J. exp. Biol. ***176****, 117–133 (1993)*
Printed in Great Britain © The Company of Biologists Limited 1993

GUANINE NUCLEOTIDES MODULATE CALCIUM CURRENTS IN A MARINE *PARAMECIUM*

JUAN BERNAL* and BARBARA E. EHRLICH

Departments of Medicine and Physiology, University of Connecticut Health Center, Farmington, CT 06030, USA and *Marine Biological Laboratory, Woods Hole, MA 02543, USA*

Accepted 22 October 1992

Summary

Voltage-dependent calcium channels play a critical role in many cell functions and in many cell types ranging from protozoa to vertebrates. We have shown previously that guanine nucleotides modulate the calcium action potential and the duration of backward swimming in *Paramecium*, both indirect measurements of calcium channel function. To determine whether guanine nucleotides do indeed alter calcium currents, the inward calcium current (I_{Ca}) in *Paramecium calkinsi* was studied. First, the calcium current was characterized. The magnitude of I_{Ca} increased as the extracellular calcium concentration was increased from 0.5 to 50mmol l^{-1}, unlike the situation in freshwater species of *Paramecium* where the inward calcium current magnitude is maximal when extracellular calcium levels reach 1mmoll^{-1}. Inorganic compounds ($NiCl_2$ at 10 μmol l^{-1} and $CdCl_2$ at 1mmol l^{-1}) and organic compounds (naphthalene sulfonamides, W-7 and W-12-Br at 100 and 2 μmol l^{-1}, respectively) reduced I_{Ca}. Regardless of the holding membrane potential (from −80 to −20mV), the threshold activation for I_{Ca} was at −10mV and the maximum value of I_{Ca} was reached at +20mV, suggesting that there is only one type of calcium channel in *P. calkinsi*. Second, we injected GTPγS, GTP and GDPβS into voltage-clamped cells while monitoring calcium and/or potassium currents. GTPγS increased the magnitude of I_{Ca} by 42±6% (mean ± S.D., N=5) and the effect was irreversible, GTP increased the magnitude of I_{Ca} by 37±4% (N=4) in a reversible manner, and GDPβS decreased I_{Ca} by 57±8% (N=3) irreversibly. The outward potassium currents did not change when GTPγS was injected into the cells. These results support the hypothesis that injection of guanine nucleotides modulates the voltage-dependent calcium channel in *P. calkinsi*, presumably by activating G-protein-dependent processes.

Introduction

Paramecium is the most primitive organism known to have voltage-dependent calcium channels (Saimi *et al.* 1988). The properties of the calcium channel of *Paramecium* are a mixture of the properties shown by the many types of calcium channels present in vertebrates (Hess, 1990) in that the *Paramecium* calcium channel has a small

*Present address: Departamento de Fisiologia y Farmacologia, Universidad Autonoma de Aguascalientes, Aguascalientes, Ags, CP 20100, Mexico.

Key words: calcium channels, *Paramecium calkinsi*, guanine nucleotides.

conductance, inactivates and is not sensitive to dihydropyridines (Ehrlich *et al.* 1984, 1988). These channels, which are located in the ciliary membrane (Dunlap, 1977; Machemer and Ogura, 1979), play an important role in the excitability of the cell and in the control of its swimming behavior (Eckert and Brehm, 1979). Indeed, there is a correlation between the duration of backward swimming and the magnitude of the voltage-dependent inward calcium current (Haga *et al.* 1982; Hennessey and Kung, 1984).

Calcium channels can be modulated by several intracellular metabolic mechanisms (Reuter, 1983; Levitan and Kaczmarek, 1987; Levitan, 1988). For example, guanine-nucleotide-activated proteins (G-proteins) modulate the calcium channels present in vertebrate cells such as neurons (Holz *et al.* 1986; Dunlap *et al.* 1987; Hescheler *et al.* 1987; Dolphin *et al.* 1988, 1989; Dolphin, 1990) and striated muscle (Yatani *et al.* 1987; Brown and Birbaumer, 1990). In addition, the backward swimming behavior and the calcium action potential of *Paramecium* are modulated by GTPγS, a compound that irreversibly activates G-proteins (Bernal *et al.* 1991).

To understand how modulation of calcium channels can control the influx of calcium in intact living cells, we studied the calcium currents of the marine ciliate *Paramecium calkinsi*. We report here that the inward calcium current (I_{Ca}) in a marine *Paramecium*, as in freshwater species, can be isolated from other types of inward and outward currents (e.g. Eckert and Brehm, 1979) and that only one kind of calcium channel appears to be present. The magnitude of this current can be increased by increasing the extracellular concentration of calcium and can be inhibited by organic and inorganic compounds. Furthermore, guanine nucleotides modulate the calcium current, but not potassium currents, in *Paramecium*. Preliminary results have already been published (Bernal and Ehrlich, 1989, 1991).

Materials and methods

The marine ciliate *Paramecium calkinsi* was used in all experiments. Cell culture conditions were similar to those reported previously (Bernal *et al.* 1991).

Because *Paramecium* swims continuously, a major technical difficulty was to immobilize individual cells before initiating electrophysiological recordings. To capture a moving cell before impaling it with two microelectrodes, we modified a preparation used to trap a *Paramecium* in agar (Schein *et al.* 1976). Briefly, cells from the culture medium (solution 1, Table 1) were centrifuged and the pellet of cells was washed twice with artificial sea water at an ionic strength 25% of normal (solution 2, Table 1). This ionic concentration was chosen because *P. calkinsi* grew well in this medium. A small drop ($\leqslant 20\,\mu$l) of solution containing concentrated *Paramecium* was placed on a recording chamber constructed from a glass coverslip under a plastic slide with a 10mm diameter hole. After a 5–10min wait to allow the drop to evaporate slightly, agarose solution (5mgml^{-1} dissolved in solution 3, Table 1) in a total volume of 100 μl was added to the suspended *Paramecium*. Immediately after the solutions had been mixed, the chamber was placed in a freezer (-20°C) for no more than 30s. As a result of this manipulation, cells were trapped in the agarose in a volume approximately the same size

Table 1. *Experimental solutions*

	1	2	3	4	5	6	7
Caseaminoacids	0.3 g l^{-1}	–	–	–	–	–	–
Stigmasterol	5 mg l^{-1}	–	–	–	–	–	–
NaCl	125	125	–	–	–	–	–
N-Methyl-D-glucamine	–	–	125	–	–	–	–
TEACl	–	–	–	125	146.7	140.5	72.5
KCl	10	10	10	–	–	–	–
CsCl	–	–	–	5	5	5	5
Citric acid	2	2	–	–	–	–	–
$CaCl_2$	5	5	15	15	0.5	5	50
$MgCl_2$	10	10	–	–	–	–	–
Mops	10	10	10	10	10	10	10
4-AP	–	–	–	2.5	2.5	2.5	2.5
3,4-Diaminopyridine	–	–	–	2.5	2.5	2.5	2.5

Except where noted, concentrations are in mmol l^{-1}.

TEACl, tetraethylammonium chloride; 4-AP, 4-aminopyridine; Mops, 3-[*N*-morpholino]propanesulfonic acid.

as the cell. The congealed agarose containing the *Paramecium* was then bathed with recording medium (solutions 3 or 4, Table 1). The total volume of the recording chamber was 250 μl. In those experiments in which the extracellular medium was changed, the perfusion solution was exchanged at the rate of 3 ml min^{-1}.

For intracellular recording experiments, the chamber containing the *Paramecium* was set on an inverted microscope (Diaphot, Nikon). To facilitate the impalement of cells with the electrodes, cells located at the very top of the agarose medium were chosen. Depending on the kind of experiments to be performed (current-clamp or voltage-clamp), one or two microelectrodes were used to impale the cell. Electrodes were prepared as reported previously (Bernal *et al.* 1991).

In current-clamp experiments, cells were clamped using the bridge configuration of the amplifier (Dagan 8500, Dagan Corp., Minneapolis, MN). The membrane potential was followed through the same electrode that was used to inject hyperpolarizing or depolarizing current pulses into the cell. In experiments where calcium and potassium currents were studied, the amplifier was used in the two-microelectrode voltage-clamp configuration. In those experiments in which compounds of interest were injected into the cell, the voltage electrode was used for this purpose. The procedure used for pressure injections is described elsewhere (Bernal *et al.* 1991).

Guanine nucleotides at a final concentration in the micropipette of 10 mmol l^{-1} were injected by pressure into the cell. GTP, GTPγS and GDPβS (Boehringer-Mannheim) with Fast Green (Sigma) as a dye indicator were injected into voltage-clamped cells. The lithium salt of the guanine nucleotides were used. Note that GTPγS and GDPβS had opposite effects on the currents (see below) and on the action potentials (Bernal *et al.* 1991) even though the amount of lithium injected into the cell was similar. Assuming that

1 % of the cell volume was injected and that the compounds were uniformly distributed in the cell, the maximal concentration of compounds injected was 100 μmol l^{-1}.

Because many different ionic currents in addition to the calcium current have been described in *Paramecium* (Saimi *et al.* 1988; Eckert and Brehm, 1979; Preston, 1990), several experimental manipulations were performed to isolate the inward calcium current (I_{Ca}). First, a sodium- and magnesium-free solution was used to avoid contamination by the calcium-dependent sodium current (Saimi and Kung, 1980) and the magnesium current (Preston, 1990). Second, several potassium channel blockers, including tetraethylammonium chloride (TEACl), 4-aminopyridine (4-AP), CsCl and 3,4-diaminopyridine, were included in the extracellular medium. In addition, electrodes used for impaling cells were filled with Cs^+-containing solutions to block potassium currents from inside the cell. Thus, the voltage electrode was filled with a solution containing 200mmol l^{-1} CsCl, 10mmol l^{-1} Mops, 2mmol l^{-1} $MgCl_2$ and 0.1mmol l^{-1} Fast Green, pH7.0, and the current electrode was filled with a solution containing 100mmol l^{-1} caesium citrate, 10mmol l^{-1} Mops and 2mmol l^{-1} $MgCl_2$, pH7.0. In those experiments in which the inward calcium current and total outward potassium currents were studied, Cs^+ in the electrodes was substituted by K^+ at the same molarity. Solutions used in the extracellular media are listed in Table 1. Figures show one of at least three similar experiments, unless otherwise noted. Means ± standard deviations are given where appropriate.

Results

Inward calcium currents can be isolated from other currents in voltage-clamped Paramecium calkinsi

Cells were held at −40mV and depolarizing pulses were applied to elicit the currents. Under control conditions, the total outward currents and the inward calcium currents can be identified (Fig. 1A). Three minutes after a solution containing potassium channel blockers had been superfused (solution 4, Table 1), the net outward current was reduced and only the inward calcium currents were seen (Fig. 1B).

There was a slight increase in the magnitude of calcium currents after application of potassium channel blockers because the voltage-dependent potassium currents and the calcium-dependent potassium currents present in *Paramecium* were inhibited (Brehm *et al.* 1978; Eckert and Brehm, 1979). The calcium-dependent sodium current (Saimi and Kung, 1980) and the magnesium current (Preston, 1990) were inhibited by using a sodium- and magnesium-free solution (solution 3, Table 1).

Calcium currents in marine Paramecium *depend on the extracellular calcium concentration*

Freshwater species of *Paramecium* live in an almost salt-free medium with an osmolarity of approximately 20mosmol l^{-1}. The calcium currents in these freshwater *Paramecium* are saturated at an extracellular calcium concentration of 1mmol l^{-1} (Satow and Kung, 1979). Initial experiments with *P. calkinsi* suggested that the calcium currents

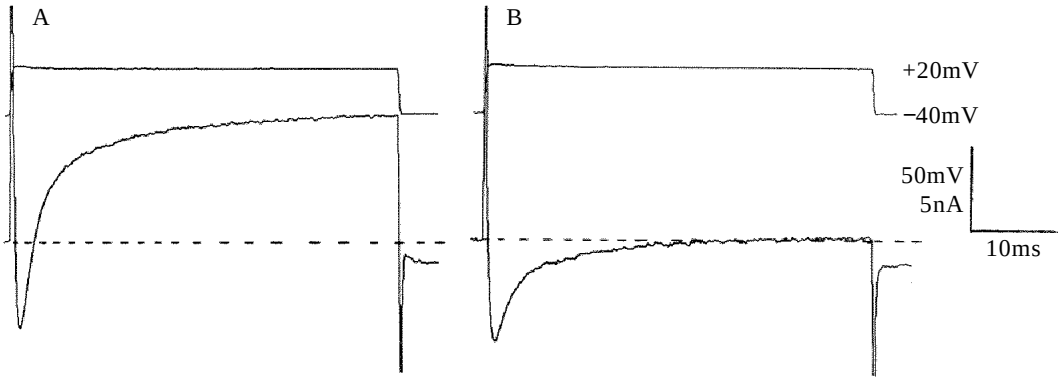

Fig. 1. Isolation of the inward calcium current from *Paramecium calkinsi.* Voltage-dependent calcium currents and outward potassium currents were monitored (A) before and (B) 3min after a cell had been superfused with a solution containing potassium channel blockers (see Materials and methods and Table 1 for description). Top traces in each panel represent the voltage monitor and bottom traces are the currents induced by depolarizing pulses; dashed lines represent zero current. The cell was depolarized to +20mV from a holding potential of −40mV.

in this marine *Paramecium* increased when the extracellular calcium concentration was increased, but in these experiments the extracellular concentration of magnesium was altered reciprocally with calcium in order to retain a constant divalent cation concentration (Deitmer and Machemer, 1982). Thus, it was not possible to determine whether the increased currents were due to the increase in calcium or the decrease in magnesium concentration, an ion known to inhibit calcium channels (Hagiwara and Byerly, 1981; Tsien *et al.* 1987).

In current-clamp experiments where the extracellular calcium concentration was increased and magnesium was absent, we found that the amplitude and duration of the calcium action potential followed the calcium concentration. For example, 3min after the extracellular calcium concentration had been changed from 15 to 0.5mmoll^{-1}, the amplitude and duration of the calcium action potential were decreased by 42±5% (N=3) and 25±7% (N=3), respectively. This reduction in the amplitude and duration of the calcium action potential was reversible. Similarly, when the extracellular calcium concentration was increased from 15 to 50mmoll^{-1}, the amplitude and duration of the calcium action potential were increased by 32±6% (N=4) and 98±17% (N=4), respectively.

Although the current-clamp experiments described above suggested that the calcium dependence of the channels in marine and freshwater *Paramecium* was different, voltage-clamp experiments were necessary to demonstrate directly that the voltage-dependent calcium current in the marine *Paramecium* depends on the extracellular calcium concentration. Cells were held at −20mV and depolarizing pulses were applied to elicit calcium currents. After the extracellular calcium concentration had been increased from 0.5 to 5mmoll^{-1} (solutions 5 and 6, Table 1) the magnitude of the calcium currents increased by 82±9% (N=4; Fig. 2A, middle trace; Fig. 2B, open circles; Fig. 3) and this effect was reversible (Fig. 2A, right trace; Fig. 2B, triangles). In addition, the maximum

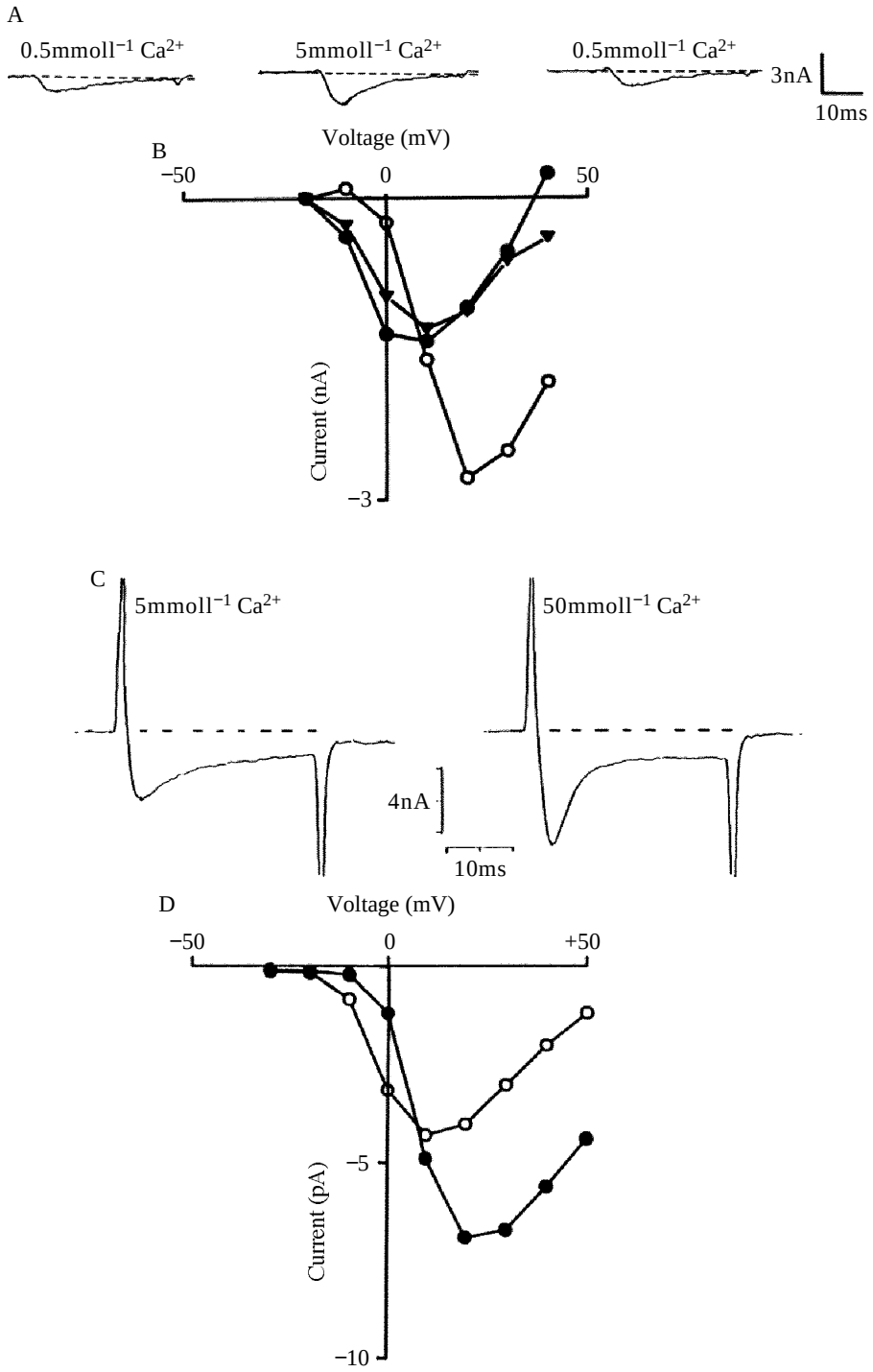
A
0.5mmoll⁻¹ Ca²⁺
5mmoll⁻¹ Ca²⁺
0.5mmoll⁻¹ Ca²⁺
3nA
10ms
B
Voltage (mV)
−50
0
50
Current (nA)
−3
C
5mmoll⁻¹ Ca²⁺
50mmoll⁻¹ Ca²⁺
4nA
10ms
D
Voltage (mV)
−50
0
+50
Current (pA)
−5
−10

Fig. 2

Fig. 2. Extracellular calcium dependence of the inward calcium current from *Paramecium calkinsi*. (A) Typical traces of calcium currents elicited at +20mV when a cell was superfused with an extracellular solution containing 0.5mmol l^{-1} Ca^{2+} (left and right traces) or 5 mmol l^{-1} Ca^{2+} (middle trace). (B) The current–voltage relationship of the calcium current at 0.5mmol l^{-1} $CaCl_2$ (filled circles and filled triangles) and after an increase in $CaCl_2$ concentration to 5mmol l^{-1} (open circles). Filled triangles in B and right-hand trace in A represent the recovery of calcium currents when calcium was returned to 0.5mmol l^{-1}. (C) Typical traces of calcium currents elicited at +20mV when a cell was superfused with an extracellular solution containing 5mmol l^{-1} (left trace) or 50mmol l^{-1} $CaCl_2$ (right trace). (D) The current–voltage relationship for the calcium current at 5mmol l^{-1} $CaCl_2$ (open circles) and after an increase in $CaCl_2$ concentration to 50mmol l^{-1} (filled circles). The holding potential was −20mV. Zero current is indicated by the dashed lines.

inward calcium current ($I_{Ca,max}$) shifted 10mV in the depolarizing direction when extracellular calcium concentration was increased 10-fold (Fig. 2B). When the extracellular calcium concentration was increased from 5 to 50mmol l^{-1} (solutions 6 and 7, Table 1), there was a further increase in the maximum calcium current by 62±5% (N=5) and a 10mV shift in the voltage at which $I_{Ca,max}$ was obtained (Fig. 2C,D; Fig. 3).

There is only one kind of calcium current in marine Paramecium

Threshold voltage

In many biological systems, more than one type of calcium channel can coexist in the same cell and these channels can be identified by their biophysical properties as well as by their sensitivity to specific calcium channel blockers (Carbone and Lux, 1984; Deitmer, 1984, 1986; Fedulova *et al.* 1985; Armstrong and Matteson, 1985; Nowycky *et al.* 1985). One biophysical characteristic that has been used to separate the different calcium channel types is the threshold voltage needed to activate the channels. We used this criterion to evaluate the number of calcium channel types that are needed to generate

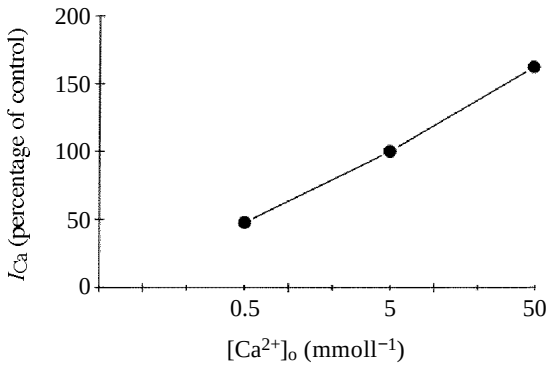

Fig. 3. Extracellular calcium dependence of calcium currents in *Paramecium calkinsi*. Maximum inward calcium current ($I_{Ca,max}$) is plotted against different extracellular calcium concentrations. Values were normalized to the current obtained at 5mmol l^{-1} extracellular calcium, a measurement made in all cells, so that currents obtained from different cells could be compared. The holding potential was −40mV. Error bars have been omitted because they fall within the symbols.

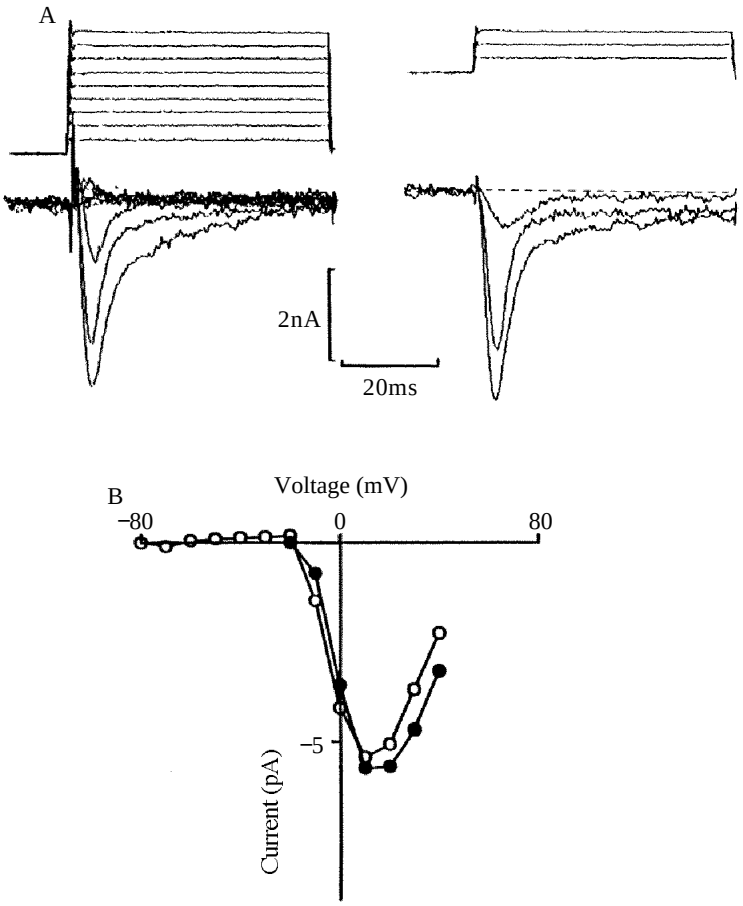

Fig. 4. Effect of the holding potential on the current–voltage relationship in *Paramecium calkinsi*. (A) Calcium currents elicited from a holding potential of −80mV (left panel) or −20mV (right panel). Zero current is indicated by the dashed line. (B) Open circles show the magnitude of the calcium currents when the holding potential was −80mV. Filled circles show the magnitude of the calcium currents when the holding potential was −20mV.

the calcium currents of *P. calkinsi*. Cells were held at two different holding potentials (−80 and −20mV) and, from these holding potentials, depolarizing command pulses were applied to elicit the calcium currents. Regardless of the holding potential, the threshold voltage for activation of the calcium current was −10mV and the $I_{Ca,max}$ was at +20mV (Fig. 4). The same result was obtained with holding potentials ranging from −100mV to −20mV.

Block by inorganic and organic compounds

Virtually all calcium channels are inhibited by inorganic compounds such as Co^{2+}, Cd^{2+} and Ni^{2+} (Hagiwara and Byerly, 1981; Tsien, 1983; Tsien *et al.* 1987). Different types of calcium channels have different sensitivities to inorganic blockers. For example,

Table 2. *Effect of inorganic and organic compounds on calcium currents from* Paramecium calkinsi

Compound	Concentration	Percentage inhibition of I_{Ca}
$NiCl_2$	10 μmol l^{-1}	42±9 (5)
$CdCl_2$	1 mmol l^{-1}	17±3 (3)
W-7	100 μmol l^{-1}	32±7 (4)
Quinacrine	100 μmol l^{-1}	51±7 (4)

The percentage inhibition of calcium current was estimated at $I_{Ca,max}$.
The numbers in parentheses indicate the number of experiments averaged for each condition.

T-type calcium channels are more sensitive to Ni^{2+} whereas L-type calcium channels are more sensitive to Cd^{2+} (Bean, 1989; Hess, 1990). In preliminary current-clamp experiments, $NiCl_2$ (at 100 μmol l^{-1}) blocked the calcium action potential and this effect was reversible. When voltage-dependent calcium currents were monitored before and after application of 10 μmol l^{-1} $NiCl_2$, $I_{Ca,max}$ was reduced (Table 2), but the current–voltage curve was not shifted along the voltage axis. Calcium currents were less sensitive to Cd^{2+} than to Ni^{2+}. Cd^{2+} at 1mmol l^{-1} was needed to produce a 17% inhibition of the calcium currents (Table 2).

Several organic compounds have been identified as calcium channel blockers and these compounds can be used as pharmacological tools to discriminate among calcium channel types (Tsien *et al.* 1987; Bean, 1989; Hess, 1990). Despite the biophysical similarities to calcium channels present in invertebrate and vertebrate systems, the pharmacology of the *Paramecium* calcium channels is different. Calcium channels in freshwater *Paramecium* can be blocked only by naphthalene sulfonamides (Hennessey and Kung, 1984; Ehrlich *et al.* 1988), compounds that were originally characterized as anticalmodulin drugs (Hidaka *et al.* 1979).

In voltage-clamp experiments the naphthalene sulfonamide W-7 at 100 μmol l^{-1} reduced the $I_{Ca,max}$ by 32±7% (N=4). This blocking effect of W-7 on the calcium current was seen at every voltage tested and there was no shift in $I_{Ca,max}$ after application of the drug (Table 2). Quinacrine, an antimalarial drug that has some structural similarities to W-7, also reduced I_{Ca} (Table 2; Barry *et al.* 1991).

Guanine nucleotides modulate calcium currents

It has been shown previously that backward swimming behavior and the calcium action potential are both modulated by GTPγS (Bernal *et al.* 1991). To test the hypothesis that guanine nucleotides do, indeed, modulate calcium currents, experiments using a two-microelectrode voltage-clamp were performed. Cells were superfused with a solution designed to inhibit potassium, sodium and magnesium currents and containing 15mmol l^{-1} extracellular calcium (solution 4, Table 1). Cells were held at −20mV and depolarizing command pulses were applied to induce calcium currents. The calcium currents were monitored both before and after pressure-injection of compounds into the

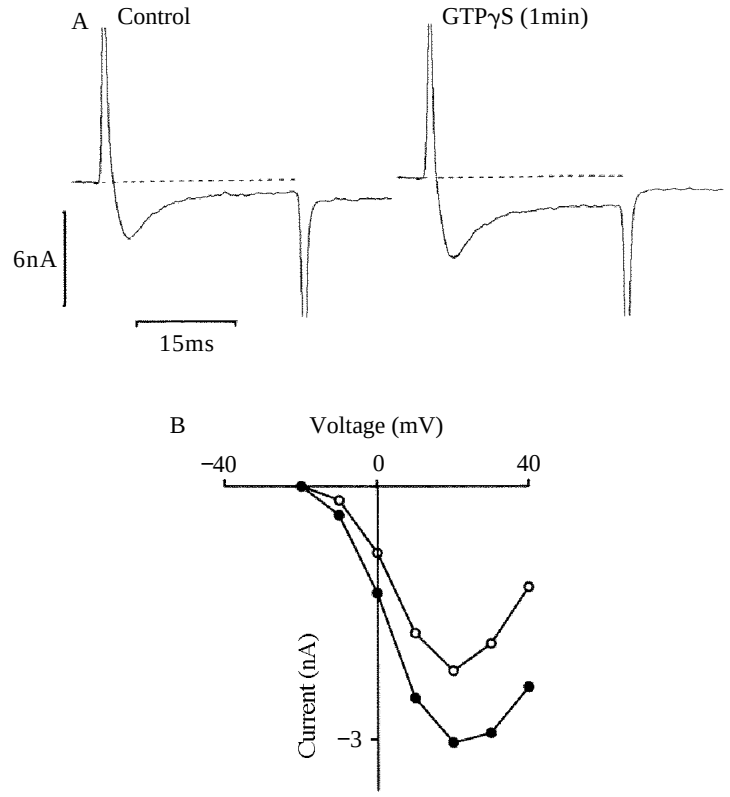

Fig. 5. GTPγS increases the magnitude of the inward calcium current in *Paramecium calkinsi.* (A) Representative traces of calcium currents elicited at +20mV in control conditions (left) and 1min after GTPγS had been pressure-injected into the cell. (B) The current–voltage relationship of the calcium currents before (open circles) and 1min after GTPγS had been pressure-injected into the cell (filled circles). The holding potential was −20mV. The dashed line represents the zero current line.

cell. In these experiments several criteria had to be fulfilled for inclusion in the analysis: (i) the cells had to have a stable holding current during the entire experiment, (ii) no changes in the leakage current could be observed before or after injection of compounds, (iii) the cells had to be stained blue after the injection of compound in order to guarantee that the compounds of interest had entered the cell.

GTPγS, an analogue of GTP that binds to and activates G-proteins, was injected into voltage-clamped cells while calcium currents were monitored. The magnitude of the calcium current increased within 1min after GTPγS had been injected into the cell (Fig. 5A right-hand record; Fig. 5B, filled circles). Five minutes after injection of GTPγS, $I_{Ca,max}$ had increased by 42±6% (N=5). The effect of GTPγS on the calcium current was seen at all membrane potentials tested, and there was no shift in the current–voltage relationship (Fig. 5B, filled circles).

GTPγS is a poorly hydrolyzable compound and, therefore, its activation of G-proteins is prolonged. Its effect on calcium currents from *P. calkinsi* was considered virtually

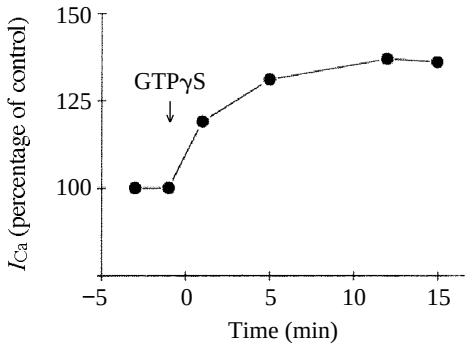

Fig. 6. The effect of GTPγS on calcium currents in *Paramecium calkinsi* is irreversible. Maximum inward calcium currents ($I_{Ca,max}$) reached at +20mV are plotted before and after a single pressure-injection of GTPγS into a voltage-clamped *Paramecium*. The arrow indicates the time when GTPγS was injected. Note that the current magnitude is elevated for over 15min.

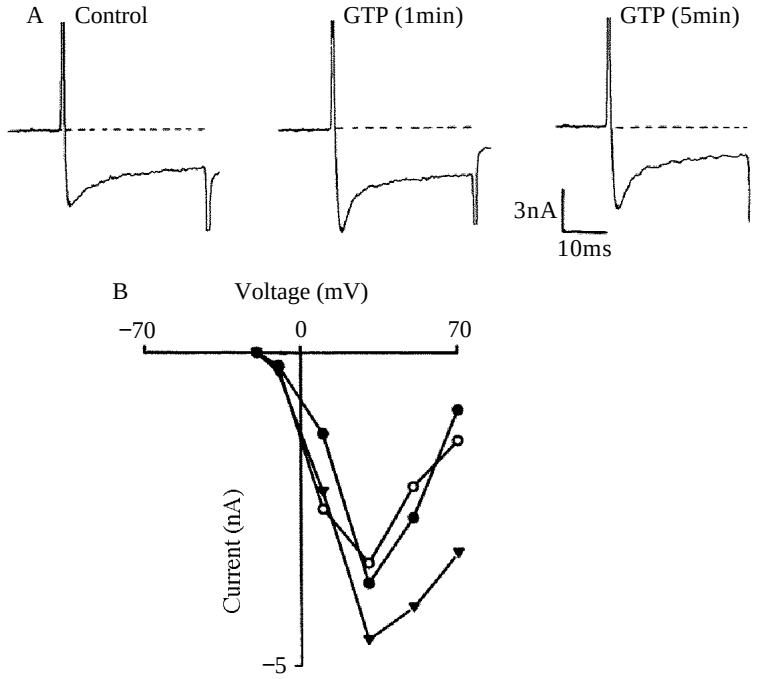

Fig. 7. The effect of GTP on calcium currents of *Paramecium calkinsi* is reversible. (A) Typical traces of calcium currents elicited at +20mV before (left trace) and 1 and 5min after injection of GTP (middle and right traces). (B) The current–voltage relationship of the calcium currents before (open circles) and 1min (filled triangles) and 5min (filled circles) after injection of GTP. The holding potential was −20mV and the dashed line indicates the zero current line.

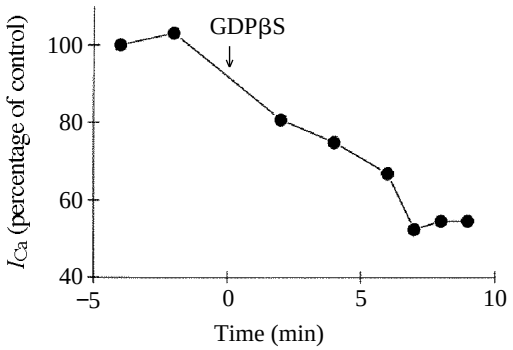

Fig. 8. GDPβS reduces the calcium currents in *Paramecium calkinsi* irreversibly. The maximum inward calcium current ($I_{Ca,max}$) elicited at +20mV is plotted before and after a single injection of GDPβS. The arrow indicates the time when a single injection of GDPβS was made.

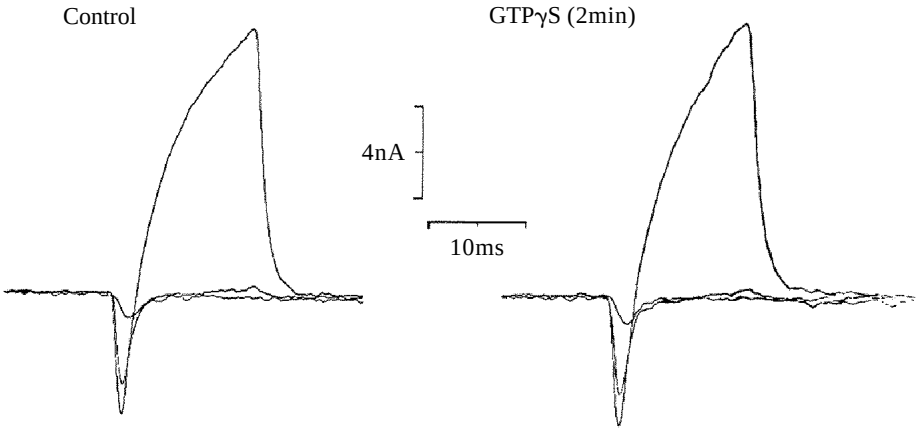

Fig. 9. The effect of GTPγS on potassium currents in Paramecium calkinsi. Typical inward calcium currents and total potassium outward currents (A) before and (B) 2min after GTPγS had been injected into the cell by pressure. Currents shown in both panels were obtained when command pulses to −10, 0 and 10mV were applied. Outward currents were only elicited when the command potential was 10mV. The holding potential was −20mV.

irreversible because the increase in the calcium current persisted for 15min after this compound had been injected into cells (Fig. 6).

Injection of GTP produces a reversible increase in the duration of the calcium action potential in *P. calkinsi* (Bernal *et al.* 1991). When hydrolyzable GTP was injected into voltage-clamped cells, the calcium current was increased initially by 37±4% (*N*=4; Fig. 7A middle trace and Fig. 7B filled triangles). Five minutes after injection of GTP, the calcium current magnitudes returned to control values (Fig. 7A, right-hand record and Fig. 7B, filled circles). In contrast, injection of GDPβS produces an irreversible decrease in I_{Ca} by 57±8% (*N*=3; Fig. 8).

Guanine nucleotides do not modify the total potassium outward currents in Paramecium calkinsi

Several ionic currents have been described in freshwater *Paramecium*; these include voltage-dependent and calcium-dependent potassium currents (Saimi *et al.* 1988; Eckert and Brehm, 1979; Preston *et al.* 1988). These potassium currents play a critical role in the behavior of the ciliate, presumably through regulation of the repolarization of the cell (Oertel *et al.* 1978; Brehm *et al.* 1978; Satow and Kung, 1979). Because backward swimming behavior and the calcium action potential are complex processes that depend on both calcium and potassium currents, it was necessary to eliminate the possibility that potassium channels present in *Paramecium* were also modified by guanine nucleotides.

Cells were superfused with a solution designed to elicit both the voltage- and the calcium-dependent potassium currents (solution 3, Table 1). In these experiments, sodium was replaced by *N*-methyl-D-glucamine to avoid contamination by the calcium-dependent sodium current (Saimi and Kung, 1980). Cells were held at -40mV and depolarizing pulses were applied to induce the potassium and calcium currents. Injection of GTPγS did not alter the total outward current (Fig. 9). Similar results were found in three other cells.

Discussion

In this paper we have shown that the calcium current in a marine *Paramecium* is carried by one type of calcium channel. The current through this channel can be increased by elevating the extracellular calcium concentration and can be inhibited by organic and inorganic compounds. In addition, we found that the calcium currents in *Paramecium calkinsi* are modulated by guanine nucleotides. In contrast to the decrease in current through mammalian L-type calcium channels after injection of GTPγS or GTP, these compounds increase the calcium current in *Paramecium.* Similarly, GDPβS reduces the calcium current in *Paramecium*, but increases the current through mammalian L-type calcium currents.

The calcium currents of freshwater *Paramecium* do not increase when the extracellular calcium concentration is elevated above $1\text{mmol}\,\text{l}^{-1}$ in intact cells (Satow and Kung, 1979) or after the channels have been incorporated into planar lipid bilayers (Ehrlich *et al.* 1984, 1988). In addition, the single-channel currents from the freshwater species were very small (2pS; Ehrlich *et al.* 1984). The experiments of Deitmer and Machemer (1982) suggested that marine species possess a calcium current whose amplitude could be increased by elevating the extracellular calcium concentration. Our experiments show directly that the currents in a marine species are not saturated under normal conditions. We expect that incorporation of channels from marine *Paramecium* into planar lipid bilayers will allow better analysis of calcium channels than analysis of channels from freshwater species because the magnitude of the calcium currents can be increased when using the channels from the marine species.

Many cells display more than one type of calcium channel (Armstrong and Matteson, 1985; Carbone and Lux, 1984; Deitmer, 1984, 1986; Fedulova *et al.* 1985; Nowycky *et al.* 1985; Tsien *et al.* 1987; Bean, 1989; Hess, 1990). Using voltage protocols that

separate different channel types, we were able to distinguish only one type of calcium channel in *Paramecium* (Fig. 4). This is different from the situation in the ciliate *Stylonychia mytilus,* which has two types of calcium channels that are separable both by voltage and by their location on the cell (Deitmer, 1984, 1986). It is possible, however, that there is a second type of channel in the cilia around the oral groove of *Paramecium.* Because the number of oral cilia is small (<5%), the current contributed by the channels on these cilia would be a small fraction of the total current and would be difficult to distinguish in voltage-clamp recordings.

The calcium currents present in *Paramecium* are increased by addition of guanine nucleotides. The regulation of cell functions by GTP-binding proteins is a well-known phenomenon (Gilman, 1987). These GTP-binding proteins are linked to many different receptors, several K^+ channel types and two different calcium channels (Yatani *et al.* 1987; Dunlap *et al.* 1987; Brown and Birnbaumer, 1988, 1990; Dolphin, 1990). We found that injection of guanine nucleotides into *Paramecium* increased the calcium current, whereas these same compounds decreased the current in vertebrate cells (e.g. Holz *et al.* 1986; Lewis *et al.* 1986; Hescheler *et al.* 1987). To test our system we injected guanine nucleotides into *Helix pomata* neurons. We found that, in these cells, injection of GTPγS decreased the calcium current and injection of GDPβS increased the current (Bernal and Ehrlich, 1990), as expected for a cell that has L-type channels (Dunlap *et al.* 1987).

Our results with GTPγS are similar to the results described by Scott and Dolphin (1989) on T-type calcium channels from rat sensory neurons exposed to GTPγS at concentrations below $6\,\mu mol\,l^{-1}$. They report that subsequent injections of GTPγS decreased the current. When using GTP, we saw an increase in the currents that declined to control levels 5min after injection of GTP. However, we never saw inhibition of the current by either GTP or GTPγS. Our results with GTP lend support to the observation that the cells were healthy in that GTP was metabolized by the cell and produced no long-term effects.

The calcium channels from *Paramecium* do not appear to fit into the categories delineated for the mammalian calcium channels (e.g. Hess, 1990). For example, using voltage protocols that alter the holding potential, the calcium channels from *Paramecium* are more L-type than T-type (Fig. 4). In addition, the calcium currents from *Paramecium* are inactivated by intracellular calcium (Brehm *et al.* 1980), another criterion used to identify channels as L-type rather than T-type. In contrast, the relative effectiveness of inorganic blockers (Table 2), the small size of the single-channel currents (Ehrlich *et al.* 1984) and the effects of injection of guanine nucleotides suggest that the channels are more T-like than L-like. For example, the calcium currents are increased by injection of GTP and GTPγS, a response typical of T-type channels.

The organic blockers also specifically inhibit different calcium channel types. The dihydropyridines block the L-type calcium channels, omega-conotoxin blocks the N-type calcium channels (Bean, 1989; Hess, 1990; Tsien *et al.* 1987), amiloride and octanol may block T-type calcium channels (Tang *et al.* 1988; Llinas and Yarom, 1986) and the naphthalene sulfonamides block calcium channels from freshwater *Paramecium* (Hennessey and Kung, 1984; Ehrlich *et al.* 1988). Only the naphthalene sulfonamides block the calcium channels from marine *Paramecium.* None of the other organic

compounds inhibits the *Paramecium* channels nor do the naphthalene sulfonamides block the calcium channels from vertebrates (Ehrlich *et al.* 1988). From these results it is not possible to fit the channels of *Paramecium* into any existing category, as was the case for the calcium channels of the squid giant synapse (Charlton and Augustine, 1990) and a variety of other invertebrates.

In nerve and cardiac cells, G-proteins modulate calcium channels by coupling to receptors for neurotransmitters and neuromodulators (Holz *et al.* 1986; Lewis *et al.* 1986; Yatani *et al.* 1987; Brown and Birnbaumer, 1988, 1990; Rosenthal *et al.* 1988; Dolphin *et al.* 1988, 1989; Dolphin, 1990). We do not know if there is a specific receptor involved in the activation of G-proteins in *Paramecium*. We speculate that the GTP-binding proteins involved in these processes are related to chemotaxis, because this is the main process that would regulate locomotion in a unicellular organism. For example, amino acid receptors have been identified in *Paramecium* (Preston and Usherwood, 1988) that may be important in chemoreception.

The biochemical processes that must occur before activation of the calcium currents are not known. From behavioral assays, we concluded that increases in cyclic AMP or cyclic GMP concentrations are not necessary events in the cascade that is stimulated by GTP (Bernal *et al.* 1991). Other pathways, however, cannot be excluded.

The observation that guanine nucleotides modulate calcium currents in *Paramecium*, the most primitive organism known to have voltage-dependent calcium channels, suggests that there is a general mechanism that has been maintained throughout evolution for the regulation of voltage-dependent calcium channels. GTP-binding proteins of the RAS family have been identified in *Paramecium* (R. Hinrichsen, personal communication). Whether these GTP-binding proteins are the link between GTP injection and channel regulation remains to be determined.

Potassium channels can also be regulated by G-proteins in a variety of cells (Brown and Birnbaumer, 1990). We did not detect any change in the total outward potassium currents in *Paramecium* after injections of GTPγS. These channels may be regulated by other intracellular pathways unrelated to the GTP-binding proteins.

In conclusion, we have shown that a marine *Paramecium* has a single type of calcium channel that can be modulated by guanine nucleotides. This primitive calcium channel may be a precursor to the whole family of calcium channels. Speculating further, different properties of the channel may have split off into the different subtypes of calcium channel as evolution progressed. Although not identified yet, the GTP-binding proteins found in *Paramecium*, and presumably associated with modulation of the channel, may also be precursors of the G-protein family found in mammalian cells.

We gratefully acknowledge Drs Susan Barry, Ilya Bezprozvanny and Milton Charlton for helpful comments on the manuscript. This work was supported by grant GM39029 from the NIH (B.E.E.) and by a fellowship from the American Heart Association, Connecticut Affiliate (J.B.). B.E.E. was a Pew Scholar in the Biomedical Sciences.

References

ARMSTRONG, C. M. AND MATTESON, D. R. (1985). Two distinct populations of calcium channels in a clonal line of pituitary cells. *Science* **202**, 65–67.

BARRY, S., BERNAL, J. AND EHRLICH, B. E. (1991). Calcium currents in *Paramecium* are blocked by antimalarial drugs. *Biophys. J.* **59**, 277a.

BEAN, B. P.(1989). Classes of calcium channels in vertebrate cells. *A. Rev. Physiol.* **51**, 367–384.

BERNAL, J. AND EHRLICH, B. E. (1989). Calcium channel modulation by G-proteins in marine *Paramecium*. *J. gen. Physiol.* **94**, 5a.

BERNAL, J. AND EHRLICH, B. E. (1990). G-proteins modulate calcium currents in *Paramecium* and *Helix* neurons. *Biophys. J.* **57**, 516a.

BERNAL, J. AND EHRLICH, B. E. (1991). Guanine nucleotides modulates calcium currents in marine *Paramecium*. *Biophys. J.* **59**, 370a.

BERNAL, J., KELSEY, A. M. AND EHRLICH, B. E. (1991). GTPγS increases the duration of the backward swimming behavior and the calcium action potential in marine *Paramecium*. *J. exp. Biol.* **155**, 505–518.

BREHM, P., DUNLAP, K. AND ECKERT, R. (1978). Calcium-dependent repolarization in *Paramecium*. *J. Physiol., Lond.* **274**, 639–654.

BREHM, P., ECKERT, R. AND TILLOTSON, D. (1980). Calcium-mediated inactivation of calcium current in *Paramecium*. *J. Physiol., Lond.* **306**, 193–203.

BROWN, A. M. AND BIRNBAUMER, L. (1988). Direct G-protein gating of ion channels. *Am. J. Physiol.* **254**, H401–H410.

BROWN, A. M. AND BIRNBAUMER, L.(1990). Ionic channels and their regulation by G-proteins subunits. *A. Rev. Physiol.* **52**, 197–213.

CARBONE, E. AND LUX, H. D.(1984). A low voltage-activated calcium conductance in embryonic chick sensory neurons. *Biophys. J.* **46**, 413–418.

CHARLTON, M. P. AND AUGUSTINE, G. J. (1990). Classification of presynaptic calcium channels at the squid giant synapse: neither T-, L-, nor N-type. *Brain Res.* **525**, 133–139.

DEITMER, J. W. (1984). Evidence for two voltage-dependent calcium currents in the membrane of the ciliate *Stylonychia*. *J. Physiol., Lond.* **355**, 137–159.

DEITMER, J. W.(1986). Voltage dependence of two inward currents carried by calcium and barium in the ciliate *Stilonychia. J. Physiol., Lond.* **380**, 551–574.

DEITMER, J. W. AND MACHEMER, H. (1982). Osmotic tolerance of calcium-dependent excitability in the marine ciliate *Parameciumcalkinsi*. *J. exp. Biol.* **97**, 311–324.

DOLPHIN, A. C. (1990). G-protein modulation of calcium currents in neurons. *A. Rev. Physiol*. **52**, 243–255.

DOLPHIN, A. C., SCOTT, R. H. AND WOOTTON, J. F. (1989). Photo-release of GTPγS inhibits the low threshold calcium channel current in cultured rat dorsal root ganglia (DRG). *J. Physiol., Lond.* **410**, 16.

DOLPHIN, A. C., WOOTTON, J. F., SCOTT, R. H. AND TRENTHAM, D. R. (1988). Photoinactivation of intracellular guanosine triphosphate anologues reduces the amplitude and slows the kinetics of voltage-activated calcium currents in sensory neurones. *Pflügers Arch.* **411**, 628–636.

DUNLAP, K. (1977). Localization of calcium channels in *Paramecium caudatum. J. Physiol., Lond*. **271**, 119–133.

DUNLAP, K., HOLZ, G. G. AND RANE, G. R. (1987). G-proteins as regulators of ion channel function. *Trends Neurosci*. **10**, 241–244.

EHRLICH, B. E., FINKELSTEIN, A., FORTE, M. AND KUNG, C. (1984). Voltage-dependent calcium channels from *Paramecium* cilia incorporated into planar lipid bilayers. *Science* **225**, 427–428.

EHRLICH, B. E., JACOBSON, A. R., HINRICHSEN, R., SAYRE, L. M. AND FORTE, M. A. (1988). *Paramecium* calcium channels are blocked by a family of calmodulin antagonists. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5718–5722.

ECKERT, B. E. AND BREHM, P. (1979). Ionic mechanisms of excitation in *Paramecium. A. Rev. Biophys. Bioeng*. **8**, 353–383.

FEDULOVA, S. A., KOSTYUK, P. G. AND VESELOVSKY, N. S.(1985). Two types of calcium channels in the somatic membrane of newborn rat dorsal root ganglion neurons. *J. Physiol., Lond.* **359**, 431–446.

GILMAN, A. G. (1987). G-proteins, transducers of receptor-generated signals. *A. Rev. Biochem*. **56**, 615–649.

HAGA, N., FORTE, M., SIAMI, Y. AND KUNG, C. (1982). Microinjection of cytoplasm as a test of complementation in *Paramecium*. *J. Cell Biol.* **96**, 1072–1080.

HAGIWARA, S. AND BYERLY, L.(1981). Calcium channel. *A. Rev. Neurosci.* **4**, 69–127.

HENNESSEY, T. AND KUNG, C. (1984). An anticalmodulin drug, W-7, inhibits the voltage-dependent calcium current in *Parameciumcaudatum*. *J. exp. Biol.* **110**, 169–181.

HESCHELER, J., ROSENTHAL, W., TRAWTWEIN, W. AND SCHULTZ, G. (1987). The GTP-binding proteins, Go, regulates neuronal calcium channels. *Nature* **325**, 445–447.

HESS, P. (1990). Calcium channels in vertebrate cells. *A. Rev. Neurosci.* **13**, 337–356.

HIDAKA, H., YAMAKI, T., TOTSUKA, T. AND ASANO, M. (1979). Selective inhibitors of Ca^{2+}-binding modulator of phosphodiesterase produce vascular relaxation and inhibit actin-myosin alteration. *Molec. Pharmac.* **15**, 15–59.

HOLZ, G. G., RANE, S. G. AND DUNLAP, K. (1986). GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature* **319**, 670–672.

LEVITAN, I. B. (1988). Modulation of ion channels in neurons and other cells. *A. Rev. Neurosci.* **11**, 119–136.

LEVITAN, I. B., AND KACZMAREK, L. K. (1987). Ion currents and ion channels: Substrate for modulation. In *Neuromodulation* (ed. L. Kaczmarek and I. B. Levitan), pp. 18–30. Oxford: Oxford University Press.

LEWIS, D. L., WEIGHT, F. F. AND LUINI, A. (1986). A guanine nucleotide-binding protein mediates the inhibition of voltage-dependent calcium current by somatostatin in a pituitary cell line. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9035–9039.

LLINAS, R. AND YAROM, Y. (1986). Specific blockade of the low threshold calcium channel by high molecular weight alcohols. *Soc. Neurosci. Abstr.* **12**, 174.

MACHEMER, H. AND OGURA, A. (1979). Ionic conductances of membranes in ciliated and deciliated. *J. Physiol., Lond.* **296**, 46–60.

NOWYCKY, M., FOX, A. AND TSIEN, R. W. (1985). Three types of neuronal calcium channels with different calcium agonist sensitivity. *Nature* **316**, 440–443.

OERTEL, D., SCHEIN, S. AND KUNG, C. (1978). A potassium conductance activated by hyperpolarization. *J. Membr. Biol.* **43**, 169–185.

PRESTON, R. R.(1990). A magnesium current in *Paramecium*. *Science* **250**, 285–287.

PRESTON, R. R. AND USHERWOOD, P. N. R. (1988). L-Glutamate induces membrane hyperpolarization and behavioral responses in *Parameciumtetraurelia. J. comp. Physiol.* **164**, 75–82.

REUTER, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* **301**, 569–574.

ROSENTHAL, W., HESCHELER, J., TRAUTWEIN, W. AND SCHULTZ, G.(1988). Control of voltage-dependent Ca^{2+} channels by G-protein-coupled receptors. *FASEB J.* **2**, 2784–2790.

SAIMI, Y. AND KUNG, C. (1980). A calcium-induced sodium current in *Paramecium*. *J. exp. Biol.* **88**, 305–325.

SAIMI, Y., MARTINAC, B., GUSTIN, M. C., CULTBEEEERTSON, M. R., ADLER, J. AND KUNG, C. (1988). Ion channels in *Paramecium*, yeast and *Escherichia coli*. *Cold Spring Harbor Symp. quant. Biol.* **8**, 667–673.

SATOW, Y. AND KUNG, C.(1979). Voltage sensitive calcium channels and the transient inward current in *Parameciumtetraurelia*. *J. exp. Biol.* **78**, 149–161.

SCHEIN, S. J., BENNET, M. V. L. AND KATZ, G.(1976). Altered calcium conductance in pawns, behavioral mutant of *Paramecium aurelia*. *J. exp. Biol.* **65**, 699–724.

SCOTT, R. H. AND DOLPHIN, A. C. (1989). G-protein regulation of neuronal voltage-activated calcium currents. *Gen. Pharmac.* **20**, 715–720.

TANG, C. M., PRESSER, F. AND MORAD, M. (1988). Amiloride selectively block the low threshold (T) calcium channel. *Science* **240**, 213–215.

TSIEN, R. W.(1983). Calcium channels in excitable cell membranes. *A. Rev. Physiol.* **45**, 341–358.

TSIEN, R. W., HESS, P., MACCLESKEY, E. W. AND ROSEMBERG, R. L. (1987). Calcium channels, mechanisms of selectivity, permeation and block. *A. Rev. biophys. Chem.* **16**, 265–290.

YATANI, A., CODINA, J., IMOTO, Y., REEVES, J. P., BIRNBAUMER, L. AND BROWN, A. M. (1987). A G-protein directly regulates mammalian cardiac calcium channels. *Science* **238**, 1288–1292.