mM KCl, 1 mM $CaCl_2$, 1 mM MOPS buffer plus Tris to pH 7.2) for 1 hr prior to experimentation. The same solution supplemented with either 0.5 mM $MgCl_2$ or 10 mM NaCl was used during intracellular recording.

Conditions used to record membrane currents under two-electrode voltage clamp were described (Preston, Saimi & Kung, 1992). The capillary intracellular microelectrodes used to establish a voltage-clamp usually contained 1 M CsCl, tip resistance *ca.* 40 MΩ. Electrodes containing 1 M Cs-glutamate were used for recording under $C1^{-}$ free conditions. The solutions used during recording under voltage clamp were designed specifically to isolate Mg^{2+}, Na^{+}, and/or Ca^{2+} currents in *Paramecium.* All solutions contained 1 mM Ca^{2+} ($CaCl_2$) and/or $Ca(OH_2)$, 10 mM EDTA, 1 mM HEPES buffer, pH 7.2. ''Ca^{2+}-solution'' additionally contained 10 mM tetraethylammonium chloride (TEA) and was used to study Ca^{2+} currents; ''Mg^{2+} solution'' additionally contained 5 mM $MgCl_2$ and 10 mM TEA and was used to study Mg^{2+} currents; and ''Na^{+} solution'' additionally contained 10 mM NaCl and 10 mM TEA and was used to study the Na^{+} current.

The Ca^{2+} conductance that underlies responses to GTP in Mg^{2+} and Na^{+} is suppressed if membrane potential is held >5 mV negative to rest (*see below*), so cells bathed in Ca^{2+} solution, TEA-free Ca^{2+}-solution, or Cl^{-}-free solutions were held at −25 mV, while cells in Na^{+} solution were held at −20 mV, and cells in Mg^{2+} solution were held at −15 mV. Cells in 10 mM Ba^{2+} were held at −5 mV. These potentials approximate resting potential in each of the respective solutions. Current traces shown were filtered at 10 Hz. All recordings were made at room temperature (22–24°C). Deciliated cells were prepared by agitation in 5% ethanol for 2 min, as described (Preston & Usherwood, 1988).

Amplitudes of GTP-induced currents were determined during a 4–6 min period approximately 2 min after applying GTP to individual cells. This observation period typically included 8–10 current oscillations. These currents were averaged and this figure was then used to calculate mean responses from the number of cells stated.

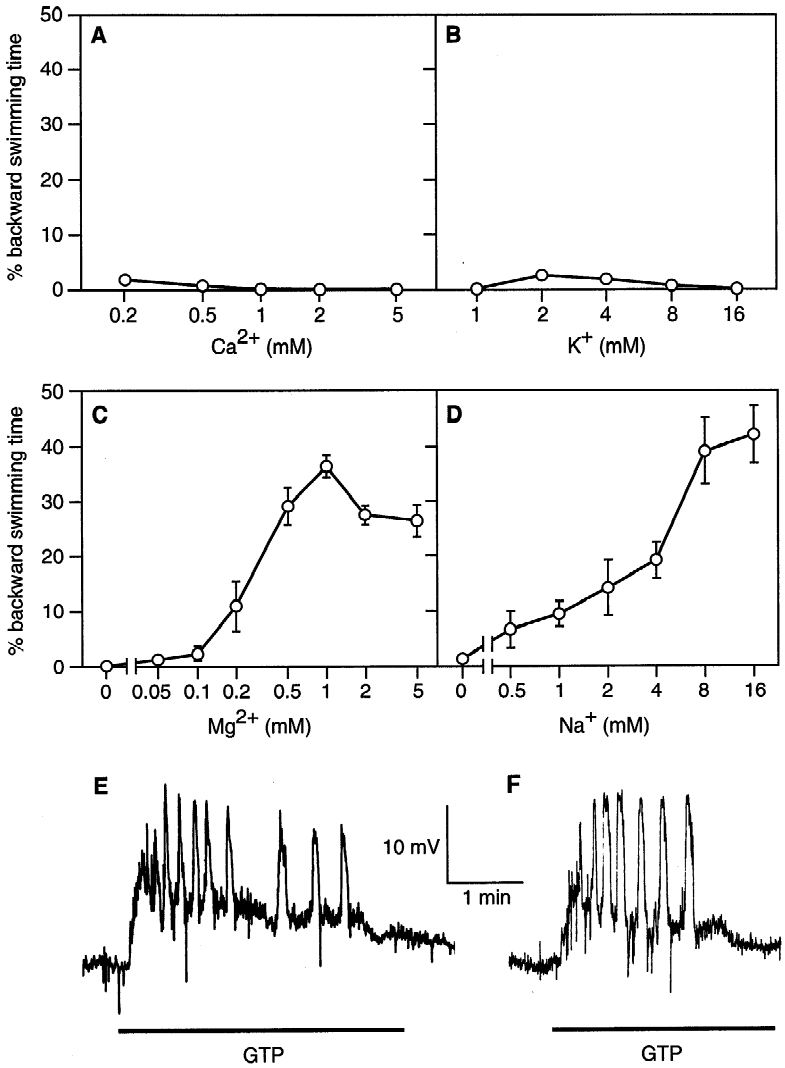

Fig. 1. Ionic requirements for backward swimming in response to GTP. Cells were viewed individually through a dissecting microscope for a period of 90 sec after GTP addition, during which time the duration of each backward swimming event was recorded using a computer. The percentage of time spent swimming backward was then calculated. Cells were bathed in buffer (10 mM HEPES buffer adjusted to pH 7.2 with Tris) plus the stated concentrations of (*A*) $CaCl_2$, (*B*) KCl plus 1 mM $CaCl_2$, (*C*) $MgCl_2$ plus 1 mM $CaCl_2$ and 4 mM KCl, or (*D*) NaCl plus 1 mM $CaCl_2$ and 4 mM KCl (*see* Behavioral Assay in Materials and Methods). The GTP concentration in all experiments was 10 μM. Each data point represents the average for five cells, while the bars represent the standard error. Membrane-potential recordings show typical responses to the addition and subsequent removal of 10 μM GTP from a cell bathed in resting solution plus (*E*) 0.5 mM $MgCl_2$ or (*F*) 10 mM NaCl.

Results

Paramecium typically responds to extracellular GTP with repeated episodes of backward swimming that each last about 8 seconds. These reversal events usually recur several times during the initial minute of exposure to GTP, but their frequency declines gradually with increasing time, and the cell ultimately resumes forward swimming without further interruption. This behavior was originally described in cells bathed in a medium containing Ca^{2+}, K^+, Mg^{2+}, and Na^+ (Clark et al., 1993). All four of these cations are membrane-permeant in *Paramecium* and thus could all be involved in the transient membrane depolarizations that underlie the characteristic behavior in GTP. Therefore, a possible dependence of the GTP response on these cations was investigated by observing the behavior of individual cells exposed to 10 μM GTP in various extracellular concentrations of Ca^{2+}, K^+, Mg^{2+}, or Na^+. The strength of the response was defined by the amount of time the cells spent swimming backward as a percentage of the total observation period (%BST). The results are shown in Fig. 1.

Neither Ca^{2+} (200 μM to 5 mM) nor K^+ (1 to 16 mM) supported GTP-induced backward swimming (Figs. 1*A*,*B*), but both Mg^{2+} and Na^+ did. Backward swimming durations rose sharply as extracellular Mg^{2+} concentrations increased from 100 μM to 1 mM (Fig. 1*C*), with the response finally attaining 36% BST. At higher concentrations of Mg^{2+} (2 to 5 mM), the response decreased slightly to a constant BST of 27%. GTP-induced backward swimming depended on extracellular Na^+ concentration with the response increasing linearly from 0.5 to 4 mM, then rising more sharply between 4 and 8 mM (Fig. 1*D*). A maximum response of 42% was attained at 16 mM Na^+. The increase in %BST that occurred with increasing concentrations of either Mg^{2+} or Na^+ was at-

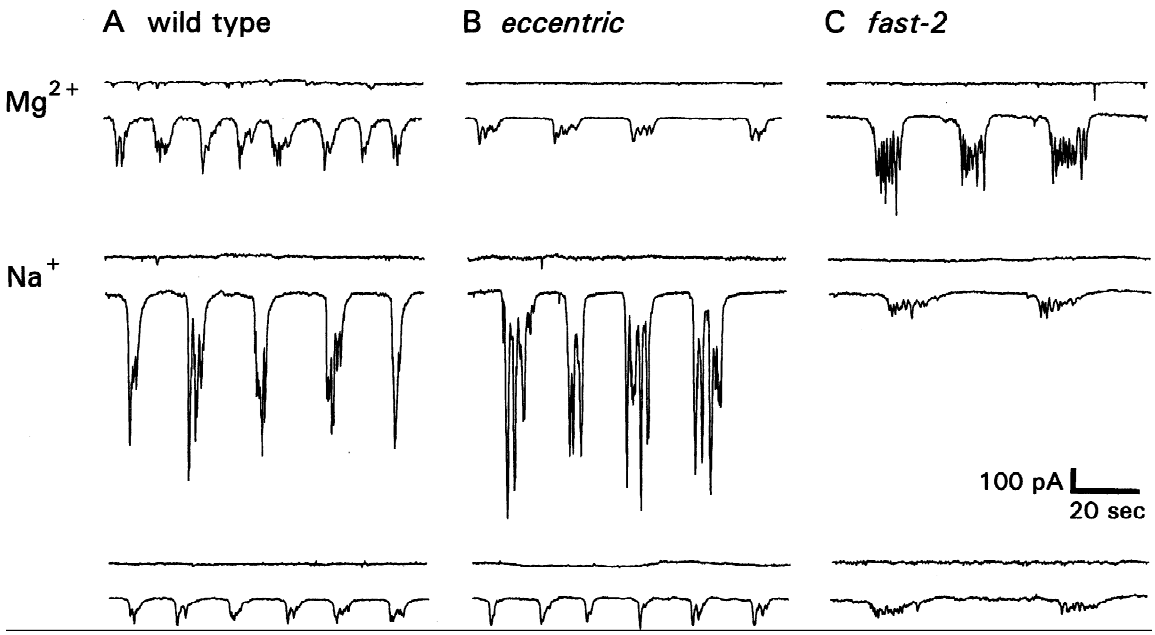

Fig. 2. GTP-induced membrane currents. Current traces were recorded under two-electrode voltage clamp from a single (*A*) wild-type, (*B*) *eccentric,* or (*C*) *fast-2* cell. Each set shows three pairs of currents recorded immediately prior to (upper trace in each pair) and approximately 2 min after (lower trace in each pair) adding 10 μM GTP. Top pairs of traces show currents recorded in the presence of 5 mM Mg^{2+} (''Mg^{2+} solution''; *see* Materials and Methods), middle pairs show currents in 10 mM Na^+ (''Na^+ solution''; *see* Materials and Methods), while bottom pairs show currents recorded in the absence of either Mg^{2+} or Na^+ (''Ca^{2+} solution''; *see* Materials and Methods). Note that 1 mM Ca^{2+} is present in all these solutions. Holding potential was −15 mV in Mg^{2+}, −20 mV in Na^+, and −25 mV in the absence of these cations (*see* Materials and Methods and text).

tributable to both an increased frequency and an increased average duration of the backward swimming events (not shown).

Examples of the membrane potential changes that underlie these behavioral responses in Mg^{2+} and Na^+ are shown in Fig. 1*E* and *F*. Adding 10 μM GTP to a cell in the presence of 0.5 mM Mg^{2+} caused a membrane response that was complex and that appeared to contain two distinct phases. The initial response was a rapid and prolonged depolarization (*ca.* 10 mV) that relaxed slowly during GTP application. Membrane recovery during removal of the stimulus also appeared to be slow, but was not investigated systematically. Superimposed upon the sustained depolarization were repeated, slow, depolarizing transients. These transients initially occurred at about 5- to 10-sec intervals, but their frequency declined with increasing time of exposure to GTP, as did the behavioral responses. When GTP was washed out of the bath, these transients ceased. Qualitatively similar responses to GTP were observed in the presence of 10 mM extracellular Na^+ (Fig. 1*F*). While both phases of the membrane response to GTP appear to be involved in generating GTP-induced swimming behavior, the observation that they can be separated by genetic mutation (J. Mimikakis, *personal communication*) suggests that two distinct mechanisms underlie the depolarizations. In the present study, we restricted our investigation to the mechanisms underlying the oscillating depolarizing transients.

Mg^{2+} and Na^+ are presumed to be able to support the behavioral responses to GTP because they are able to traverse the plasma membrane and, in so doing, to depolarize the cell. This depolarization would be sufficient to activate voltage-dependent Ca^{2+} channels that reside in the ciliary membrane (Ogura & Takahashi, 1976), causing Ca^{2+} influx, ciliary reversal and backward swimming. Two distinct pathways for Mg^{2+} and Na^+ influx are known to exist in *Paramecium:* a Mg^{2+}-specific current, I_{Mg} (Preston, 1990), and a Na^+-specific current, I_{Na} (Saimi & Kung, 1980). To assess the possible involvement of these conductances in the GTP response, membrane currents were recorded under voltage clamp. The clamp was established using experimental conditions that had been used previously to isolate and characterize I_{Mg} and I_{Na}. In the presence of 5 mM Mg^{2+} extracellularly, 10 μM GTP elicited inward current oscillations (Fig. 2*A*, top). The frequency of the current oscillations varied from cell to cell and declined slightly over the first two minutes of exposure to GTP, but approximated that of the behavioral events and of the depolarizations. The form of the oscillations also showed considerable cell-to-cell variation, but generally comprised an inward current that was sustained for 5–10 sec, superimposed upon which were repeated, fast, inward-current tran-

Table 1. Persistence of the GTP-evoked inward current despite mutational and pharmacological inhibition of known Mg^{2+}, Na^+, and Ca^{2+} conductances in *Paramecium.*

	Amplitude (pA)	*n*
A		
Wild type	73 ± 16	11
eccentric	102 ± 36	4
fast-2	79 ± 20	6
B		
10 mM TEA^+	79 ± 14	4
0 mM TEA^+	67 ± 18	4
0 mM Cl^-	93 ± 33	5
30 mM Cl^-	78 ± 17	5
C		
pawn B	96 ± 43	6
Deciliated wild type	81 ± 31	5
Dancer	106 ± 49	5
D		
Control	91 ± 14	6
2 mM Amiloride	64 ± 15	6
Control	68 ± 12	4
10 mM Ba^{2+}	104 ± 23	4

Inward currents were elicited by 10 μM GTP in cells bathed in Ca^{2+} solution (no Mg^{2+} or Na^+). (*A*) The periodic inward current was observed in the wild type and in mutant lacking I_{Mg} (*eccentric*) or I_{Na} (*fast-2*). (*B*) The current observed in the wild type in the presence of 10 mM TEA^+ persisted after removing this cation from the bath solution, and was also detected under Cl^- free conditions. The current was not affected significantly by imposing an inward Cl^- gradient (30 mM Cl^- added extracellularly). (*C*) A GTP-induced inward current was recorded from *pawn B*, a mutant that lacks $I_{Ca,d}$, and from wild-type cells after inhibiting this Ca^{2+} current by deciliation. The current was unaffected in *Dancer*, whose mutation hinders inactivation of $I_{Ca,D}$. (*D*) The inward current remained after adding 2 mM amiloride or 10 mM Ba^{2+} to the bath solution. Both treatments fully inhibit $I_{Ca,H}$. Data represent mean peak inward currents (± standared deviation) recorded from *n* cells.

sients. The maximum amplitude of these responses to GTP in Mg^{2+} was 206 pA (±26 pA, $n = 10$). Similar currents were observed in the presence of 10 mM Na^+, although their magnitude was increased (586 ± 64 pA, $n = 5$) compared with those in Mg^{2+}, and the fast inward transients were a more prominent feature of the response (Fig. 2*A*, middle). We also examined the effects of GTP in the absence of either Mg^{2+} or Na^+, with Ca^{2+} as the sole extracellular cation (*see* Fig. 1*A*). Under these conditions, there is no behavioral response to GTP. Surprisingly, inward current oscillations were observed under these conditions (Fig. 2*A*, bottom), although their magnitude was reduced considerably compared with Mg^{2+} or Na^+ currents (73 pA: Table 1*A*).

The inward currents in Mg^{2+} and Na^+ might reflect either activation of I_{Mg} and I_{Na} or activation of a previously unknown Mg^{2+}- or Na^+-permeant pathway(s). To distinguish between the two possibilities, we used two mutants that specifically lack either I_{Mg} (*eccentric;* Preston & Kung, 1994) or I_{Na} (*fast-2;* Kink et al., 1990). Applying 10 μM GTP to the I_{Mg}-deficient mutant *eccentric* in the presence of Na^+ yielded inward currents that were similar in form and magnitude to those of the wild type (860 ± 137 pA, $n = 3$; Fig. 2*B*, middle). Inward currents seen when Mg^{2+} was the predominant extracellular cation were reduced considerably compared with those of the wild type (94 ± 41 pA, $n = 4$; Fig. 2*B*, top), but were of similar magnitude to those observed in the absence of either Mg^{2+} or Na^+ (102 pA; Fig. 2*B*, lower; Table 1*A*). Conversely, applying GTP to the I_{Na}-deficient mutant *fast-2* in the presence of Mg^{2+} elicited inward currents that were comparable with those of the wild type (214 ± 101 pA, $n = 6$; Fig. 2*C*, top), but the currents elicited in the presence of Na^+ were reduced to the extent that they were indistinguishable from those observed in the absence of Na^+ (95 ± 19 pA, $n = 5$; *cf.* 79 pA, Table 1*A;* Fig. 2*C* middle and bottom).

Consistent with these observations was the finding that by inhibiting I_{Mg}, the *eccentric* mutation deprived *Paramecium* of the ability to respond behaviorally to GTP in Mg^{2+} (Fig. 3*A*), while responses in the presence of Na^+ (Fig. 3*B*) were indistinguishable from those of the wild type (Fig. 1*D*). Similarly, by inhibiting I_{Na}, the *fast-2* mutation specifically inhibited backward swimming in response to GTP in Na^+ solutions (Fig. 3*B*), while responses in Mg^{2+} solutions were unaffected (Fig. 3*A*).

These data are consistent with the idea that GTP induces backward swimming in the wild type through the parallel activation of I_{Mg} and I_{Na}. Both of these currents are Ca^{2+}-dependent; under physiological conditions they activate following an increase in intracellular Ca^{2+}-concentration ($[Ca^{2+}]_{cyto}$; Preston, 1990). This raises the possibility that the inward current elicited by GTP in the absence of Mg^{2+} and Na^+ is a GTP-induced Ca^{2+} conductance that, in turn, elicits I_{Mg} and I_{Na}. Several Ca^{2+} currents have been described in *Paramecium:* one elicited upon depolarization ($I_{Ca,d}$; Oertel, Schein & Kung, 1977), a second activated upon hyperpolarization ($I_{Ca,h}$; Preston, Saimi & Kung, 1992), and a third seen in response to anterior mechanical conductance ($I_{ca,m}$; Machemer, 1988). There is preliminary evidence for a fourth, ligand-activated Ca^{2+} conductance ($I_{ca,l}$; Hennessey, Kim & Satir, 1995). The $I_{Ca/d}$ can be suppressed genetically (Oertel et al., 1977), pharmacologically (Gustin & Hennessey, 1988; Hennessey & Kung, 1984), and by deciliation, the latter suggesting an association with the ciliary membrane (Dunlap, 1977). The other three Ca^{2+} currents appear to be localized on the body (somatic) membrane. These differences allowed us to investigate their possible roles in the GTP response.

First, we excluded the involvement of the only other

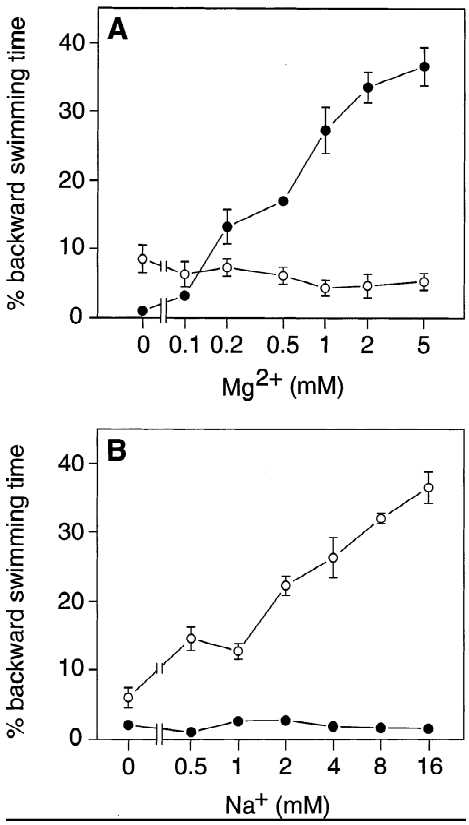

Fig. 3. Ion-dependence of the behavioral response to GTP in *eccentric* and *fast-2*. Response of the I_{Mg}-deficient mutant *eccentric* (○) and the I_{Na}-deficient mutant *fast-2* (●) to 10 μM GTP (*A*) as a function of increasing Mg^{2+} concentration or (*B*) as a function of Na^+ concentration. The behavioral test solution contained, in addition to the indicated Mg^{2+} or Na^+ concentration, 1 mM $CaCl_2$ and 4 mM KCl (as in Fig. 1*C* and *D; see* Materials and Methods). Points represent mean responses of five cells with standard errors.

significant ions in the bathing medium, TEA^+ and Cl^-, in generating the inward current. TEA^+ involvement was excluded by removing it from the bathing medium with no apparent effect on the inward current (Fig. 4*A;* Table 1*B*). The contribution of Cl^- was examined by replacing this anion with glutamate in both the capillary microelectrodes and in the bathing medium. Glutamate was chosen because it does not permeate Cl^- channels in *Paramecium* (R.R. Preston and Y. Saimi, *unpublished results*). Not only did the inward current persist under these conditions (Fig. 4*A;* Table 1*B*), it remained unaltered when an inward Cl^- gradient was imposed (30 mM TEA-Cl added extracellularly: Fig. 4*A;* Table 1*B*). Thus, by elimination, Ca^{2+} is the likely carrier of the oscillating inward current.

The *pawn B* mutant lacks a functional $I_{Ca,d}$ (Oertel et al., 1977) but GTP-induced currents were observed in this mutant (Fig. 4*B;* Table 1*C*). These observations are consistent with earlier findings that GTP induces apparently normal membrane-potential oscillations in *pawn B*, despite its inability to respond behaviorally with backward swimming (Clark et al., 1993). $I_{Ca,d}$ can also be inhibited in the wild type by deciliation (Dunlap, 1977), but this treatment had no significant effect on the GTP-induced current (Fig. 4*B;* Table 1*C*). The *Dancer* mutation also affects $I_{Ca,d}$, by enhancing it (Hinrichsen & Saimi, 1984). Again, the mutation had no apparent effect on the GTP response (Fig. 4*B;* Table 1*C*). $I_{Ca,h}$ can be inhibited using amiloride or Ba^{2+} (Preston et al., 1992). The GTP-induced inward currents were found to persist in the presence of either 2 mM amiloride or 10 mM Ba^{2+}, concentrations that are adequate to inhibit $I_{ca,h}$ fully (Fig. 4*C:* Table 1*D*).

While these data suggest that GTP increases $[Ca^{2+}]_{cyto}$ by a mechanism that is distinct from these two voltage-activated Ca^{2+} currents, the GTP-induced current *is* voltage sensitive. This is demonstrated in Fig. 4*D*. Throughout this investigation, in each of the various solutions, membrane potential was clamped at resting potential (*see* Materials and Methods) rather than at the −40 mV value used in most previous studies. However, holding at potentials >5 mV negative to rest caused the GTP-induced Ca^{2+} conductance to inactivate reversibly but fully (Fig. 4*D*, middle).

Discussion

The results described here suggest that the oscillating depolarizations that underlie repulsion from GTP in *Paramecium* are caused by the parallel activation of two independent ion currents, I_{Mg} and I_{Na}. Behavioral observations have demonstrated that either Mg^{2+} or Na^+ can support the backward swimming episodes and that the extent of backward swimming increases with extracellular Mg^{2+} and Na^+ in a concentration-dependent manner. Behavioral data cannot tell us whether these cations enter the cell via a single, nonspecific pathway or by way of separate Mg^{2+}- and Na^+-specific conductances. But by specifically inhibiting Mg^{2+}-dependent responses to GTP with a mutation that blocks I_{Mg} and inhibiting Na^+-dependent responses with a mutation that suppresses I_{Na} we have shown the single, nonspecific channel hypothesis to be untenable.

For largely historical reasons, discussion of the possible functions of I_{Mg} and I_{Na} in *Paramecium* has focused on their contribution to the overall form of the action potential (Kung & Saimi, 1982; Preston & Kung, 1994). Action potentials are initiated when the membrane depolarizes beyond the threshold required for opening voltage-sensitive Ca^{2+} channels in the ciliary membrane. The Ca^{2+} that enters the cilia at this time is the primary impetus for ciliary reversal, but it may also allow for activation of I_{Mg} and I_{Na}. Both currents are depolarizing under physiological conditions, which has led to the suggestion that their role is simply to prolong the spike and

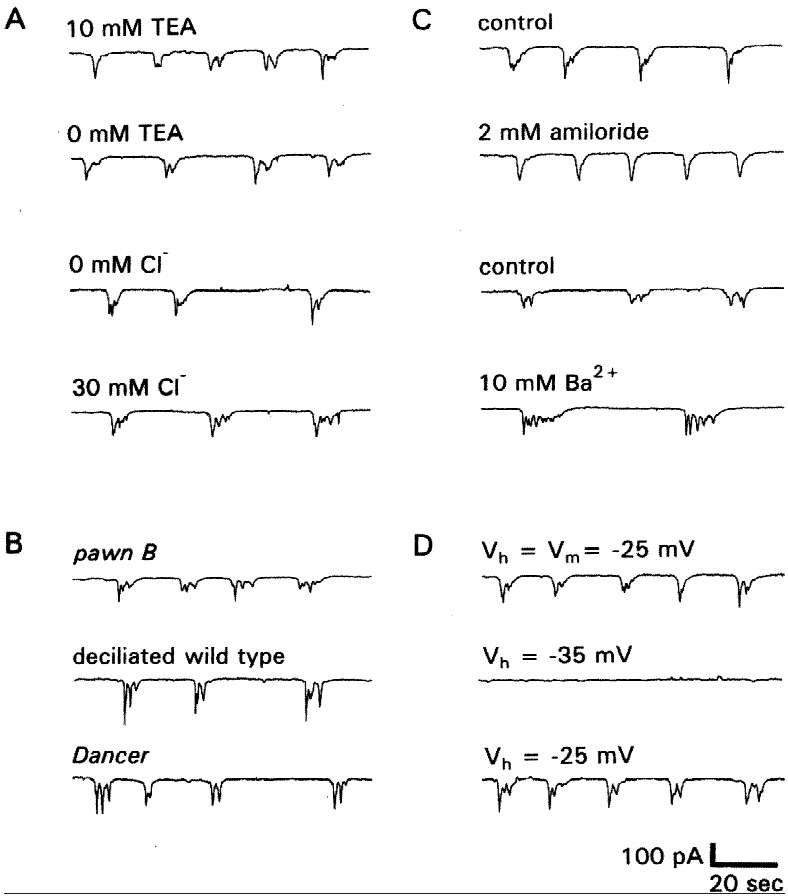

Fig. 4. Properties of the current induced by GTP in the absence of extracellular Mg^{2+} or Na^{+}. Voltage-clamp records of currents elicited by 10 μM GTP from cells bathed in ''Ca^{2+} solution'' (no Mg^{2+} or Na^{+}; *see* Materials and Methods). (*A*) Current oscillations were unaffected by removing TEA, a common K^{+}-channel inhibitor, from the bath solution (upper two traces) or by reversing the Cl^{-} gradient (lower two traces) from outward (0 mM Cl^{-}) to inward (30 mM Cl^{-}). (*B*) GTP-induced inward currents were observed in *pawn B* and in deciliated wild-type cells, both of which lack the depolarization-activated Ca^{2+} current, and in *Dancer*, a mutant in which this Ca^{2+} current is enhanced. (*C*) The GTP-induced inward current was not inhibited by either 2 mM amiloride (upper two traces) or 10 mM Ba^{2+} (lower two traces), conditions that fully suppress the hyperpolarization-activated Ca^{2+} current. (*D*) The GTP-induced inward current was inactivated at membrane potentials negative to rest. The top trace shows the GTP-induced current when the holding potential (V_h) approximates resting potential (V_m) in Ca^{2+}/TEA-solution, −25 mV. Holding membrane potential at 10 mV negative to rest (−35 mV) inhibited this current fully (middle trace) but reversibly (lower trace).

resultant backward swimming events. Subsequent membrane repolarization and recovery of forward swimming is effected by a fast outward rectifying K^{+} current and by a slower Ca^{2+}-dependent K^{+} current (Saimi et al., 1983). Our studies have revealed an important additional and previously unsuspected role for I_{Mg} and I_{Na}. Because the inward current oscillations shown in Fig. 3 were recorded under voltage-clamp, GTP must activate these currents by a mechanism that is completely independent of membrane depolarization. Thus I_{Mg} and I_{Na} must now be viewed in their own right as physiologically relevant intermediates in a signal-transduction pathway that is initiated by the binding of GTP to its putative receptor and that results in cell repulsion from the stimulus.

The oscillation of I_{Mg} and I_{Na} is particularly interesting. The two conductances may be controlled by independent oscillators, but a simpler and more plausible explanation is that their activation is governed by a single, common oscillating regulator. As these inward currents were recorded under voltage clamp, this regulator must be chemical rather than electrical. Both I_{Mg} and I_{Na} are $[Ca^{2+}]$-dependent currents, so it is reasonable to propose that they are activated in response to GTP-induced oscillations in $[Ca^{2+}]_{cyto}$. Confirming such changes in $[Ca^{2+}]_{cyto}$ is beyond the scope of the present report, but it would be consistent with our findings and with the numerous reports of cytoplasmic Ca^{2+} oscillations in other cell types (Tsien & Tsien, 1990).

Previous studies on metazoa have suggested that contributions to oscillations in intracellular $[Ca^{2+}]$ can come from intracellular Ca^{2+} stores and/or from the extracellular space via ion channels (Tsien & Tsien, 1990). *Paramecium*, like most cells, has extensive intracellular Ca^{2+} stores. One is located within a system of membrane-bound ''alveolar sacs'' that lie just beneath the plasma membrane throughout the entire cell (Stelly et al., 1991). The function of the alveolar stores is unknown, but one suggestion is that they are the protozoan equivalent of the sarcoplasmic reticulum of myocytes (Erxleben &Plattner, 1994). According to this model, when stimulated appropriately, alveolar sacs release Ca^{2+} into the small space between plasma and alveolar membranes and trigger trichocyst secretion (trichocysts are defensive organelles). The close proximity of this Ca^{2+} store to the Mg^{2+} and Na^{+} conductances of the plasma membrane also makes it an attractive candidate as a source of a possible GTP-induced rise in $[Ca^{2+}]_{cyto}$.

The second possibility, that I_{Mg} and I_{Na} activate in response to periodic increases in cell permeability to extracellular Ca^{2+}, is strengthened by the discovery of a

residual GTP-induced inward current that persists when all cations other than Ca^{2+} have been removed from the bathing medium (Figs. 2 and 4). Showing unequivocally that the residual GTP-induced current is indeed a Ca^{2+} conductance is beyond the scope of the present report and may be difficult because *Paramecium* dies within seconds of removing extracellular Ca^{2+}. This thwarts demonstration of an absolute dependence on Ca^{2+}. However, it may prove possible to show reversal of the current at the equilibrium potential for Ca^{2+}. There is a precedent for such purinergic control of ciliary activity among higher organisms. Extracellular application of ATP to mucus-transporting epithelial cells from amphibians and mammals causes their cilia to beat faster (Villalón, Hinds & Verdugo, 1989). ATP is believed to bind to a P_2-purinoceptor on the cell surface and to cause Ca^{2+} influx. However, in these cases, the rise in intracellular $[Ca^{2+}]$ elicits Ca^{2+}-dependent K^+ efflux to hyperpolarize the membrane and stimulate ciliary beating (Villalón et al., 1989; Weiss et al., 1992; Länge, Klauke & Plattner, 1995).

In the absence of a specific pharmacological or genetic inhibitor of the residual GTP-induced current, it is impossible to determine whether the current is *required* for the Mg^{2+} and Na^+ oscillations or whether it is a third (Ca^{2+}-permeant) conductance that is elicited by a common oscillator. Since this conductance persists after inhibiting the two well-characterized Ca^{2+} currents in *Paramecium,* it may represent a novel conductance. Its persistence after deciliation suggests that it must be restricted largely to the somatic membrane. This would be consistent with the observation that *pawn B* does not swim backward in GTP (Clark et al., 1993), for if the suggested Ca^{2+} influx is restricted to the cell body, it is most likely buffered by the cytoplasm before it ever reaches the ciliary reversal mechanism. Thus in the wild type, GTP-induced backward swimming is dependent on the depolarization associated with Mg^{2+} and Na^+ influx, which in turn activates the voltage-sensitive Ca^{2+} channels in the ciliary membrane. The apparent localization of the residual GTP-induced conductance to the cell body membrane (Fig. 4*B*) and its inactivation by membrane hyperpolarization (Fig. 4*D*) should aid in fully describing and understanding its properties.

In summary, we suggest that the binding of extracellular GTP to a putative surface receptor in *Paramecium* induces a rise in $[Ca^{2+}]$ in the cell body ($[Ca^{2+}]_{cyto}$) through release from an intracellular store, activation of a plasma membrane Ca^{2+} channel, or perhaps by the combination of both mechanisms. Increasing $[Ca^{2+}]_{cyto}$ then elicits the two Ca^{2+}-dependent (and in the case of I_{Na}, Ca^{2+}/calmodulin-dependent (Saimi & Ling, 1990)) conductances. Entry of Mg^{2+} and Na^+ into the cell causes membrane depolarization, activation of voltage-dependent Ca^{2+} channels in the ciliary membrane, elevation of $[Ca^{2+}]_{cil}$, and hence backward swimming. The behavioral response presumably terminates when $[Ca^{2+}]_{cyto}$ falls, I_{Mg} and I_{Na} deactivate, and a repolarizing conductance and/or electrogenic Mg^{2+} and Na^+ pumps return the membrane potential to resting level. With repolarization, ciliary Ca^{2+} channels close and $[Ca^{2+}]_{cil}$ drops, due to Ca^{2+} extrusion through the ciliary membrane and/or diffusion of Ca^{2+} away from the ciliary compartment.

Although we have seen no evidence for a repolarizing conductance, the membrane currents shown above were recorded under conditions designed specifically to inhibit K^+ currents. The slow activation of a Ca^{2+}-dependent K^+ current (of which there are two in *Paramecium;* Preston et al., 1990) in response to a rise in $[Ca^{2+}]_{cyto}$ would be sufficient to serve this purpose.

Why design a signal-transduction pathway that contains two different currents (I_{Mg} and I_{Na}) that each produce the same end effect? Perhaps the environmental conditions (in particular the salt concentrations) that *Paramecium* experiences in a fresh-water pond change quite drastically according to rainfall, drought, and influx of water from different mineral sources. It may be that these varied conditions have selected for redundancy in chemosensory and locomotor response mechanisms, which would allow a cell to respond under wide-ranging conditions. The considerable machinery devoted to this signal-transduction pathway suggests that the GTP response is important to *Paramecium.*

The literature offers many examples of oscillating cytosolic Ca^{2+} levels, but in most cases their function remains to be explained. The response of *Paramecium* to extracellular GTP may be the first clearly defined case of intracellular Ca^{2+} oscillations linked with a corresponding physiological ‘‘output’’: periodic backward swimming.

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