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Paramecium Na^+ Channels Activated by Ca^{2+}-Calmodulin: Calmodulin is the Ca^{2+} Sensor in the Channel Gating Mechanism

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Abstract. *Paramecium* Na^+ channels, which were Ca^{2+}-calmodulin activated, were studied in the inside-out mode of patch clamp. After excision of the membrane patch, they were active in the presence of 10^{-5} to 10^{-3} M Ca^{2+} in the bath. They became much less active in the presence of 10^{-6} M Ca^{2+}, and their activity subsided completely at 10^{-8} M Ca^{2+}. A Hill plot showed a dissociation constant of 6 μM for Ca^{2+} binding. This dissociation constant shifted to a submicromolar range in the presence of 1 mM Mg^{2+}. The channels also exhibited a mild voltage dependence. When exposed to 10^{-8} M Ca^{2+} for an extended period of 2–4 min, channels were further inactivated even after bath Ca^{2+} was restored to 10^{-4} M. Whereas neither high voltage (+100 mV) nor high Ca^{2+} (10^{-3} M) was effective in reactivation of the inactive channels, addition of *Paramecium* wild-type calmodulin together with high Ca^{2+} to the bath restored channel activity without a requirement of additional Mg^{2+} and metabolites such as ATP. The channels reactivated by calmodulin had the same ion conductance, ion selectivity and Ca^{2+} sensitivity as those prior to inactivation. These inactivation and reactivation of the channels could be repeated, indicating that the direct calmodulin effect on the Na^+ channel was reversible. Thus, calmodulin is a physiological factor critically required for Na^+ channel activation, and is the Ca^{2+} sensor of the Na^+-channel gating machinery.

Key words: *Paramecium* — Patch clamp — Calmodulin — Ion channels — Channel regulation — Ca^{2+} sensor

Introduction

In Ca^{2+} signal transduction, Ca^{2+} appears in the cytoplasm from external media through Ca^{2+}-permeating ion channels in the plasma membrane, or is released from internal stores through Ca^{2+}-release channels (Tsien & Tsien, 1990; Berridge, 1993; Meissner, 1994). While Ca^{2+} flux is governed by Ca^{2+}-permeating channels, Ca^{2+} in turn modulates activities of a variety of ion channels.

Ca^{2+} is capable of activating a multitude of enzymes and also interacting with other cytoplasmic components, such as cytoskeletal elements (Lee & Wolff, 1984; Bennett, 1990; Aderem, 1992; Pirollet et al., 1992; Cross, Vial & Maccioni, 1993). Often such Ca^{2+} effects are mediated by various Ca^{2+}-binding proteins (Heizmann & Hunziker, 1991). One such Ca^{2+}-binding protein is calmodulin, a 17-kD protein, which is known to activate more than twenty enzymes (Cohen & Klee, 1988). There is also growing evidence that calmodulin directly regulates several ion channels in a variety of systems including *Paramecium* Na^+ channels (Saimi & Ling, 1990), ryanodine receptor/Ca^{2+}-release channels of sarcoplasmic reticulum (Meissner, 1986; Smith, Rousseau & Meissner, 1989; Fuentes et al., 1994), cyclic nucleotide-activated channels (Hsu & Molday, 1993; Chen & Yau, 1994), and perhaps others (*see* Saimi, Ling & Kung, 1994*a*).

Involvement of calmodulin in channel regulation in *Paramecium* was first demonstrated through transforming mutants in their behavioral phenotype by microinjection with wild-type calmodulin (Hinrichsen et al., 1986). Later, Kink et al. (1990) and Ling et al. (1994) cloned the *Paramecium* calmodulin gene and showed that there are two classes of calmodulin mutants. Mutants of the pantophobiac type over-react to various stimuli including Na^+, and have greatly reduced Ca^{2+}-activated K^+ currents. All pantophobiac-type mutations map to the C-terminal lobe of the calmodulin molecule. Mutants of the fast-2 type exhibit shortened avoiding responses to stimulation with Na^+, and lacks a Ca^{2+}-activated inward Na^+ current when examined under whole-cell voltage clamp.

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All of these fast-2 type mutations map to the N-terminal lobe. The link between calmodulin and Na^+ channels thus identified through mutant analyses has been further indicated to be a direct action of calmodulin on the Na^+ channel in excised membrane patches. In these experiments, calmodulin is required for activation of the Na^+ channel and its action apparently requires no metabolites such as ATP. Therefore, a membrane-delimited interaction between calmodulin and the channel has been proposed (Saimi & Ling, 1990).

This report examines Ca^{2+} dependence of the single Na^+ channel of *Paramecium* in the presence and absence of 1 mM Mg^{2+}, an important physiological ion. It also shows voltage dependence of the channel. However, after inactivation of the channel by exposure to low Ca^{2+}, the channel is no longer responsive to either voltage or Ca^{2+} until calmodulin is included in the bath. Thus, we conclude that calmodulin is a prerequisite physiological factor for the Na^+ channel regulation, and that calmodulin is the Ca^{2+} sensor of the Na^+-channel gating machinery.

Material and Methods

Cell Preparation and Patch Clamp Conditions

Paramecium tetraurelia with an nd-6 background (trichocyst-nondischarge; Lefort-Tran et al., 1981) was reared in the medium M at 31° C before patch clamp experiments were carried out (Saimi & Martinac, 1989). Solutions used in the experiments comprised the following basic ingredients unless mentioned otherwise: 100 mM Na^+, 20 mM Cl^-, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.0 adjusted with glutamic acid as much as 80 mM). All the bath solutions contained 1 mM dithiothreitol to avoid possible oxidation. Free Ca^{2+}, if lower than 100 μM, was buffered with 1 mM EGTA, and not buffered for higher concentrations (Saimi & Martinac, 1989). Addition of 10 mM Mg^{2+} affected free Ca^{2+} estimations only little. All experiments were carried out at 19–23° C, and were repeated at least three times. Data were given as means ± SD wherever applicable.

The protocol for patch clamp recording from Na^+ channels was the same as that previously described (Saimi & Martinac, 1989). Briefly, blisters of the plasma membrane from paramecia were induced by incubating cells in a high ionic solution containing 100–150 mM Na^+ glutamate and 10^{-5} M free Ca^{2+}. Vesicles derived from the blisters were collected with a micropipet, and gigaohm seals were formed on the vesicles with patch pipettes in a bath solution containing 10 mM $MgCl_2$ and 10^{-8} to 10^{-5} M free Ca^{2+}. After the formation of gigaohm seal, the patch membrane was excised by tapping on the micromanipulator, achieving the inside-out excised patch clamp mode. The chamber, ~300-μl in volume, was then perfused with a solution containing 10^{-5} or 10^{-4} M free Ca^{2+}, and 10 μM tetrapentylammonium (TPA). Unless otherwise noted, TPA was added to reduce the activity of nonspecific channels which was not Ca^{2+} dependent. TPA additionally caused flickers in the Na^+ channel activity (Saimi & Ling, 1990). The contaminating nonspecific channel activity was as much as 0.02 in terms of NP_o; all data with higher contamination were discarded. Unless otherwise noted, the pipette included a solution with no added Mg^{2+}, 10^{-5} M free Ca^{2+} and 10 μM TPA, thus establishing symmetric conditions for major ions, under which the preamplifier was adjusted for zero current. Mg^{2+} was not included except in the solutions to form gigaohm seals and to test Mg^{2+} effects.

An EPC7 (List/Medical Systems, NY) was used for single-channel recording, and pCLAMP software (Axon Instruments, CA) was used for data acquisition in both online and offline modes. The data were filtered at 5 kHz using a 4-pole filter (Frequency Devices, MA) and then stored either directly in a PC computer or in a PCM/VCR (Indec, CA) for later retrieval. The data taken into the computer were further filtered digitally at either 500 Hz or 1 kHz using a pCLAMP function before final analyses. Channel activity, if high, was analyzed with pCLAMP to generate amplitude histograms which were then fitted with Gaussian curves, and channel activity (NP_o) was calculated from the fit parameters. This method allowed minor contamination by unwanted channel activities. Among few other contaminating channels, the nonselective channels were most often encountered that had a larger conductance and much shorter open durations than the Na^+ channel (Saimi & Ling, 1990). However, low channel activity was estimated by the event listing procedure of pCLAMP to collect all correct events and eliminate extraneous channel activities. Durations of analyzed data were 15 to 26 sec in all cases. Hard copies of fast events were made through pCLAMP and slower ones on a chart recorder (Gould 220, OH).

Calmodulin Samples

Native *Paramecium* wild-type calmodulin was isolated and purified from cytosol extracts of the nd-6 mutant as described previously (Ling et al., 1992). However, large quantities of wild-type calmodulin were produced in *E. coli* using a plasmid, pOK2, bearing a wild-type gene for *Paramecium* calmodulin (Kink et al., 1991). Also a plasmid bearing a half wild-type calmodulin gene which included amino acid residues from 1 through 79 was constructed by insertion of a stop codon. Both wild-type and half recombinant calmodulins were overexpressed in JM109 upon induction with 0.1 mM isopropyl-1-thio-B-D-galactopyranoside at 37° C (Kink et al., 1991). Calmodulins were extracted from bacterial lysates and further purified to near homogeneity (>99% as judged in SDS gels) as the native calmodulins described above.

In the channel reactivation experiments, 2 μl of calmodulin suspensions (1 mg/ml) in the basic solution were directly added to the bath (~300 μl). The bath was then vigorously mixed with pipettes 2–3 times more than needed for complete mixing of dye indicators in test trials. The final calmodulin concentration added to the bath was estimated to be ~0.4 μM, which was approximately an order of magnitude less than that in vivo, assuming all retrieved calmodulin was free in the cytoplasm (K.-Y. Ling and Y. Saimi, *unpublished*).

Results

Excised membrane patches from *Paramecium* contained a variety of ion channels (Saimi et al., 1994*b*). When Na^+ was the major cation in both the pipette solution and the bath solution, only a few types of channels displayed activities. One of them was Ca^{2+}-activated Na^+ channels (Saimi & Ling, 1990). These Na^+ channels in excised patches varied in number and activity from patch to patch. For practical reasons of data analyses, we only studied selected patches exhibiting reasonable Na^+-channel activity (NP_o of 0.12 to 2.29) in the presence of 10^{-4} M Ca^{2+} in the bath, to which the cytoplasmic face of the membrane patch was exposed.

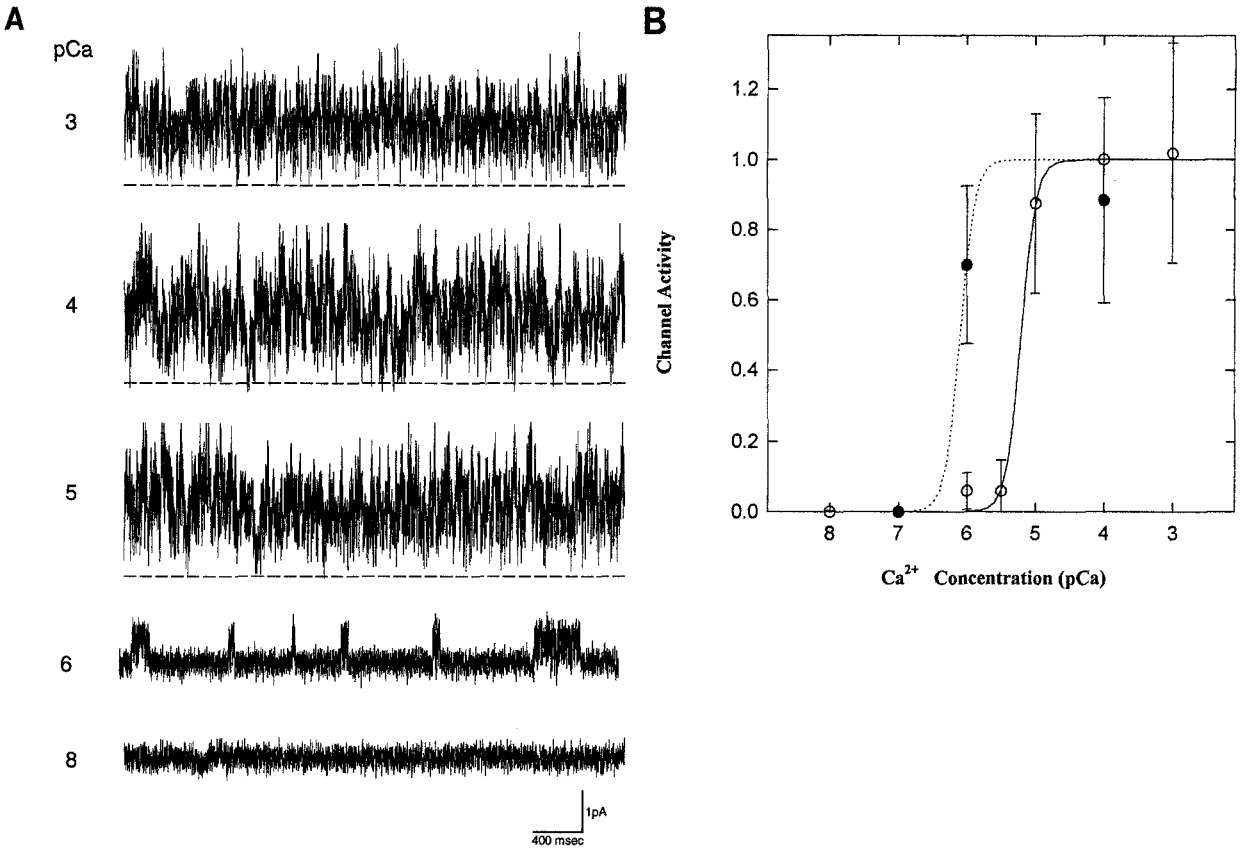

Fig. 1. Ca^{2+} dependence of Na^+ channels. (*A*) A family of traces of single Na^+-channel activity at different Ca^{2+} concentrations (pCa, indicated on the left) in the bath. Because of channel inactivation upon exposure to low Ca^{2+}, the channel activity in pCa = 8 was recorded last. The voltage across the membrane patch was maintained at +50 mV. The flickery appearance compared to those previously published was due to the presence of 10 μM TPA (*see* Material and Methods). The broken lines indicate closed levels of single-channel current. (*B*) Normalized channel activities plotted against Ca^{2+} concentrations (pCa). Channel activities at various Ca^{2+} concentrations in the absence of Mg^{2+} were normalized to those at pCa = 4 for individual patches and then plotted (continuous line and open circles, mean ± SD, 3–9 patches). Similarly, these in the presence of 1 mM Mg^{2+} (broken line and filled circles, 3–6 patches) were normalized to those at pCa = 4 without Mg^{2+}. In order to extract parameters, the data points were first fitted to Hill curves, yielding dissociation constants (K_d: 6.2 and 0.79 μM) and Hill coefficients (n: 4.1 and 3.6), in the absence and in the presence of Mg^{2+}, respectively. Using these K_d's and 4 for n, lines were then drawn through the data points.

Ca^{2+} and Voltage Dependence of Na^+ Channels

When examined under the symmetric condition of 100 mM Na^+ in both pipette and bath, *Paramecium* Na^+ channels were active in the presence of high Ca^{2+} (10^{-5} to 10^{-3} M) in the bath, while the patch was kept depolarized to +50 mV (Fig. 1*A*). Channel activity reduced to a low level when the bath was perfused with a solution containing 10^{-6} M Ca^{2+} and disappeared at 10^{-8} M Ca^{2+}. Channel activities (NP_o) at various Ca^{2+} concentrations were normalized to those at 10^{-4} M Ca^{2+} for individual patches, and plotted against Ca^{2+} concentrations (Fig. 1*B*, open circles). A Hill plot (Fig. 1*B*, continuous line) best fitting the data points revealed a dissociation constant (K_d) of 6.2 μM for Ca^{2+} and a Hill coefficient (n) of 4.1, though an accurate estimation of the Hill coefficient was difficult because of inherent large variability of the single-channel activity.

It has been shown that activities of some, if not all Ca^{2+}-activated K^+ channels in reconstituted systems increase when nonpermeant divalent ions such as Mg^{2+} and Ni^{2+} are included at millimolar concentrations in the bath (Golowasch, Kirkwood & Miller, 1986; Oberhauser, Alvarez & Latorre, 1988). In the present study, we found that the affinity of Ca^{2+} for the *Paramecium* Na^+ channel increased almost tenfold in the presence of 1 mM Mg^{2+} (K_d: 0.8 μM; Fig. 1*B*, filled circles, broken line). While Mg^{2+} modulated the Ca^{2+} sensitivity of the channel, Mg^{2+} alone (1–10 mM) was not sufficient to activate the channel in the presence of 10^{-8} M Ca^{2+} in the bath. Divalent ions also had additional effects on the channel. Both Ca^{2+} and Mg^{2+} at concentrations of 1 mM or above reduced the conductance slightly. Ten mM Mg^{2+} inhibited the channel activity completely in the presence of 10^{-4} M Ca^{2+} in the bath (*data not shown*).

Ca^{2+}-dependent channels shown here were highly selective for Na^+ as previously shown (Saimi & Ling, 1990). The estimated conductance of the Na^+ channel in

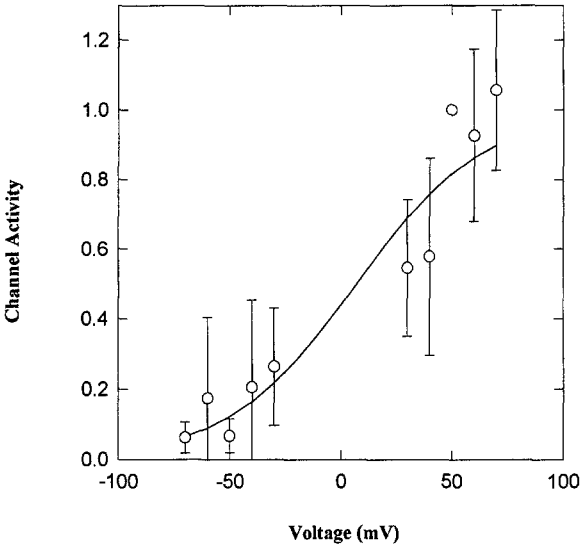

Fig. 2. Voltage dependence of Na^+ channels. Channel activities at variable voltages were normalized to those at +50 mV for individual patches (3–4 patches) in the presence of 10^{-5} M Ca^{2+} in the bath. Mg^{2+} and TPA were omitted in both the pipette and the bath to avoid its possible voltage-dependent inhibition. The data points were fitted to a Boltzmann equation as follows: normalized activity = $1/[1 + \exp\{-z\delta F(V - V_{1/2})/RT\}]$ where F is the Faraday constant, R the gas constant and T the temperature with a $V_{1/2}$ of 6.9 mV and a $z\delta$ (effective valency) of 34.7.

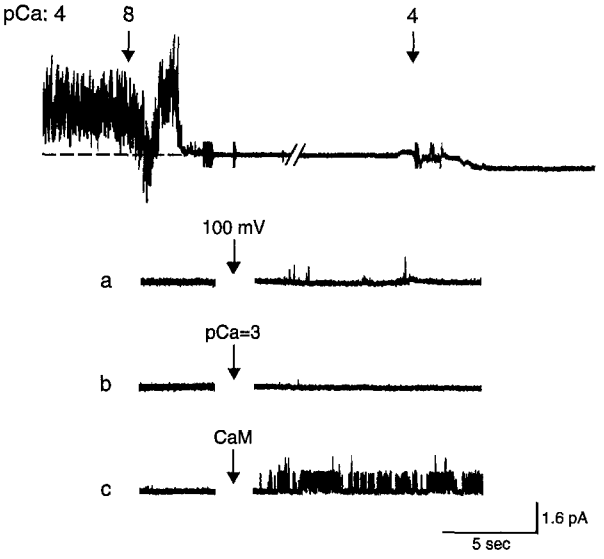

Fig. 3. Inactivation and reactivation of Na^+ channels. First, Na^+ channel activity was recorded in the presence of high Ca^{2+} (pCa = 4; top trace, left), and then the bath was perfused with low Ca^{2+} (pCa = 8; top trace, middle) for 2 min. The rebounding activity upon perfusion was due to the mixing process of the solutions. Also note other perfusion artifacts in the trace. Restoring high Ca^{2+} in the bath (pCa = 4; top trace, right) failed to reactivate Na^+ channels. After channel inactivation, raising voltage across the membrane patch from +50 mV to +100 mV (trace a), or increasing the Ca^{2+} concentration of the bath to pCa = 3 (trace b) also failed to reactivate the channels. However, some channel activity returned upon addition of wild-type *Paramecium* calmodulin produced in *E. coli* (~0.4 μM final concentration; trace c) after a lag time of several seconds in this example.

the presence of 10^{-4} M Ca^{2+} and 10 μM tetrapentylammonium (TPA) was 12 ± 0.85 pS (n = 9), which was slightly smaller, perhaps because of high Ca^{2+}, than that previously noted (17–19 pS) in the presence of 10^{-5} M Ca^{2+} (Saimi & Ling, 1990).

Activities of Na^+ channels were also modulated by voltage. These channels were more active upon depolarization of the membrane patch in reference to the pipette voltage. Channel activity in the presence of 10^{-5} M Ca^{2+} was first normalized to that at +50 mV, and then plotted against the membrane voltage (Fig. 2). A Boltzmann curve fitting revealed a voltage dependence of the channel (29 mV for e-fold activity change) much shallower than those of the voltage-dependent Na^+ channels in mammals.

Inactivation and Reactivation of the Na^+ Channel

When the bath was perfused with solutions containing 10^{-8} M Ca^{2+}, the Na^+ channel activity subsided (Fig. 1*A*). An immediate return to high Ca^{2+} within seconds always restored near full channel activity. However, extended superfusion of low Ca^{2+} solutions for 2–4 min further inactivated channels because even high Ca^{2+} (10^{-4} M) in the bath was no longer able to restore channel activity (Fig. 3, top trace). The possibility of a reduced Ca^{2+} sensitivity of the channel after inactivation was examined by exposing the patch to a yet higher concentration of Ca^{2+} (10^{-3} M) without channel activation (Fig. 3, trace b). Since active channels exhibited some voltage dependence (Fig. 2), we tested whether high voltage could elicit channel activity in the presence of 10^{-4} M Ca^{2+}. Attempts at reactivation with high voltage (+100 mV) imposed across the patch membrane were to no avail (Fig. 3, trace a). However, as demonstrated before (Saimi & Ling, 1990), addition of wild-type *Paramecium* calmodulin (~0.4 μM, final concentration) to the bath restored channel activity. Fig. 3 (trace c) shows channel reactivation by recombinant calmodulin produced in *E. coli* (*see* Material and Methods). In this example, the time lag between the addition of calmodulin and reactivation of channel was only several seconds. In many cases, though varied from patch to patch and relatively consistent within patches, time lag was a few minutes; in others, it was longer than 5 min despite careful mixing of the bath (*see* Material and Methods). The time lag might have reflected diffusion of calmodulin through the narrow aperture of pipettes and perhaps through cytoskeleton lining of the patch membrane. These slow acting patches were excluded from the present report; an estimated percentage of such successful reactivation was greater than 50%, similar to that in the previous study

(Saimi & Ling, 1990). Channel activation appeared to be effected equally well by both native and recombinant *Paramecium* calmodulins. For the sake of simplicity, the experiments described below used both calmodulins interchangeably. Unlike intact calmodulin, however, 2 μM or a higher concentration of truncated recombinant calmodulin molecules encompassing the N-terminal half and part of the central helix (residues 1 to 79) did not reactivate Na^+ channels, implying that an intact structure of calmodulin was necessary.

Once reactivated, these channels remained active upon perfusion of the chamber with calmodulin-free solutions containing 10^{-4} M Ca^{2+} (Fig. 4, top trace, right). This suggested that once calmodulin was attached to the channel, it remained tightly attached over tens of minutes in the presence of high Ca^{2+}. Based on these observations, the rate of calmodulin dissociation at 10^{-4} M Ca^{2+} in the bath was estimated to be on the order of 10^{-3} sec^{-1} or lower, whereas that at 10^{-8} M Ca^{2+} was 5–10-fold higher as estimated from the time required (2–4 min) for inactivation of the channel (*see above*). These dissociation rates of calmodulin were similar to those for a variety of Ca^{2+}-calmodulin activated enzymes (Klee, 1988). A low dissociation rate of calmodulin from the Na^+ channel in the presence of 10^{-4} M Ca^{2+} accounted for the occurrences of active channels immediately after patch excision from the *Paramecium* membrane vesicles (*above*).

In order to confirm that the channels reactivated by calmodulin were in fact Na^+ channels, their ion selectivity was examined by replacing Na^+ with K^+ in the bath, maintaining the pipette solution of 100 mM Na^+. Figure 4 shows that channels reactivated by native *Paramecium* wild-type calmodulin passed current in both directions when the bath contained Na^+ (lower, left panel). By contrast, only current going into the bath from the pipette was observed when K^+ was included in the bath (right). Thus, these channels selectively passed Na^+ and not K^+, and were indeed Na^+ channels. Again channels reactivated by either native or recombinant *Paramecium* calmodulin were confirmed to be Na^+ selective. The conductance of these channels reactivated by recombinant *Paramecium* calmodulin was 13 ± 0.46 pS (n = 5), a figure similar to that prior to inactivation (*above*). Ca^{2+} dependence of channels reactivated by calmodulin was examined by perfusion of the bath with 10^{-8} M Ca^{2+} solutions. Reactivated channels became silent again upon low Ca^{2+} perfusion, thus indicating Ca^{2+} dependence, and they were further inactivated upon longer exposure to low Ca^{2+}. These channels were inactivated and reactivated repeatedly by low Ca^{2+} exposure and calmodulin addition to the bath (Fig. 5).

Thus, the calmodulin-reactivated channels had the same conductance, ion selectivity and Ca^{2+} sensitivity as those Na^+ channels first encountered upon patch excision. These results indicated that calmodulin is a crucial factor for activation of the Na^+ channel, and that decalcified calmodulin dissociates from the channel.

Discussion

We report here that the Na^+ channel in *Paramecium* is regulated by Ca^{2+} and voltage. However, binding of calcified calmodulin to this channel from the cytoplasmic side, which can be reversed in low Ca^{2+}, is a prerequisite for the channel activation.

Ca^{2+} and Calmodulin Sensitivity

With a dissociation constant of 6 μM, the Ca^{2+} sensitivity of the Na^+ channel in *Paramecium* is only moderate when compared to those of other Ca^{2+}-activated channels (Latorre et al., 1989). However, this figure is comparable to that (3.7–7.1 μM) for the olfactory cyclic-nucleotide activated channel (Chen & Yau, 1994), whose sensitivity to cyclic nucleotide is reduced 20-fold in the presence of Ca^{2+} calmodulin. Like most other calmodulins, *Paramecium* calmodulin binds a maximum of 4 Ca^{2+} (Rao et al., 1993). In general, the C-terminal Ca^{2+} binding loops, III and IV, have a higher affinity ($K_d \approx 1$ μM) than those (I and II loops, ≈10 μM) in the N-terminus (Klee, 1988). These Ca^{2+} dissociation constants of calmodulin are similar to the Ca^{2+} affinity for the Na^+ channel observed in the present report. Haiech et al. (1991) showed that certain mutant calmodulins bind Ca^{2+} with a higher affinity when they first bind target peptides. Therefore, it is possible that the affinity of these Ca^{2+}-binding loops of calmodulin improves after calmodulin binding to the Na^+ channel, explaining the small discrepancy of the dissociation constants.

Most calmodulins bind 4 Ca^{2+}. In the present study, the Hill coefficient of Ca^{2+} binding for the Na^+ channel activation is consistent with four binding sites, though more accurate measurements are required to define this parameter. In olfactory cyclic-nucleotide activated channels, a Hill coefficient of 1 to 2 for Ca^{2+} binding has been reported (Chen & Yau, 1994). However, the authors were cautious of these estimations since these channels are homomultimeric and there may be multiple calmodulin-binding sites in the functional unit. For calmodulin binding to these channels, a Hill coefficient of 1.1 and a dissociation constant of 2.1 nM were also reported. Ryanodine receptor/Ca^{2+}-release channels in sarcoplasmic reticulum are inhibited by Ca^{2+}-calmodulin (Meissner, 1986; Smith et al., 1989; Fuentes et al., 1994). Yang et al. (1994) reported multiple calmodulin binding sites for the homotetrameric ryanodine receptor channel in pig skeletal muscle: one per subunit with a dissociation constant of 4.3 nM and four per subunit with 239 nM. In an electrophysiological experiment, Fuentes et al. (1994) measured a dissociation constant of 150 nM for calmod-

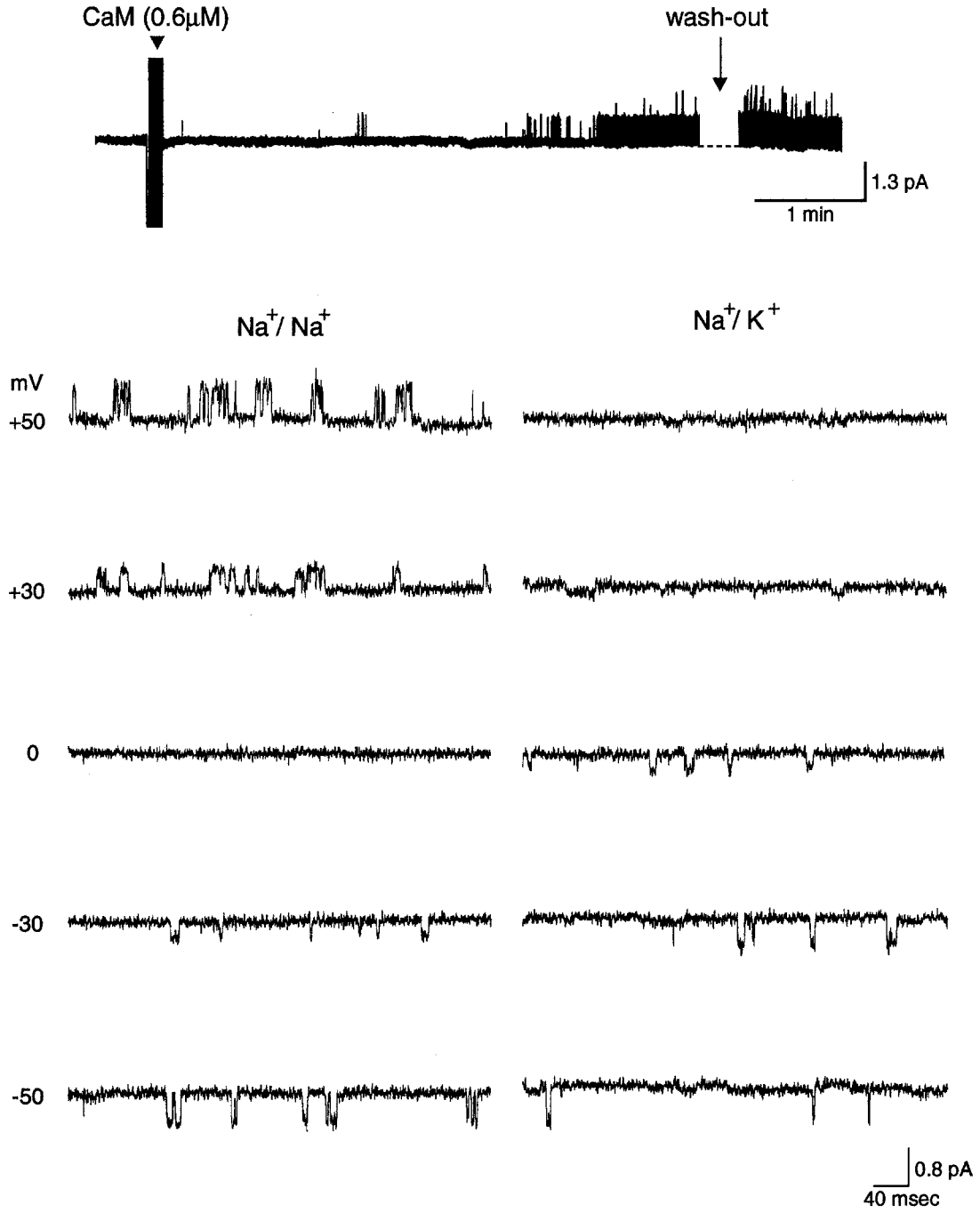

Fig. 4. Reactivation of Na^+ channels and ion selectivity. The channels were first inactivated by subjecting them to low Ca^{2+} (pCa = 8) for 2 min. After perfusion with a solution containing high Ca^{2+} (pCa = 4; top trace, left), native wild-type *Paramecium* calmodulin (~0.4 μM final concentration) was added to the bath. The channel activity was restored after a few minutes (top trace, middle). The channel stayed active even after the bath was washed with a solution devoid of calmodulin (top trace, right), while voltage across the patch was kept at +50 mV. Then, single channel activities were recorded at variable voltages in the presence of 100 mM Na^+ (lower left traces) or in the presence of 100 mM K^+ (lower right traces) in the bath. While single channel current was observed in both inward and outward directions in the presence of Na^+ in the bath, only inward current from the pipette into the bath was detected in the presence of K^+ in the bath, indicating that K^+ was not as permeable through the channel.

ulin inhibition of the ryanodine receptor channel. It is also suggested, though not proven, that *Drosophila* putative photoreceptor channels, *trpl,* (Hardie & Minke, 1993) have two potential calmodulin binding sites (Phillips, Bull & Kelly, 1992). These multiple calmodulin-binding sites may be involved in further intricate regulation of the channels, such as activation-inactivation control.

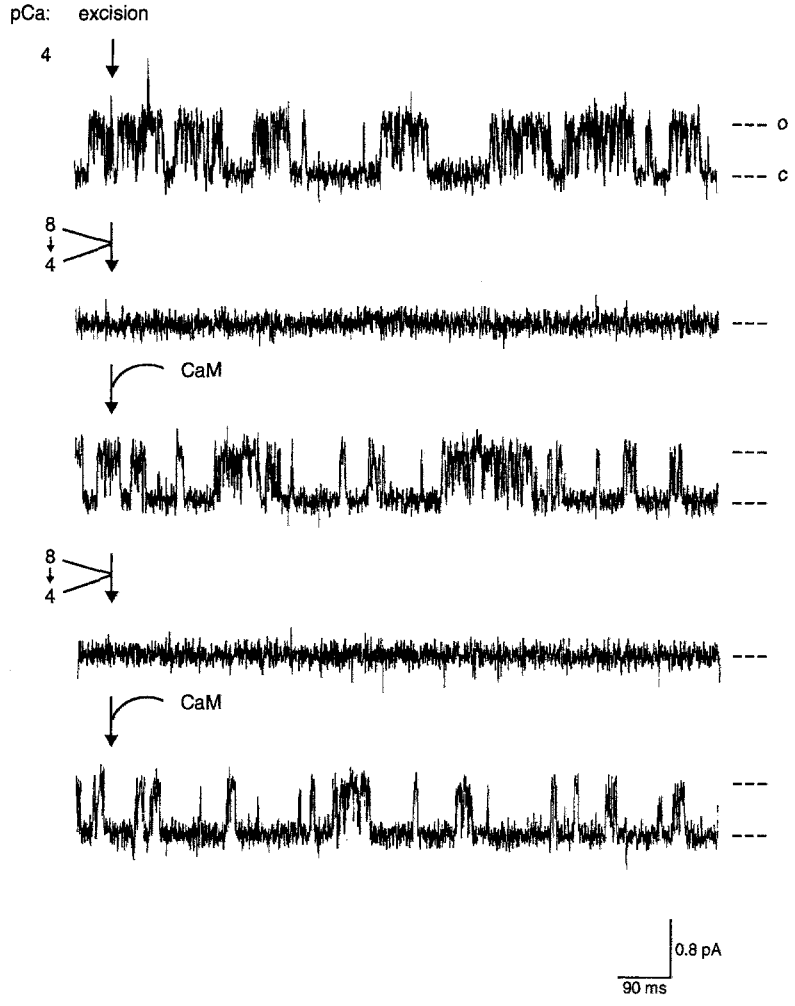

Fig. 5. Repeated inactivation and activation of Na^+ channels. Na^+ channels were recorded in the presence of 10^{-4} M Ca^{2+} in the bath after excision of the patch (top trace). The patch was then exposed to 10^{-8} M Ca^{2+} for 3 min, and channel activity was recorded after the bath Ca^{2+} was increased to 10^{-4} M (2nd trace). Channel activity was also recorded 5 min after addition of wild-type recombinant calmodulin at ~0.4 μM (3rd trace). The patch was again exposed to 10^{-8} M Ca^{2+} for 4 min and the bath Ca^{2+} was restored to 10^{-4} M where channel activity was recorded (4th trace). Again, channel activity was recorded 5 min after inclusion of calmodulin at the same concentration (5th trace). The membrane voltage was maintained at +50 mV throughout the experiment.

Though most calmodulins can bind 4 Ca^{2+}, a half calmodulin molecule is enough to support yeast growth (Sun, Ohoya & Anraku, 1991). Proteolytic half calmodulin molecules also activate some enzymes and interact with others (Klee, 1988). In the present study, half calmodulin molecules, which consisted of residues 1 to 79 encompassing the N-terminus and part of the central helix, were not capable of channel activation even in the presence of high Ca^{2+}. Whereas the mutations resulting in the loss of Na^+ current in *Paramecium* are clustered in the N-terminal lobe (Kink et al., 1990; Ling et al., 1994), the interacting site with the Na^+ channel may not be restricted only to the N-terminal half. The intact calmodulin structure may be required for its binding to the channel, while its N-terminus half further interacts with the channel and finally activates it. However, Na^+ channels can be activated equally well by native *Paramecium* calmodulin or by *E. coli*-produced (recombinant) calmodulin which lacks all post-translational modifications. Such modifications including N-terminal acetylation and methylation of Lys at 13 and 115 residues are apparently not important for Na^+ channel activation.

Golowasch et al. (1986) and Oberhauser et al. (1989) observed that Mg^{2+} and other divalent ions enhanced activity of Ca^{2+}-activated K^+ channels. In these examples, Hill coefficients for Ca^{2+} activation of K^+ channels increased from ~2 up to 10 in the presence of divalent ions. Besides an increase in Ca^{2+} bindings, they also observed that the affinity for Ca^{2+}-binding increased in the presence of these divalent ions. Though Mg^{2+} alone does not activate the channel, Ca^{2+} sensitivity of the *Paramecium* Na^+ channel (Fig. 1*B*) improves from 6 μM to 0.8 μM in the presence of 1 mM Mg^{2+} which is only 2–3 times higher than the estimated physiological Mg^{2+} concentration (Preston, 1990). For calmodulin-activated channels in particular, Mg^{2+} may substitute for Ca^{2+} in calmodulin binding. It is, however, unlikely that Mg^{2+} increases Ca^{2+} binding to calmodulin (Ohki et al., 1993). Mg^{2+} is known to bind to calmodulin, possibly to the Ca^{2+}-binding pockets I and II with dissociation constants of 0.5 mM (Tsai et al., 1987), or to other portions of the calmodulin molecule (Klee, 1988). Alternatively, Ca^{2+}-calmodulin affinity for channels may increase in the presence of Mg^{2+}. Mg^{2+} increases calmodulin binding

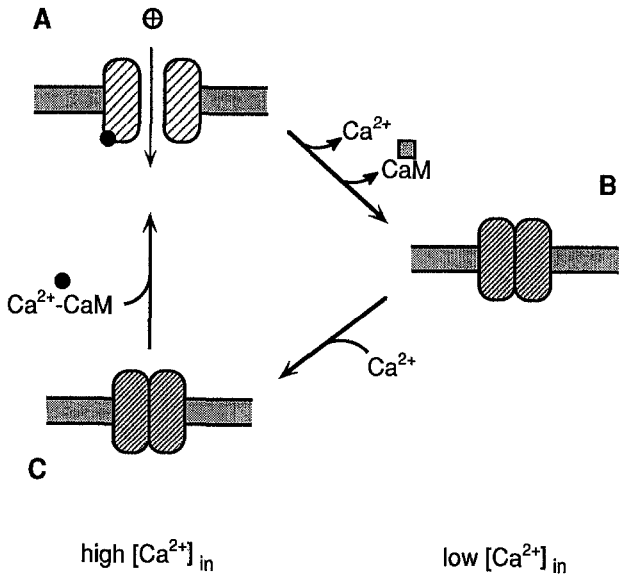

Fig. 6. A diagram on inactivation and activation of the Na^+ channel. In the beginning of the experiment, Na^+ channels are captured in their active form in the presence of high Ca^{2+}. (*A*) Low Ca^{2+} exposure not only decalcifies calmodulin immediately, thereby quieting channel activity, but also detaches decalcified calmodulin from the channel after low Ca^{2+} perfusion for a few more minutes, rendering the channel inactivated. (*B*) The channel deprived of calmodulin remains inactive even after high Ca^{2+} is replenished (*C*), until Ca^{2+}-calmodulin is provided (*A*).

affinity for ryanodine receptor channels, whereas the number of calmodulin bindings to the channel decreases (Yang et al., 1994), though the binding sites have not yet been identified definitively. In the example of calcineurin, a Ca^{2+}-calmodulin dependent protein phosphatase, the presence of Mg^{2+} increases its activity. This effect remains after limited proteolysis rendering calcineurin calmodulin independent. This indicates that the Mg^{2+} binding site is on the catalytic subunit itself and perhaps not on calmodulin (Hubbard & Klee, 1989).

Mechanisms of Channel Regulation

There is no molecular information on the Na^+ channel structure of *Paramecium* available to date. Although possibilities remain that calmodulin sensitivity of channels can be conferred by calmodulin binding to cytoskeletal elements as has been suggested for vertebrate photoreceptor channels (Hsu & Molday, 1993), here we draw a parallel with other calmodulin-dependent enzymes. Figure 6 depicts our view on the gating mechanism of the Ca^{2+}-calmodulin dependent channel. For the sake of simplicity, we invoke direct binding of calmodulin to the Na^+ channel protein, either the pore-forming subunit or another subunit. In low Ca^{2+}, decalcified calmodulin comes off the Na^+ channel (Fig. 6, right). The channel without bound calmodulin normally is inactivated even in the presence of high Ca^{2+} (Fig. 6, low left). Neither high voltage nor high Ca^{2+} alone can restore channel activity. When calmodulin is present together with 10^{-5} M or higher Ca^{2+}, Ca^{2+}-calmodulin then binds to the calmodulin-binding domain of the channel and activates the channel (Fig. 6, upper left). Once bound, calmodulin remains attached to the channel for an extended period even after the bulk calmodulin is washed away as long as Ca^{2+} is maintained at a high concentration. In the case of calmodulin regulated enzymes, calmodulin-binding domains sterically interfere with enzymatic reaction centers, dubbed autoinhibitory domains. They can be cleaved by limited proteolysis, leading to permanently active forms of enzymes without Ca^{2+}-calmodulin requirement.

Besides channel regulation through dephosphorylation and phosphorylation in the cascade of Ca^{2+} signal transduction, the Na^+ channel in *Paramecium* and the other calmodulin-sensitive channels present a new mechanism of channel regulation through protein-protein interactions. This direct calmodulin-channel interaction parallels the G-protein regulation of various ion channels (Brown, 1993; Clapham & Neer, 1993).

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