J. Membrane Biol. 112, 79–89 (1989)

The Journal of
Membrane Biology

Calcium-Dependent Potassium Channel in *Paramecium* Studied Under Patch Clamp

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Summary. We have studied a class of Ca_i^{2+}-dependent K channels in inside-out excised membrane patches from *Paramecium* under patch clamp. Single channels had a conductance of 72 ± 9.0 pS in a solution containing 100 mM K^+. The channels were selective for K^+ over Rb^+ with the permeability ratio of 1 : 0.56, and over Na^+, Cs^+ or NH_4^+ with a ratio 1 : <0.1. The channel activity was dependent on Ca_i^{2+}, which was applied to the cytoplasmic side; the Ca_i^{2+} concentration for the half maximal activation was 2 μM. The Hill coefficient for the Ca_i^{2+} dependence of the channel activity was 2.58, indicating that more than two Ca_i^{2+} bindings are necessary for full activation. Unlike most Ca_i^{2+}-dependent K channels in other organisms, the channels in *Paramecium* were slightly more active upon hyperpolarization than upon depolarization. The voltage dependence was fitted to a Boltzmann curve with 41.2 mV per *e*-fold change in channel activity. While a high Ca_i^{2+} concentration activated the channels, it also irreversibly reduced the channel activity over time. The decay of channel activity occurred faster at higher Ca_i^{2+} concentrations. Quaternary ammonium ions suppressed ion passage through the channel; more highly alkylated quaternary ammonium ions were more efficient in blocking. Ba_i^{2+} and Ca_i^{2+} were relatively ineffective in blockage. It was concluded that these Ca_i^{2+}-dependent K channels in *Paramecium* are different from the previously described Ca_i^{2+}-dependent K channels, and are perhaps of a novel class.

Key Words *Paramecium* · patch clamp · K channels · Ca_i^{2+} dependence · hyperpolarization activated · run-down

Introduction

Among many separate ion currents found under two-electrode voltage clamp in *Paramecium* (Machemer, 1988), there are two Ca_i^{2+}-dependent K currents, one upon depolarization (Satow & Kung, 1980), and the other upon hyperpolarization (Richard, Saimi & Kung, 1986; Preston, Saimi & Kung[1]). In order to understand properties of ion channels in *Paramecium,* single-channel activities have been recorded using the patch-clamp method (Martinac et al., 1986, 1988). One of them is a 150-pS K channel in *Paramecium* that is activated by Ca_i^{2+} and depolarization and similar to those found in other organisms (BK channels; Latorre, 1986; Blatz & Magleby, 1987). Because of its voltage sensitivity, this 150-pS K conductance appears to represent the Ca_i^{2+}-dependent K conductance which is observed upon depolarization of the whole cell.

We report here another type of Ca_i^{2+}-dependent K channels from *Paramecium* recorded with the patch-clamp method. The channels are slightly more active upon hyperpolarization than upon depolarization. In addition, the channel activity declines irreversibly in high Ca_i^{2+} concentrations over time.

Materials and Methods

Cell Culture

A trichocyst-non-discharge mutant, *nd-6,* of *Paramecium tetraurelia* was used. The *nd-6* mutant has no obvious electrical membrane property different from that of the wild type (51s; Y. Saimi, *unpublished;* R.R. Preston & Y. Saimi, *unpublished*). This mutation has been used as the background for various membrane-current mutants (Richard et al., 1986; Saimi, 1986). Cells were grown at 31°C in casaminoacid media, similar in composition to that used by Forte, Hennessey and Kung (1986). One medium consisted of 0.3 g/l casaminoacids, 4 mM KCl, 0.44 mM Na citrate, 0.1 mM $CaCl_2$, 0.08 mM $MgCl_2$, 5 mg/l stigmasterol, some trace metals, 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), adjusted to pH 7.0 with Tris[hydroxymethyl]aminomethane (Tris) base. An alternative medium comprised 0.1 g/liter casaminoacids, 0.1 g/liter glucose, 0.5 mM K_2HPO_4, 0.2 mM $MgSO_4$, 0.2–0.5 mM $CaCl_2$, 5 mg/liter stigmasterol, 7.5 mg/liter phenol red, 5 mM HEPES, adjusted to pH 7.0–7.4 with ~3 mM NaOH. Both media were bacterized with *Aerobacter aerogenes* and then inoculated with *Paramecium* the day before the experiment. Paramecia were kept in log-phase growth; when necessary, more bacterized medium was added to the culture. These two media produced indistinguishable channel recordings.

[1] Preston, R.R., Saimi, Y., Kung, C. Two potassium currents activated upon hyperpolarization of *Paramecium tetraurelia. (submitted)*

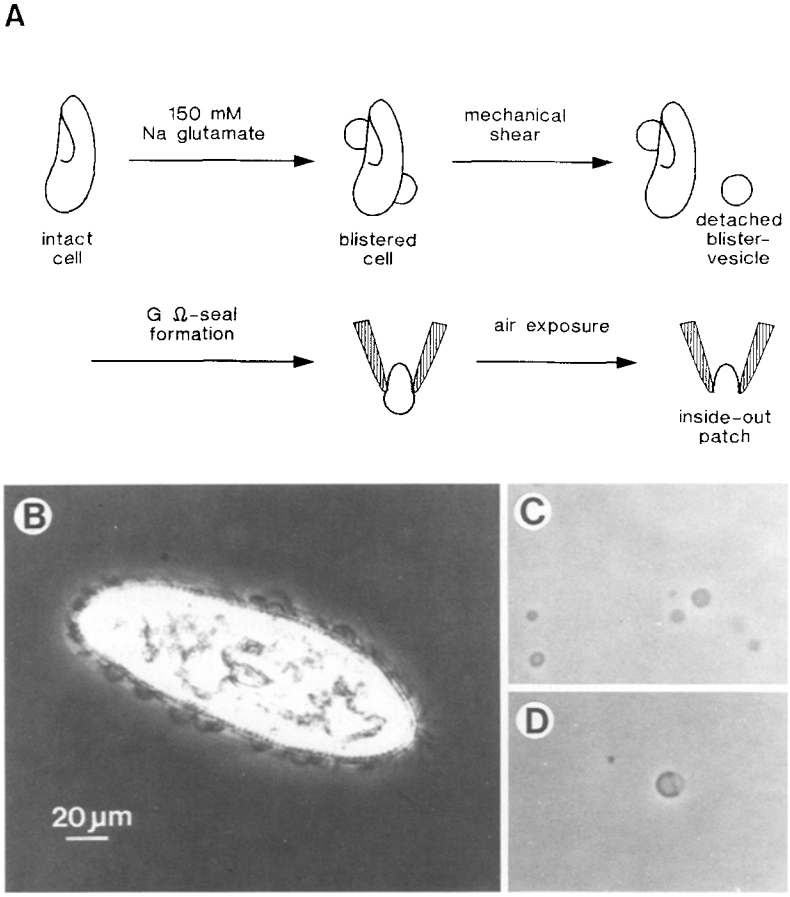

Fig. 1. (*A*) A scheme of the method for the isolation of the plasma membrane from *Paramecium* and giga-seal formation. (*B*) (*C*), (*D*) Light micrographs of a "blistered" paramecium and isolated blisters. (*B*) Blisters were induced by soaking cells in the "blistering" solution for less than 10 min. (*C*) Blisters were detached from the body by mechanical shearing and then transferred to the chamber for giga-seal formation. (*D*) A vesicle derived from the blister underwent separation of its outer and inner membranes. Giga-seals with the patch pipette were formed on the outer membrane, either peeled or not

Isolation of Plasma Membrane

Attempts to patch clamp intact, or live deciliated *Paramecium* were not successful (B. Martinac & Y. Saimi, *unpublished*). Instead we prepared isolated plasma membranes as depicted in Fig. 1*A*. Paramecia were withdrawn from the culture and put in a solution that induced membrane blisters (blistering solution). The blistering solution contained 100–150 mM Na glutamate, 10^{-5} M free Ca^{2+} (buffered with 1 mM ethylene glycol *bis*-(*p*-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA)), 5 mM HEPES, 0.01 mM ethylenediamine-tetraacetic acid (EDTA). Within 10 min the cells, though alive, were covered with blisters of 10–20 μm in diameter (Fig. 1*B*). Typically, we took those live cells and stripped blisters from the body by forcing the cell through a glass micropipette drawn to a bore slightly narrower than the cell diameter. The isolated membrane vesicles derived from the blisters were immediately transferred to another chamber filled with the "giga-seal forming" solution (*see below*) for patch-clamp recording (Fig. 1*C*).

Giga-Seal Formation

A giga-seal (5–20 GΩ) was formed in a similar manner to that by Hamill et al. (1981). It was formed between the tip of a glass pipette (Boralex, Rochester, NY) and the surface membrane of the isolated blister vesicle by applying light suction. Most membrane vesicles, if not all, apparently consisted of an inner and an outer membrane, presumably with the plasma membrane outside. When placed in a solution slightly hypotonic to the blistering solution, the outer layer of the vesicle often peeled from the inner membrane (Fig. 1*D*); in these cases giga-seals were formed on the outer membrane. We were not able to detect any channel activity from the inner membrane (*unpublished observation*). The inner membrane might be the alveolar membrane (*see* Jurand & Selman (1969) for *Paramecium* cortical structures) that was removed along with the plasma membrane during mechanical shearing of blisters.

During giga-seal formation, the blisters were kept in a solution containing 10 mM $MgCl_2$ and about 10^{-8} M Ca^{2+} buffered with 1 mM EGTA. After seal formation, the membrane patch was excised from the membrane vesicle by air exposure. All the above processes, from blistering of the cell to excision of the membrane patch, required less than 20 min.

Solutions

Experimental bath solutions consisted of 100 mM K^+, 10^{-4} to 10^{-8} M free Ca^{2+}, 5 mM HEPES, 0.01 mM EDTA with 20 mM Cl^- and approximately 80 mM L-glutamic acid at pH ~7.0 adjusted with L-glutamic acid. Ca^{2+} (50, 326, 828, 941 or 979 μM total) was added and buffered with 1 mM EGTA to give rise to a final free Ca^{2+} concentration of 10^{-8}, 10^{-7}, 10^{-6}, 3.2×10^{-6} or 10^{-5} M, respectively. The free-Ca^{2+} concentrations were calculated according to the method by Portzehl, Caldwell and Rüegg (1964),

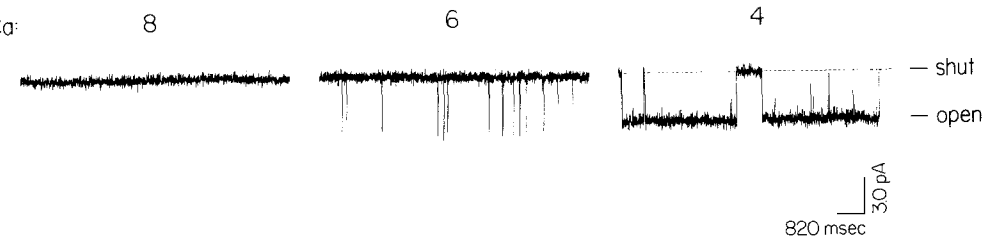

Fig. 2. Single-channel activity in 100 mM K solutions with three different Ca^{2+} concentrations. Little activity was observed at 10^{-8} M Ca^{2+} (left). Only some short openings were detected at 10^{-6} M Ca^{2+} (middle) in this case. The channel was very active at 10^{-4} M Ca^{2+} (right) showing long openings. There was no sign of a second channel in this patch. The patch potential was held at −50 mV throughout. Ca^{2+} concentration was changed in the order of 10^{-6}, 10^{-8} and then 10^{-4} M

assuming an apparent binding constant of $10^{6.68}$ M^{-1}. Solutions of 10^{-4} M Ca^{2+} contained no EGTA. In most cases, the bath solutions also included protease inhibitors such as leupeptin, aprotinin and antipain at 1 μg/ml to avoid possible proteolysis (Chad & Eckert, 1986). A mixture of these three protease inhibitors had a slight, but readily reversible effect on the channel kinetics. They induced more short closures, and thus reduced the summed current from multiple channels by about 5%. To inhibit possible oxidation, a reducing agent, dithiothreitol (DTT), was included in most solutions at 1 mM without causing any alterations in the channel activity. These chemicals were added to the blistering solution in some cases. Other chemicals, such as quaternary ammonium ions, were added to the bath solution when needed. Other monovalent cations replaced K^+ in the bath solution for the ion selectivity experiments.

Pipettes were filled with solutions similar to the bath solutions: 100 mM K^+, a low concentration of free Ca^{2+} (10^{-5} to 10^{-8} M), 5 mM HEPES, 0 or 10 mM Mg^{2+}, 0.01 mM EDTA with 20 mM Cl^- and approximately 80 mM L-glutamic acid, adjusted to pH ~7.0 with L-glutamic acid. No protease inhibitor or DTT was added to the pipette. The resistance of the patch electrode was between 10 and 20 MΩ. In the experiments where the ion selectivity of the channel was determined, Mg^{2+} was omitted from the pipette solution.

Perfusion

As described below, the channel activity declined rapidly in the continued presence of Ca^{2+} concentrations higher than 10^{-6} M. In order to minimize these effects of Ca^{2+}, we devised a special chamber to exchange solutions rapidly. A main chamber (about 200 μl in volume) was connected to a small canal with a slanted bed (5–10 mm long; ca. 2 mm wide; 1–2 mm deep; less than 40 μl in volume). The other end of the canal was widened, where several hoses were placed to guide solutions from separate reservoirs. During the experiment, the pipette with an excised membrane patch was moved into the canal. When a solution was switched on, fluid ran through the canal into the main chamber; thus the solution exchange was very rapid (approximately 2 sec to clear the canal).

Recording and Data Processing

Recordings were made with a List EPC-7 or a Dagan 8900 patch-clamp preamplifier. The reference level for the membrane potential was set under symmetric K^+ concentrations. For experiments to determine the ion selectivity of the channel, both the bath and the pipette were filled with exactly the same solution containing 100 mM K^+ and 10^{-5} M Ca^{2+} without Mg^{2+}. Under this condition, the potential of the membrane patch was corrected to null membrane current, and was defined as the zero potential of the membrane patch. The reference Ag-AgCl electrode was encased in a glass capillary filled with 1 or 3 M KCl. This capillary was plugged with agar at the other end and immersed in the bath. Membrane potentials are presented as the bath voltage with respect to the pipette voltage, so that they refer to the potential that the channel experiences in vivo, provided the membrane patch was in an inside-out configuration.

Data were stored on a Gould FM tape recorder (Model 6500) or a PCM-1-video tape system. The data were digitized off-line with an INDEX System at sampling rates of 1 to 10 kHz after refiltration through an 8-pole Bessel filter (at 1 kHz). In most cases, channel activity is expressed as *NPo* (integral of current during a certain time period divided with a single-channel current amplitude). All experiments were done at room temperature (20–23°C).

Results

Single-Channel Activities

Ca_i^{2+}-dependent K channels were encountered in more than 30% of the patches examined. Excluding empty patches, the maximal number of current steps was 17 ± 9.1 (mean ± SD; n = 19), suggesting that a patch contained many Ca_i^{2+}-dependent K channels. There were several cases in which only one or a few channels in a patch were detected.

After excision of a membrane patch with one or a few channels and replenishment of the bath with a solution containing 10^{-8} M Ca_i^{2+} and no Mg_i^{2+}, the patch remained silent (Fig. 2, left). When the Ca_i^{2+} concentration was raised to 10^{-6} M, frequent, short openings were observed (Fig. 2, middle). A higher Ca_i^{2+} concentration (10^{-4} or 10^{-5} M) further activated channels (Fig. 2, right). The opening probability at 10^{-4} M Ca_i^{2+} was as high as 0.8 at −50 mV. However, in the continued presence of high Ca_i^{2+} concentrations, the channel activity declined abruptly to a state where only infrequent, short

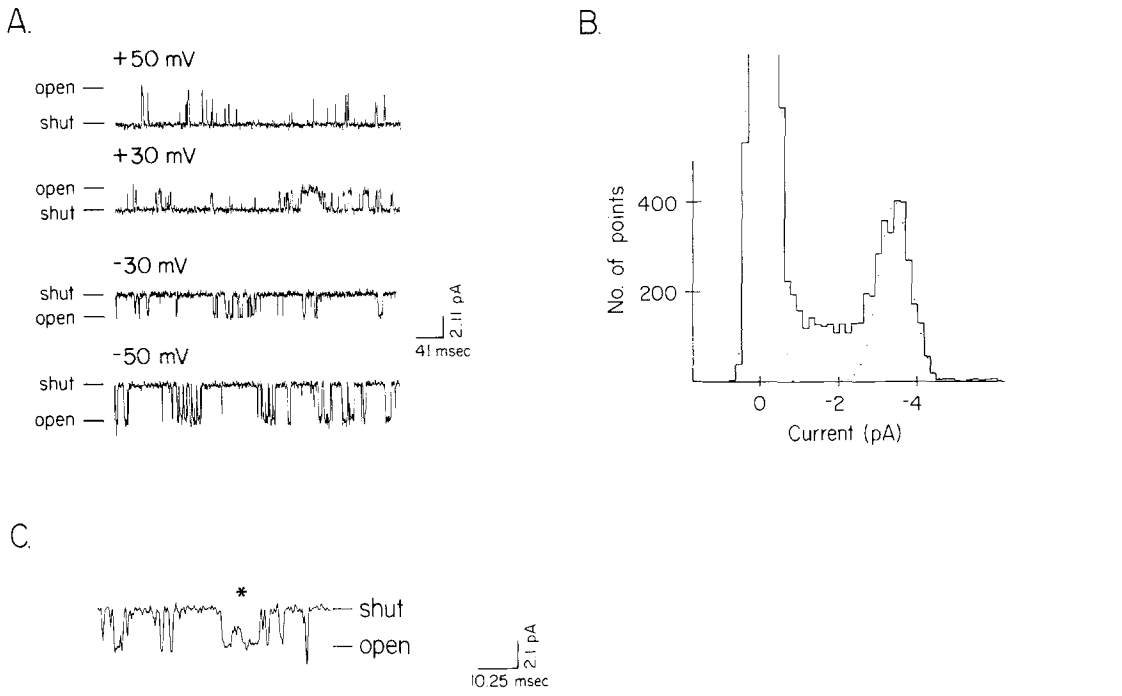

Fig. 3. A family of current traces at various membrane voltages in the 100 mM K solution with 10^{-5} M Ca^{2+}. (*A*) Channel activities at various voltages indicated above each trace. (*B*) An amplitude histogram made from a current trace at −50 mV, a segment of which is shown in (*A*). The left peak indicates the closed state, and the right one the open state. The dotted line indicates a sum of two Gaussian curves fitted to the peaks, leaving the intermediate region unfitted. (*C*) A current trace from another patch at −50 mV. The asterisk indicates apparent transitions between the predominant open state and another open state

openings were observed; thereafter, the opening probability became as low as 0.05 or lower. A similar step-wise decay of the channel activity was seen in patches of multiple channels with approximately the same time course (*see below*). Though the mode of the channel activity changed in high Ca_i^{2+} concentrations, the current amplitude of the single channel was not affected by the decay of the channel activity (2 ± 3% change after reduction in channel activity; $n = 6$).

Single-Channel Conductance

Figure 3*A* shows typical current traces at various voltages in the presence of 10^{-5} M Ca_i^{2+} from channels whose activity declined already. In most cases we examined, there was one main conducting state that was most often visited (predominant state). This is evident in the amplitude histogram (Fig. 3*B*) that can be fitted to a sum of Gaussian curves. The middle portion of the curve did not fit well, perhaps because of unresolved short events. There were also other open states of different conductances; among others, there were open states of 1/3 and 2/3 of the predominant conductance. There appeared to be direct transitions between the predominant open state and those open states (Fig. 3*C*, asterisk), suggesting that those might be substates of the predominant conductance. Although a statistical analysis was necessary to confirm that, an uncertain number of channels in the patch prevented further analysis because of the low open probability. We measured current amplitudes through single channels dwelling longer than 2 msec in the predominant conducting state at various voltages in the presence of 10^{-5} M Ca_i^{2+}. The single-channel currents are plotted against voltages in Fig. 4*A* (circles). The mean conductance in the presence of 100 mM K^+ was 72 ± 9.0 pS ($n = 7$).

Ion Selectivity

The ion selectivity of the channel was determined by replacing only K^+ of the bath solution with another monovalent cation at the same concentration. The reversal potential estimated by interpolation to zero current in the Rb^+ solution was +14.6 ± 0.41 mV (Fig. 4*A*, squares), which suggested a Rb^+-to-K^+ permeability ratio of 0.56, according to the Goldman-Hodgkin-Katz equation for the bi-ionic condition. The single-channel current in Rb^+ solution showed slight rectification upon depolarization,

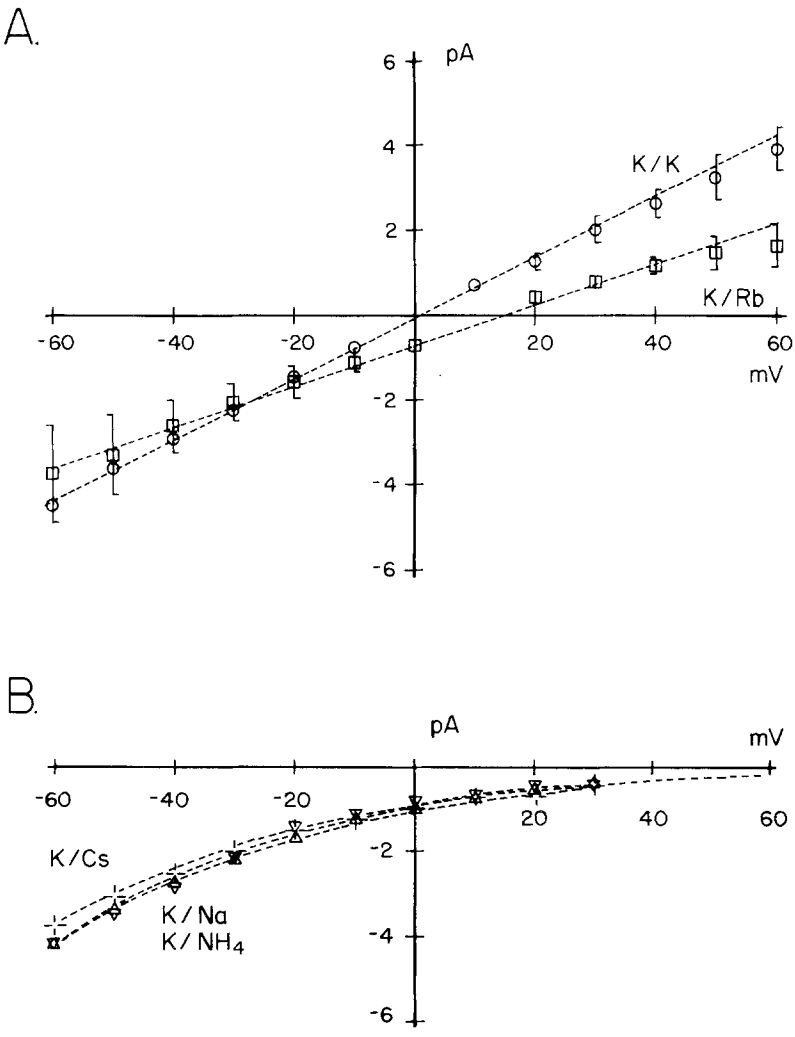

Fig. 4. Unit current and membrane voltage relationship in the presence of 10^{-5} M Ca^{2+}. (*A*) Single-channel currents (mean ± SD) from five patches were plotted against membrane voltages. Circles represent those for the symmetric K solution (100 mM K^+ in both pipette and bath) with a dashed line indicating the result of a linear regression. The slope conductance was 72 pS. Squares represent unit currents for the Rb^+ solution (100 mM Rb^+ in the bath and 100 mM K^+ in the pipette). The linear regression for points lower than +40 mV is shown. The intercept of the voltage axis was +14.6 mV. (*B*) Single-channel currents in 100 mM Na^+ (triangles), NH_4^+ (inverted triangles) or Cs^+ (cursors) in the bath solution (with 100 mM K^+ in the pipette) were always inward within the voltage range of −60 to +30 mV. No outward current was detected up to +60 mV. Though not shown for clarity, standard deviations were similar to those in (*A*)

which was also seen to a lesser extent in K^+ solution. When the bath was perfused with a solution containing Na^+, Cs^+ or NH_4^+, we were not able to detect a reversal potential within the range of +60 to −60 mV (Fig. 4*B*), indicating that permeabilities for these alkali metal ions were at least 10 times smaller than that for K^+.

Ca^{2+} Dependence of Channel Activity: Activation and Inactivation

In the solution containing 10^{-8} M Ca_i^{2+}, there were only a few, very short channel openings. When the chamber was perfused with a solution of 10^{-5} or 10^{-4} M Ca_i^{2+}, there was an increase in the activity of channels (Fig. 5). This channel activity, however, gradually declined during exposure to high Ca_i^{2+} (*see below* for details).

Because of this inactivation, we designed the following protocol in order to examine the Ca_i^{2+} dependence of the channel. A membrane patch was first kept in the low Ca_i^{2+} solution (10^{-8} M) and then intermittently exposed to different solutions with

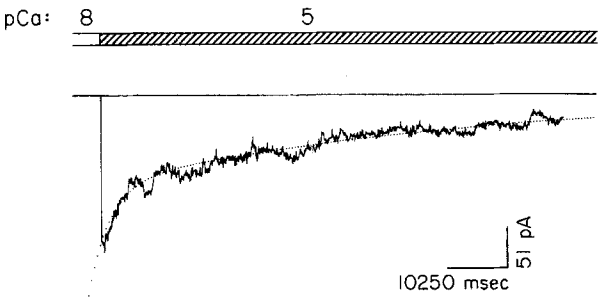

Fig. 5. Ca^{2+}-dependent activation and inactivation of channels. The patch was subjected to a solution of 10^{-8} M Ca^{2+} and then to that of 10^{-5} M Ca^{2+}. The patch voltage was maintained at −50 mV. The data were filtered first at 1 kHz sampling rate and then every 50 points were averaged. Sum of two exponential curves (dotted line) fitted by eye to the curve of these averages is shown

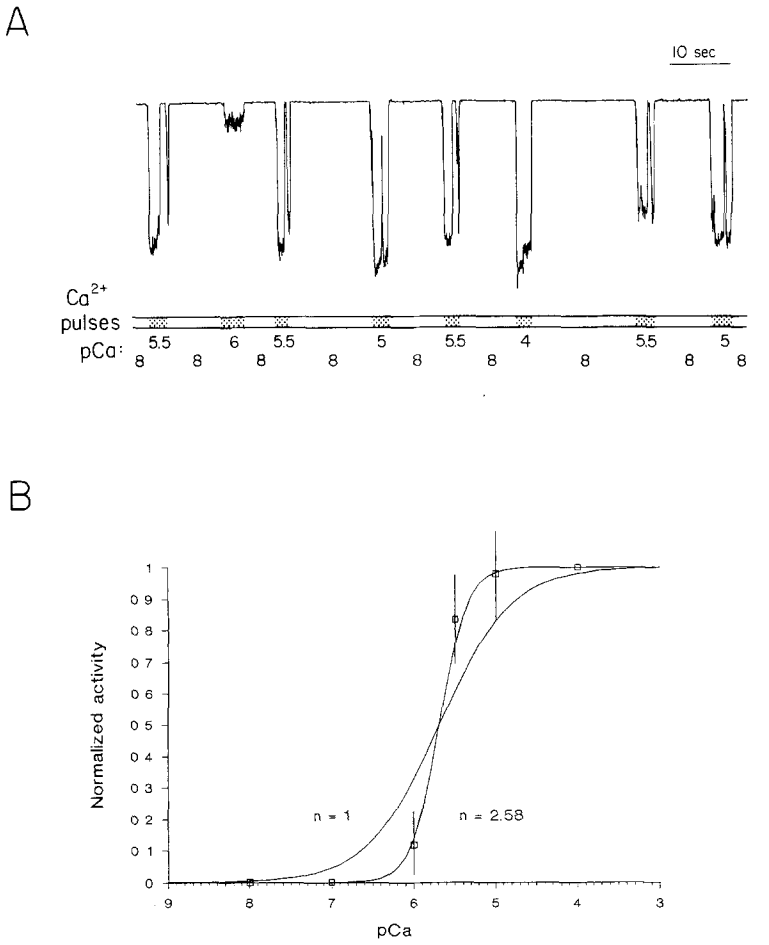

Fig. 6. Channel activity at various Ca^{2+} concentrations. (*A*) The patch was bathed in and continually superfused with the solution of 10^{-8} M Ca^{2+}, except for brief exposures to solutions with higher Ca^{2+} concentrations. The course of the experiment is diagramed beneath the current trace, while the membrane potential was kept at −50 mV in the presence of 100 mM K^+ in both bath and pipette. The perfusion caused some "ringings" of the Ca^{2+} concentration, resulting in the current fluctuation at the time of high Ca^{2+} exposure in some cases. (*B*) Ca^{2+} dependence of channel activity averaged from five patches. The current amplitude averaged for 1 sec at various Ca^{2+} concentrations obtained from experiments such as in (*A*) were first corrected for the decay of activity according to the frequently checked current amplitude of 3.2×10^{-6} M Ca^{2+}, and then normalized to that at 10^{-4} M Ca^{2+} for each patch. Finally normalized current amplitudes from five patches (except for 10^{-7} M where n was 2) were averaged and are plotted as the normalized activity. These points (mean ± SD) were best fitted with a sigmoidal curve (labeled as $n = 2.58$) with Eq. (1) described in the text. A curve calculated for $n = 1$ with the same mid-point is also drawn for comparison

10^{-4} to 10^{-7} M Ca_i^{2+} for 5 to 10 sec. Such exposures to high Ca_i^{2+} were followed by returns to 3.2×10^{-6} M Ca_i^{2+} to monitor the remaining channel activity (Fig. 6*A*). As in the case of the whole-cell voltage clamp, the activity of the channels was estimated from the current amplitude; for this purpose, only patches with at least 10 channels were analyzed. The Ca^{2+} effects on the channel activity were estimated at −50 mV in order to minimize possible contamination of the activity of Ca_i^{2+}-, depolarization-dependent channels (perhaps BK channels, though rarely encountered). Furthermore, after the series of Ca^{2+} concentrations, decays of the channel activity to very low levels were confirmed in the presence of a high Ca_i^{2+} concentration, suggesting that at least most of the current was through the channels of interest. The normalized channel activities were averaged for five patches (except two patches for 10^{-7} M), and are plotted against Ca_i^{2+} concentrations in Fig. 6*B*. The data points in the middle range (10^{-5} to 10^{-7} M) were used to extract the Hill coefficient ($n = 2.58$) and the half-saturation concentration ($C_o = 2 \times 10^{-6}$ M), according to the equation as follows:

$$\text{normalized activity} = C^n/(C^n + C_o^n) \qquad (1)$$

where C is the Ca_i^{2+} concentration (in M). The best fitted curve (marked as $n = 2.58$) and also the curve with $n = 1$ are drawn, showing that the experimental data clearly deviated from the curve with a Hill coefficient of 1. Because of the rapid decay of the channel activity in the presence of high Ca_i^{2+}, the estimates for the current amplitude at higher Ca_i^{2+} concentrations were, if any, underestimated; this would imply that the curve could be steeper than that with n being 2.58. Thus, this result suggested that there are more than two Ca_i^{2+} bindings for full channel activation. More than two Ca_i^{2+} bindings are commonly found for Ca_i^{2+}-dependent K channels (Magleby & Pallotta, 1983; Golowasch, Kirkwood & Miller, 1986).

Table. Ca^{2+}-dependent decay of channel activity

pCa	1st component		2nd component		n
	τ (sec)	Relative amplitude	τ (sec)	Relative amplitude	
4	5.6 ± 1.3	0.41 ± 0.23	65.7 ± 29.7	0.59 ± 0.23	3
5	11.4 ± 9.6	0.32 ± 0.20	102.7 ± 34.3	0.68 ± 0.20	4
6	721 ± 270	—	—	—	5
8	2344 ± 1437	—	—	—	4

Exponential components for decay of the channel activity at 10^{-4} and 10^{-5} M Ca^{2+} were directly extracted by fitting current traces to two exponential curves (*see* Fig. 5). The numbers for 10^{-6} and 10^{-8} M Ca^{2+} were obtained with a different procedure (*see* text).

Ca^{2+} Dependence of Decay of Channel Activity

Immediately after channel activation in a high Ca_i^{2+} solution, this activity started to decline. When the Ca_i^{2+} concentration was lowered to, e.g., 10^{-8} M, the channels became quiescent. These silent channels could be re-activated by high Ca_i^{2+} but only to the level before quiescence, and not to the level they attained upon first exposure to high Ca_i^{2+}. Thus the decay of channel activity was irreversible. When the channels were subjected to high Ca_i^{2+} for an extended period, their activity declined within 10 min to a very low level, which persisted over half an hour. The channel activity did not increase further from the level in 10^{-4} M Ca_i^{2+} even when the Ca_i^{2+} concentration was raised to a much higher level, e.g., 1 mM, which indicated that the decline of channel activity was not due to a simple shift in Ca_i^{2+} sensitivity.

The decay of the total current could be fitted with two exponential curves by eye (Fig. 5, dotted line); a single exponential fitting was unsatisfactory. The extracted time constants and relative amplitudes of both components are listed in the Table (entries for pCa = 4 and 5). There was, however, invariably residual channel activity remaining over half an hour. Though it is possible that there were more than two decaying components, perhaps such residual activity represented the basal activity of the channel. The residual channel activities also showed Ca_i^{2+} dependence. A similar decay of channel activity was observed during depolarization. Addition of 10 mM Mg_i^{2+} to a low Ca_i^{2+} solution did not effect a decline of the channel activity.

Since there was little activity at 10^{-6} M or lower Ca_i^{2+}, we used a different procedure to estimate the decay of channel activity in low Ca_i^{2+} solutions. The patch was continuously superfused with a low Ca_i^{2+} solution (10^{-6} or 10^{-8} M), which was occasionally (~100 sec apart) interrupted with short pulses of a high Ca_i^{2+} solution (10^{-5} M) to assess the remaining channel activity. The change of the channel activity (current amplitude) in low Ca_i^{2+} was monitored up to 715 sec, and the time constant of an exponential decay was estimated (Table; entries for pCa = 6 and 8). Though the sampling period might not have been long enough to extract exact time constants, the decay of the channel activity was clearly slower in low Ca_i^{2+} concentrations.

Voltage Dependence of Channel Activity

The channel activity elicited by 10^{-6} M or higher Ca_i^{2+} was higher upon hyperpolarization than depolarization (Fig. 7*A*). At 10^{-6} M Ca_i^{2+}, even a depolarization higher than +60 mV could elicit very little channel activity; this was quite different from Ca_i^{2+}-dependent K channels in other organisms (Latorre, 1986). Since the activity of the channel in high Ca_i^{2+} changed over time, it was not practical to quantify the voltage dependence of the channel before the channel activity became stable. Thus after the activity became stable at 10^{-5} M Ca_i^{2+}, we examined the voltage dependence of the channel with a series of voltage pulses from −60 to +60 mV for about 15 sec each. The channel activity expressed as *NPo* was calculated and normalized to the maximal *NPo* within a set of data from each patch. The averages from five patches are plotted against voltages (Fig. 7*B*), showing more channel activity upon hyperpolarization than depolarization. The data were fitted with a Boltzmann distribution as follows:

$$\text{normalized activity} = 1/\{1 + \exp(-z\delta F(V - V_{1/2})/RT)\} \quad (2)$$

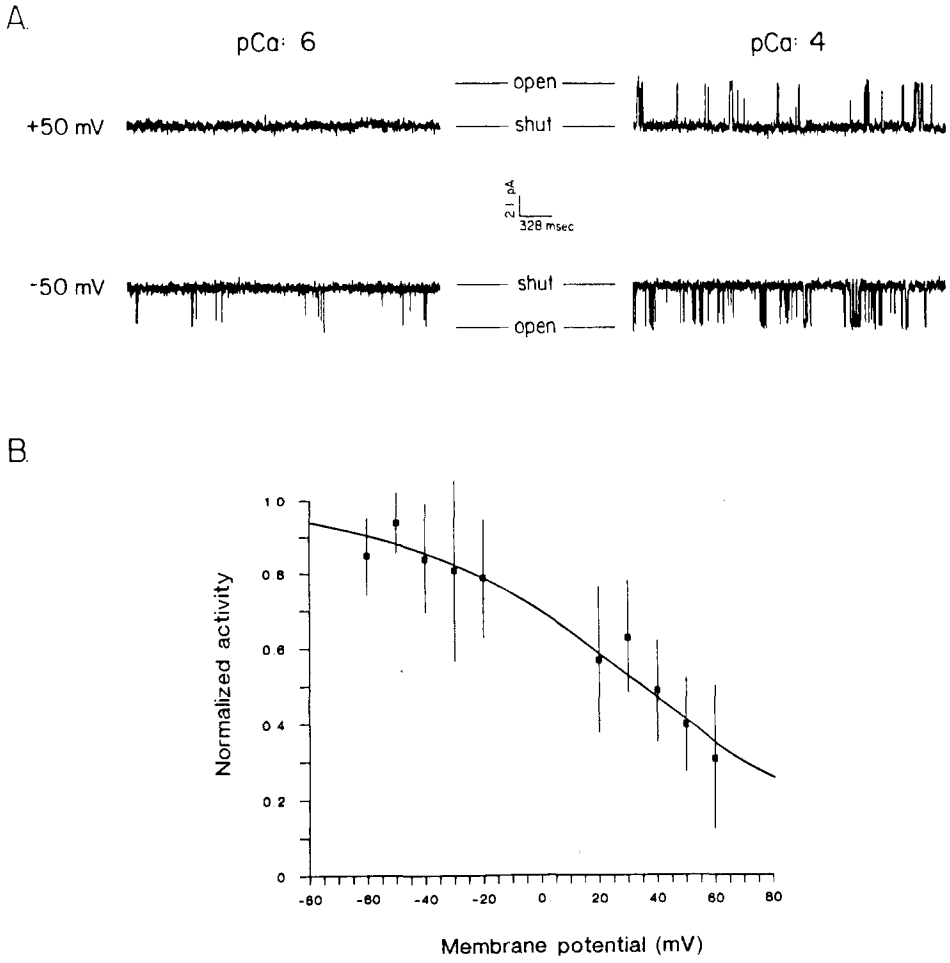

Fig. 7. Voltage dependence of channel activity. (*A*) Channel activities at 10^{-6} M Ca^{2+} (left) and 10^{-4} M (right) at voltages indicated beside the traces. (*B*) The channel activity expressed as *NPo* at 10^{-5} M Ca^{2+} was calculated from approximately 15-sec segments of current traces upon voltage steps to various levels. *NPo* was normalized to the maximum within the batch of data from a patch. Normalized *NPo*'s from five patches were averaged and plotted as the normalized activity (mean ± SD). These points were fitted with the Boltzmann curve (Eq. (2)) described in the text

where R, T and F have the usual meanings; z is valency of the charged particle; $z\delta$ (effective valency) = -0.61; V is the membrane potential in mV; $V_{1/2}$ = 36.9 mV. The negativity of the effective valency ($z\delta$) signifies more activity upon hyperpolarization, a distinct feature of this *Paramecium* channel.

Channel Blockers

Potassium channel blockers are commonly used to classify channel types and to probe the channel architecture (*see* review by Yellen, 1987). We examined the effects of K channel blockers on the Ca_i^{2+}-dependent K channels in *Paramecium* by presenting them to the cytoplasmic side of the membrane patch. Among quarternary ammonium (QA) ions there was a rank in effectiveness: the longer the alkylation, the more potent in blocking. Figure 8 (top) shows the blockage of the channel by 10 mM tetraethylammonium (TEA); the blockage was less effective upon hyperpolarization. A higher QA, such as tetrapentylammonium (TPA), was more effective, though the blockage was perhaps still voltage dependent (Fig. 8, middle). TPA blockage was reversible after an extensive wash. Tetrahexylammonium was an even better blocker, though the current record became noisier and the blockage was only partially reversible, possibly due to a hydrophobic interaction with the patch membrane. QA's with intermediate chain lengths were intermediate in the blocking efficacy. Blockages of this channel by QA's were similar to those of other reported Ca_i^{2+}-dependent K channels, indicating that this

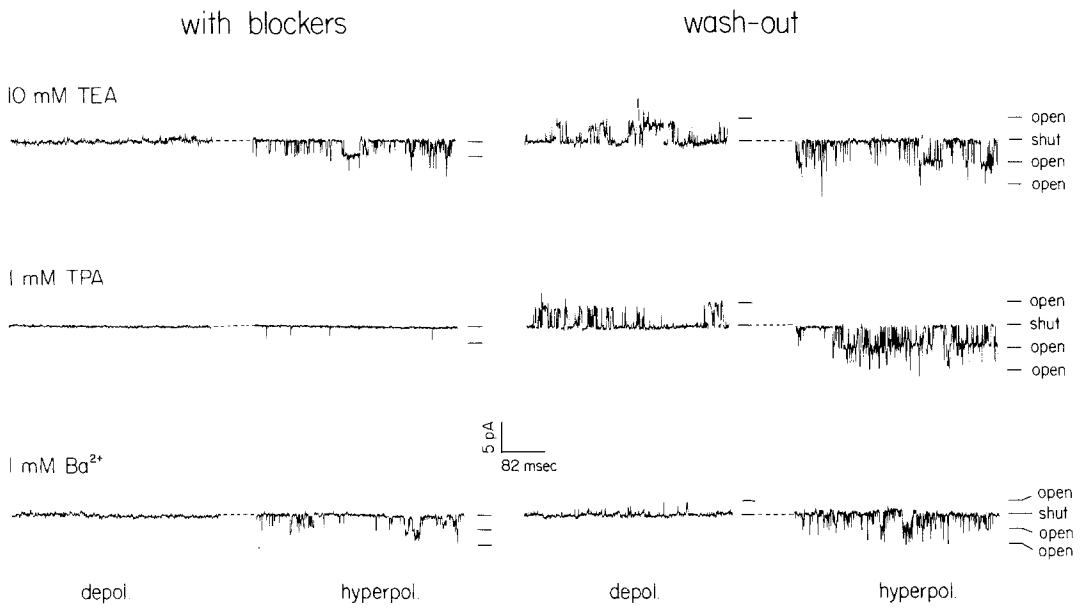

Fig. 8. Effects of K channel blockers. Top: TEA (10 mM) reduced the current in a voltage-dependent manner (left) compared to the current after TEA wash-out (right). Middle: TPA (1 mM) showed a more effective suppression of the current though still voltage dependent (left). The effect was reversible after an extensive wash-out (right). Bottom: Ba^{2+} (1 mM) showed incomplete suppression of the current (left) and readily reversible (right). All the traces were recorded at 10^{-5} M Ca^{2+} from one (top, middle) and from a second (bottom) patch. Test voltages were +50 mV for all depolarizations and −50 mV for hyperpolarizations

channel has also a large "mouth," at least on its cytoplasmic side (Yellen, 1987).

Ba_i^{2+} at micromolar concentrations is known to block K channels by occupying a site in the channel (Vergara & Latorre, 1983, Yellen, 1987), perhaps more deeply and tightly binding than QA's. However, Ba_i^{2+} was an ineffective blocker of this channel (Fig. 8, bottom); 1 mM Ba_i^{2+} inhibited the K^+ current upon depolarization but to a lesser extent upon hyperpolarization. At least 10 mM Ba_i^{2+} was required for nearly complete blockage upon both depolarization and hyperpolarization, and its effect was readily reversible. Similarly, Ca_i^{2+} was ineffective in blockage. We also tested quinidine and quinine; both caused partial blockage at 1 mM.

Discussion

A Ca^{2+}-Dependent K Channel in *Paramecium*

Single channels from protozoa have previously been studied by incorporating semi-purified ciliary membranes into lipid bilayers (Hanke, Eibl & Boheim, 1981; Ehrlich et al., 1982; Oosawa & Sokabe, 1985; Oosawa & Kasai, 1988). In this patch-clamp study, we used a fresh plasma-membrane preparation in the form of isolated vesicles derived from blisters of cells, which was probably of the somatic membrane. We show a Ca_i^{2+}-dependent K channel under patch clamp which has a conductance of 72 pS, has apparently more than two Ca_i^{2+} binding sites, shows decline of its activity in the presence of high Ca_i^{2+}, and has very weak voltage sensitivity. The function of the Ca_i^{2+}-dependent K channels in *Paramecium*, however, is not clear. It may control the repolarization process of the Ca action potential like the Ca_i^{2+}-dependent K channels in other organisms. Alternatively, it might contribute to the setting of the "resting" potential. A Ca_i^{2+}-dependent K current has been reported upon hyperpolarization under two-electrode, whole-cell voltage clamp (Richard et al., 1986; Preston et al.[2]), and is thought to regulate the "resting" potential since a mutational increase in this whole-cell current leads to a more negative "resting" potential in low K^+ solutions (Richard, Hinrichsen & Kung, 1985).

Novelty of the Ca^{2+}-Dependent K Channel in *Paramecium*

The Ca_i^{2+}-dependent K channel described in the present report is significantly different on mainly two accounts from Ca_i^{2+}-dependent K channels, so

[2] *See* footnote 1, p. 79.

called BK channels (Latorre, 1986; Blatz & Magleby, 1987), which are found in many organisms and usually have conductances larger than 150 pS. First, unlike BK channels which can be activated upon depolarization, the *Paramecium* channel has a very weak voltage sensitivity (the effective valency ($z\delta$) being −0.61, or 41.2 mV per *e*-fold change in the channel activity), and is slightly more active upon hyperpolarization than upon depolarization. Such an unusual voltage dependence of the Ca_i^{2+}-dependent K channels is not merely due to voltage-dependent channel blockage by ions, which has been seen in some ion channels (Nowak et al., 1984; Matsuda, Saigusa & Irisawa, 1987). On the contrary, it appears to be a genuine channel property because neither Ba_i^{2+} nor Ca_i^{2+} is expected to block much of the channel activity at micromolar concentrations (*see* Fig. 7). A similarly weak voltage dependence has been reported for some of the Ca_i^{2+}-dependent K channels in red blood cells (Grygorczyk, Schwarz & Passow, 1984), in rat skeletal muscle (Blatz & Magleby, 1986, 1987), and in GH_3 cells (Lang & Ritchie, 1987). Such a negative effective valency may mean, though certainly not proving, that the Ca_i^{2+}-binding sites for the activation of this *Paramecium* channel is located outside of the voltage drop across the membrane, since Ca_i^{2+} binding within the voltage drop should be enhanced by depolarization. Such a voltage-dependent Ca_i^{2+} binding has been raised as one possible mechanism for the BK channels (Moczydlowski & Latorre, 1983; Latorre, 1986).

Second, the activity of *Paramecium* channels decays irreversibly in the presence of high Ca_i^{2+} (Fig. 5). The higher the Ca_i^{2+} concentration, the faster the decay (Table). This Ca_i^{2+} effect cannot be reproduced by Mg_i^{2+}, although it is not clear whether channel opening is a prerequisite for the decay of its activity. This irreversible loss of channel activity differentiates the *Paramecium* K channel from not only the BK channels, but also other Ca_i^{2+}-dependent K channels including SK channels (Blatz & Magleby, 1986, 1987). "Run-down" of Ca_i^{2+}-dependent channel activities has been reported for nonselective cation channels in pancreatic acini (Maruyama & Petersen, 1982) and for FV channels in a plant cell (Hedrich & Neher, 1987). The activity of a variety of channels is known to deteriorate, particularly Ca channels when the patch is excised (Fenwick, Marty & Neher, 1982). The decay of *Paramecium* channel activity was not affected by a reducing agent (DTT) or protease inhibitors, suggesting that loss of channel activity may not be due to proteolytic activity or oxidation/reduction. In fact when a protease is included in the bath, the channel activity increases (Kubalski, Martinac & Saimi, 1989). In a few experiments, we also included phosphatase inhibitors (30 mM β-glycerophosphate or 1 mM molybdate), but could not prevent the decay (*unpublished data*).

Presence of Multiple Types of Ca^{2+}-Dependent K Channels in Single Cells

It is likely that *Paramecium* possesses at least two Ca_i^{2+}-dependent K channels which are distinct in kinetics, voltage dependence, Ca_i^{2+} sensitivity and pharmacology. Apart from the novel type of the Ca_i^{2+}-dependent K channels described in this report, a Ca_i^{2+}-dependent K channel of approximately 150 pS in 100 mM K^+ has been reported. Although infrequently encountered, this Ca_i^{2+}-dependent K channel is preferentially activated upon depolarization, has an apparent one Ca_i^{2+} binding for activation (Martinac et al., 1986, 1988), and is completely blocked by 1 mM Ba_i^{2+} in the bath solution (B. Martinac & Y. Saimi, *unpublished data*). These properties are quite distinct from those of the Ca_i^{2+}-dependent K channels described in this report. Under two-electrode, whole-cell voltage clamp, two pharmacologically separable Ca_i^{2+}-dependent K currents have been identified (Preston et al.[3]).

Occurrences of two or more different Ca_i^{2+}-dependent K channels in one cell may not be unusual; at the level of single-channel recording, multiple types of Ca_i^{2+}-dependent K channels are found in pituitary GH_3 cells (Lang & Ritchie, 1987) and in rat muscle (Blatz & Magleby, 1986). These various types of Ca_i^{2+}-dependent K channels are likely to represent different molecular entities.

The authors thank Dr. C. Kung for his continued support of this work, and Drs. H. Machemer, R.R. Preston and M.C. Gustin for their critical reading of this manuscript. This work was supported by grants from NIH (GM22714 and GM36386) and from the Lucille P. Markey Trust.

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Received 13 March 1989; revised 19 June 1989