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Two Ion-Selecting Filters in the Calcium Channel of the Somatic Membrane of Mollusc Neurons

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Summary. The slow inward current carried by Na^+ through potential-dependent calcium channels in conditions when divalent cations were removed from the extracellular solution by EDTA has been investigated on isolated internally perfused neurons of the snail *Helix pomatia*. The calcium channels also acquire the capability to pass monovalent cations if other calcium-binding substances are added to the extracellular solution. Based on these facts the conclusion is made that the immediate reason for the modification of the channel selectivity is the absence of divalent cations in the extracellular medium. All potential-dependent characteristics of the modified calcium channel are shifted by 60 to 70 mV in the hyperpolarizing direction compared with those of the original calcium channel. The series of relative permeabilities for modified calcium channels towards monovalent cations ($P_{Na^+}:P_{Li^+}:P_{N_2H_5^+}:P_{NH_3OH^+}=1.0:0.8:0.55:0.21$) is close to that of common "fast" sodium channels ($P_{Na^+}:P_{Li^+}:P_{N_2H_5^+}:P_{NH_3OH^+}=1.0:1.04:0.44:0.21$). The induced sodium current decreases immediately when the concentration of divalent cations in the extracellular solution is elevated. This decrease is not potential dependent and can be approximated by Langmuir's isotherm with dissociation constants $pK_{Ca}:pK_{Sr}:pK_{Ba}:pK_{Mg}=6.6:5.5:4.8:4.2$. The conclusion is drawn that the calcium channels in the somatic membrane have two ion-selecting filters with different functions – an external one consisting, probably, of several carboxylic groups which bind divalent cations in a highly specific manner and determine the impermeability of the channel to monovalent cations in physiological conditions, and the channel ion-selecting filter including a single carboxylic group normally determining the channel selectivity for different divalent cations.

Key Words ion selectivity · calcium channel · mollusc neurons

Introduction

Recently it has been shown on several electrically excitable membranes which possess a specific calcium conductance that the addition of calcium-chelating substances to the extracellular calcium-free solution containing Na^+ induces a peculiar slowly developing potential-dependent inward current. In the experiments of Kostyuk and Krishtal (1977*b*) and Krishtal (1978) on intracellularly perfused isolated neurons from the mollusc *Helix pomatia* this current was directly recorded after addition of EDTA or EGTA. Their effective concentrations were 0.1 to 1.0 mM. This "EGTA-induced current" decreased by 40 to 60% after addition of verapamil (5×10^{-5} M) or its derivative D-600 (10^{-5} M) to the extracellular solution. As these substances are known as calcium-channel blockers, a conclusion was drawn that the induced sodium current passes through calcium channels which acquire the capability to pass monovalent ions in such experimental conditions.

Indirect data indicating that Na^+ can carry considerable currents through calcium channels after addition of chelating substances to the extracellular solution have been obtained by Curtis and Prosser (1977) who observed large action potentials in smooth muscle fibers after substitution for Ca^{2+} by Na^+ in the extracellular solution in the presence of millimolar concentrations of EGTA or EDTA. These action potentials were TTX-insensitive but they could be blocked by addition of Mn^{2+}, Co^{2+}, La^{3+} or verapamil. They also disappeared after replacement of Na^+ by Li^+ or $Tris^+$. Palade and Almers (1978) on frog striated muscle fibers observed in calcium-free extracellular solutions a slowly rising sodium current which disappeared after substitution for Na^+ by TMA^+ or TEA^+ and could be blocked by 1 mM Ca^{2+}. When 10 mM Ca^{2+} were introduced into the extracellular solution, this current was replaced by another inward current which did not change after replacement of Na^+ by TMA^+ but depended on the external concentration of Ca^{2+}. Yamamoto and Washio (1979) observed on muscle fibers of the mealworm, *Tenebrio molitor*, the disappearance of the action potential after removal of Ca^{2+} from the extracellular solution. Addition of sodium ions restored the excitability; however, it could be depressed again by small concentration of Ca^{2+}. The authors suggested the presence of calcium channels

Table. Composition of basic extracellular solutions

Salts	Solutions (mM)			
	Normal	Ca-free EDTA-containing	Ca-free	Na-free
NaCl	100	30–50	120	–
KCl	2.5	2.5	2.5	2.5
$CaCl_2$	10	–	–	10–30
$MgCl_2$	5	–	13	5
Tris-HCl	25	120–100	10	125–95
EDTA	–	1	–	–

in this preparation which become permeable to Na^+ in the absence of extracellular Ca^{2+}.

Obviously, more detailed investigations of this modification and especially of the mechanisms which determine the changes in channel selectivity are very important, as they may throw light upon the structure of both calcium and sodium channels. In the present work a complex study of the properties of the modified calcium channels in the somatic membrane of snail neurons has been made using the intracellular perfusion technique which allows a precise separation of necessary components of the transmembrane current.

Materials and Methods

Experiments were made on nonidentified isolated intracellularly perfused neurons of the snail *Helix pomatia* under voltage clamp. The technique of cell isolation and intracellular perfusion did not differ from that already described (Kostyuk, Krishtal & Doroshenko, 1974; Kostyuk & Krishtal, 1977*a*; Kostyuk, Krishtal & Pidoplichko, 1981). The composition of the main extracellular solutions is given in the Table. To exclude the outward currents, K^+ in the perfusing solution was replaced by $Tris^+$. Intracellular pH was kept at 7.2 to 7.3 by Tris-aspartate, and extracellular pH was fixed at 7.6 to 7.7 by Tris-HCl buffers. For measurements of sodium current reversal potentials, 30 mM NaCl were added to the intracellular perfusing solution.

It should be noted that the cell membrane becomes extremely unstable in solutions without divalent cations. In most cases a progressive increase in leakage current started after placing the cell in a calcium-free EDTA-containing solution. A partial stabilizing effect could be obtained by lowering the temperature to 8 to 12 °C. In such conditions a stable functioning of the cell membrane could be obtained in some cases for 30 to 40 min and all experiments were done in this temperature interval.

The voltage clamping was performed, using on Ag/AgCl/3 M KCl intracellular electrode, through a perfusing pore of the plastic suction pipette; a similar electrode connecting the extracellular solution to the virtual ground amplifier was used for current measurements. It must be noted that we always used pores of maximum possible diameters and the cells with relatively small dimensions (average diameter 100 μm). The series resistance in our experiments was usually in the range of 100 kΩ which could give an error in potential measurements for currents ranging from 50 to 100 nA equal to 5 to 10 mV.

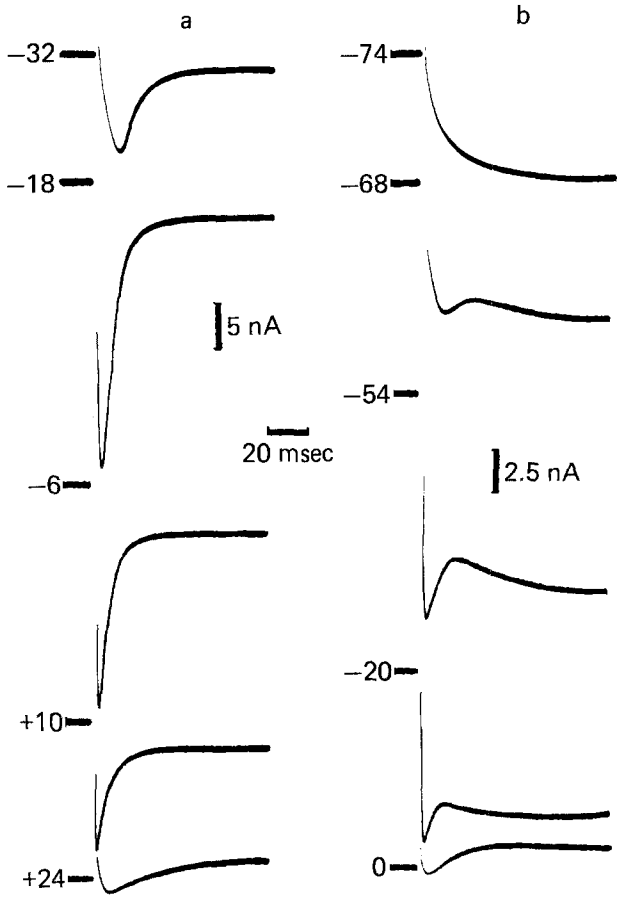


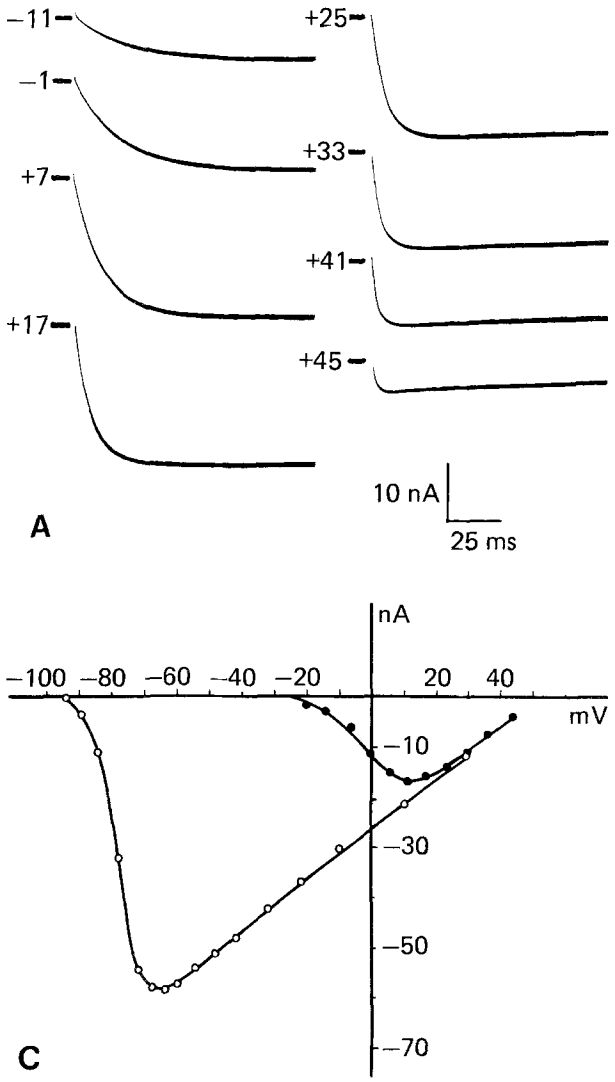
Fig. 1. Effect of replacement of normal external solution (*a*) by Ca-free EDTA-containing solution (*b*). Holding potentials −60 mV (*a*) and −100 mV (*b*). Numbers near current curves indicate corresponding membrane potentials

In some cells the induced current was so large that it became impossible to control the membrane potential by the voltage-clamp system; the loss of control was manifested by the appearance of "all-or-none" current responses. In this case a decrease in $[Na^+]_{ext}$ could help in decreasing the induced current and clamping the system. Since for the recording of induced sodium currents the holding potential had to be kept at −100 mV (*see* Results), it was impossible to use summation of the equal de- and hyperpolarizing potential shift responses to eliminate the capacitative and leakage currents: at strong hyperpolarizing shifts the membrane became irreversibly damaged. Therefore the leakage current was compensated by analog technique using an additional current amplifier (Kostyuk & Krishtal 1977*a*).

Results

Potential-Dependence of the Induced Sodium Current

In most cells two inward sodium current components could be observed after replacement of normal external saline by Ca-free EDTA-containing solution – a fast one reaching the maximum in about 5 msec and a slow "noninactivating" one. Figure 1 presents inward currents recorded from



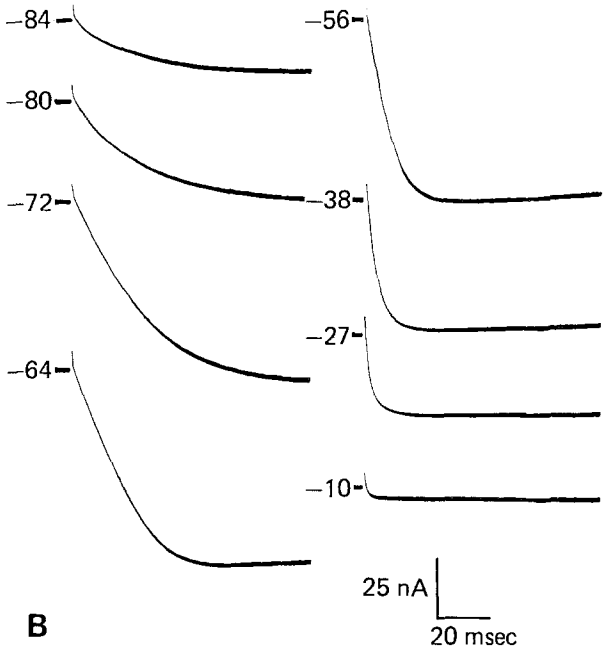


Fig. 2. Potential dependence of the induced sodium current. Records of currents obtained in Na-free solution with 30 mM $CaCl_2$ (*A*) and in Ca-free EDTA-containing external solution with 30 mM NaCl (*B*). Intracellular solution: 120 mM Tris-aspartate + 30 mM NaCl. Holding potential −100 mV. (*C*) Current-voltage characteristics of the calcium (30 mM $CaCl_2$ outside, dots) and induced sodium (30 mM NaCl outside, circles) currents in the same cell

the same cell first in Ringer's solution containing 100 mM Na^+ + 10 mM Ca^{2+}, and then in Ca-free EDTA-containing solution with 30 mM Na^+. The initial fast current component decreased in amplitude after such a change (due to a decrease of the external sodium concentration); it represents the activation of common fast sodium channels. The slow component in the former case corresponds to the flow of calcium ions through the calcium channels (*cf.* Kostyuk & Krishtal, 1977*a*; Akaike, Lee & Brown, 1978). Its amplitude highly increased (despite the decrease in $[Na^+]_{ext}$) in Ca-free EDTA-containing solution when it became a sodium current. In the present paper this component will be designated as the "induced sodium current." The amplitude of the slow component of the sodium current depended on the amplitude of the calcium current present in the same cell when bathed in normal Ringer's saline. In cells which normally demonstrated negligible calcium currents, the induced sodium currents were also very small. On the contrary, in other cells the fast sodium conductance was very small and the calcium conductance was high; so, practically pure, slow induced currents could be observed. These types of cells were especially convenient for the analysis of the induced sodium currents. An example of such pure, slow inward currents evoked by depolarization of the membrane potential is shown in Fig. 2*A*.

The induced currents were activated at membrane potential 60 to 70 mV more negative than that necessary to activate the calcium currents in the same cell (*see* Fig. 2*B*), and reach maximum value at potentials of about −60 mV. At increasing depolarizations the induced currents diminished approaching zero at potentials near +50 mV. No current reversal could be observed

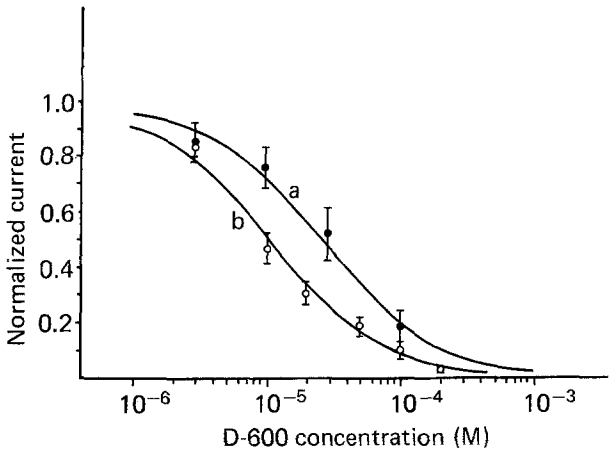


Fig. 3. Dose-effect dependence for the blocking action of D-600 on the calcium (*a*) and induced sodium (*b*) currents. Measurements on four cells are presented. Vertical bars – SD of values. Solid lines – Langmuir's isotherms with $K_M = 2.6 \times 10^{-5}$ M and $K_M = 10^{-5}$ M, respectively, for the calcium and induced sodium currents

at more positive potentials. However, clear reversal could be observed if Na^+ ions were introduced into the cell. When both extra- and intracellular solutions contained 30 mM Na^+, the reversal of a pure sodium current (according to the Nernst equation) should appear at zero potential. For five cells the average value was -6 ± 2 mV ($\pm$SEM), which is in the range of our experimental error due to possible effects of diffusion potentials at electrodes and to series resistance.

Blocking of the Induced Sodium Current

The use of such competitive calcium channel blockers as Co^{2+}, Cd^{2+} and Mn^{2+} for the induced sodium current was impossible, because EDTA binds these ions much stronger than does Ca^{2+}. After addition to the extracellular solution these ions would form complexes with EDTA and as a result their blocking effect would disappear. For this reason we used in our experiments only organic calcium channel blockers as D-600 (derivative of verapamil) and niphedipine.

Figure 3 shows the concentration dependence of the blocking effect of D-600 on the calcium and induced sodium currents. The experimental data are approximated by Langmuir's isotherms with $K_d = 2.6 \times 10^{-5}$ M and 10^{-5} M for calcium and induced sodium currents, respectively. The results indicate that after modification the calcium channel still possesses a binding site which seems to bind D-600 even more effectively. If should be noted that in both cases the blocking effect was potential dependent decreasing with the increase in the depolarizing membrane potential shift. This finding seems to indicate that the site which binds D-600 is located not at the outer surface of the membrane but inside the channel.

The difference observed in the effectiveness of blocking the induced sodium and calcium currents may have several reasons. (1) The removal of Ca^{2+} from the external solution greatly increases the negative surface charge of the membrane and, consequently, the near-membrane concentration of D-600 (which is positively charged) may increase producing stronger block. (2) The removal of Ca^{2+} may alter the binding of D-600 in the channel if both compete for the same binding site.

The estimation of the former possibility can be made assuming that the near-membrane concentration of D-600 (a single charged cation) should change exp $(-\varphi F/RT)$ times compared with the concentration in the bulk solution, where φ is the surface potential and F, R, and T have their usual meanings (*cf.* Kostyuk, Mironov & Doroshenko, 1982*a*). As a result, the dissociation constant should also increase exp $(-\varphi F/RT)$ times. Taking the surface potential values in the presence of 30 mM Ca^{2+} and in the absence of these ions as -7 and -25 mV (Kostyuk et al., 1982*b*), the real dissociation constants should be 3.38×10^{-5} M and 2.8×10^{-5} M for the calcium and the induced sodium currents, respectively. Such a small difference in the blocking constants is in the range of experimental error and may indicate that the first possibility is correct; therefore it seems that there is no need to suggest that D-600 and Ca^{2+} compete for the same binding site in the channel.

Similar results have been obtained with niphedipine. The experimental blocking constants for the calcium and the induced sodium currents were 2.3×10^{-5} and 0.8×10^{-5} M. After correction for the effect of surface charges they became 3×10^{-5} and 2.25×10^{-5} M, respectively.

A highly specific way to block the calcium conductance in the somatic membrane is the replacement of usual intracellular anions by fluoride (Kostyuk & Krishtal, 1977*a*). It was important to investigate the effect of these anions on the induced sodium currents. Figure 4*A* presents the changes of the amplitude of this current after the beginning of cell perfusion with fluoride-containing solution. In about 25 min the induced sodium current disappeared completely. This relatively slow decline might be due to low temperature (~ 10 °C); it has been shown that the blocking effect of internal fluoride on calcium conductance is extremely temperature dependent (Kostyuk et al., 1981; Doroshenko, Kostyuk & Martynyuk, 1982).

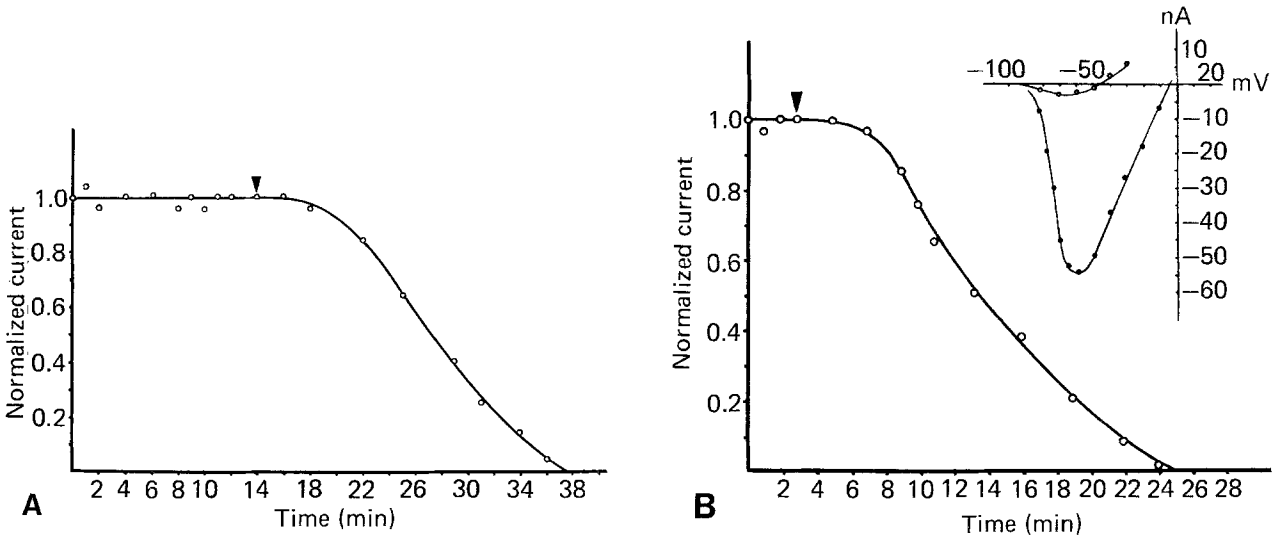


Fig. 4. Kinetics of reduction of the induced sodium current during cell perfusion with 150 mM Tris-F (*A*) and 150 mM Tris-aspartate + 5×10^{-4} M $CaCl_2$ (*B*) solutions. Arrow shows the start of perfusion. Inset demonstrates the current-voltage characteristics of the sodium current before cell perfusion with Ca-containing solution (dots) and 20 min after its beginning (circles). Solid lines are drawn by eye

The calcium channels in the somatic membrane are also very sensitive to the increase in intracellular Ca^{2+} concentration (Kostyuk & Krishtal, 1977*b*; Akaike et al., 1978). Elevation of $[Ca^{2+}]_{int}$ to 10^{-6} M completely blocks the calcium conductance. A similar effect was also observed for the induced sodium currents. They disappeared in about 20 min (*see* Fig. 4*B*) during cell perfusion with a solution containing 5×10^{-4} M Ca^{2+}.

Selectivity of the Modified Calcium Channels

The relative selectivity of the modified calcium channels and the common original "fast" sodium channels to different monovalent cations has been determined from the shifts of the reversal potentials of the corresponding inward currents after the replacement of sodium by other ions in the external solution using the following equation (Hille, 1975):

$$E_{Na} - E_X = \frac{RT}{F} \ln \frac{P_X[X]_{ext}}{P_{Na}[Na]_{ext}} \quad (1)$$

where the symbol X denotes the substituting ions and E_X is the reversal potential. Since it is difficult to block potassium channels in the somatic membrane of mollusc neurons we chose those monovalent cations which, according to Hille (1975), do not pass through potassium channels but are permeable through sodium ones. The most suitable of them are Li^+, $N_2H^+_5$ (hydrazynium) and NH_3OH^+ (hydroxylammonium).

Figure 5 presents records of the fast and induced sodium currents and the corresponding current-voltage relations used for the selectivity calculations. The relative permeabilities calculated according to Eq. (1) were for the fast sodium channels $P_{Li^+}/P_{Na^+} = 1.04 \pm 0.14$; $P_{N_2H_5^+}/P_{Na^+} = 0.44 \pm 0.03$; $P_{NH_3OH^+}/P_{Na^+} = 0.19 \pm 0.02$; and for the modified calcium channels $P_{Li^+}/P_{Na^+} = 0.8 \pm 0.15$; $P_{N_2H_5^+}/P_{Na^+} = 0.55 \pm 0.07$; $P_{NH_3OH^+}/P_{Na^+} = 0.21 \pm 0.02$ (mean values from measurements on five cells).

The obtained permeability series have only some quantitative differences indicating that the transport of monovalent cations through sodium and modified calcium channels may be based on similar mechanisms.

Effect of Divalent Cations on Modified Calcium Channels

To clarify the mechanism of modification of the calcium channels, we studied the effect of several EDTA homologs – EGTA (ethylenglycoldiaminotetraacetic acid), HMDTA (hexamethylendiaminotetraacetic acid), NTA (nitryltriacetic acid). All these substances being introduced into the extracellular solution modified the calcium channels and induced a slow inward sodium current with characteristic kinetics and selectivity. The amplitude of the induced currents correlated with the affinity of these substances to Ca^{2+} ions. This finding allowed us to suggest that the described modification

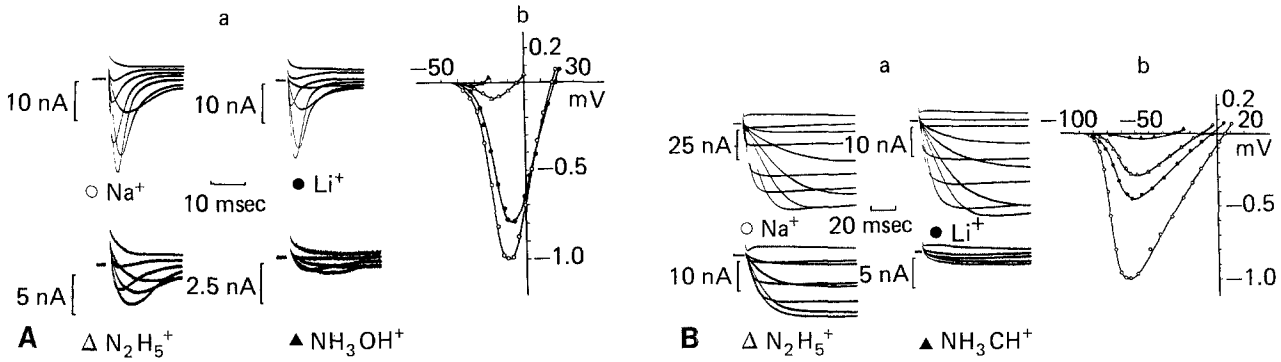


Fig. 5. Selectivity of fast sodium and modified calcium channels to monovalent cations. *A*. Records of currents through the fast sodium channels in solutions containing 120 mM Na^+, 120 mM Li^+, 120 mM $N_2H_5^+$ and 120 mM NH_3OH^+ (*a*). Intracellular solution: 100 mM Tris-F + 50 mM NaF. (*b*). Corresponding current-voltage characteristics. *B*. Records of currents through modified calcium channels in solutions with 50 mM Na^+, 50 mM Li^+, 50 mM $N_2H_5^+$ and 50 mM NH_3OH^+ (*a*). Intracellular solution: 120 mM Tris-aspartate + 30 mM NaCl. Holding potential −100 mV. (*b*) Corresponding current-voltage characteristics

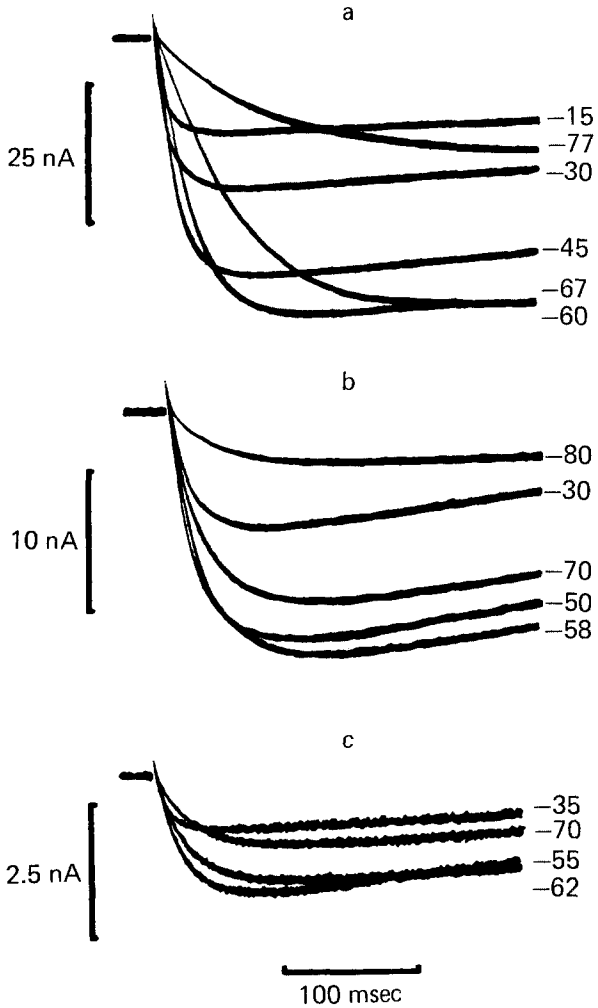


Fig. 6. Induction of sodium currents by different Ca-binding substances. Current records obtained from the same cell in solutions containing 1 mM EDTA (*a*), 120 mM Tris-citrate (*b*) and 120 mM Tris-phosphate (*c*). Extracellular solutions contained 30 mM NaCl in all cases. Intracellular solution: 120 mM Tris-aspartate + 30 mM NaCl. Membrane potentials are indicated near current curves. Holding potential −100 mV

of calcium channels is caused just by a drastic decrease of the level of free calcium ions in the extracellular medium.

To verify this suggestion, we also tested several other substances which effectively bind Ca^{2+} but differ in their structure from EDTA and its homologs. Figure 6 presents the records of currents obtained in solutions containing EDTA (*a*), citrate (*b*) and phosphate (*c*) ions. In all cases the concentration of Na^+ was kept constant (30 mM). A slow sodium current was induced in all cases, but its maximum amplitude was different. The measurement of free calcium level in these solutions using metallochromic indicator Arsenazo III (*cf.* Butler, 1964) has shown that the inverse value of the current amplitude was approximately linearly related to $[Ca^{2+}]_{ext}$, which varied between 10^{-5} and 10^{-7} M. Thus, it seems that the calcium channels possess a specific site which can bind Ca^{2+} in this range of concentrations.

It was therefore important to measure the affinity of this site to Ca^{2+} and to find out if it can also bind other divalent cations. For this purpose we investigated the dependence of the maximum amplitude of the induced sodium corrent on the extracellular concentration of Ca^{2+}, Sr^{2+}, Mg^{2+} or Ba^{2+}. The necessary free concentration of these cations was established by addition of EDTA to the stock solution containing a given initial concentration of divalent cation under investigation. The resulting concentration of the ion M^{2+} was calculated according to the equation

$$K'_{M\text{-EDTA}}[M\text{-EDTA}] = [M][\text{EDTA}] \qquad (2)$$

where symbols in brackets are the concentrations of the corresponding substances, and $K'_{M\text{-EDTA}}$ is the apparent dissociation constant for the complex

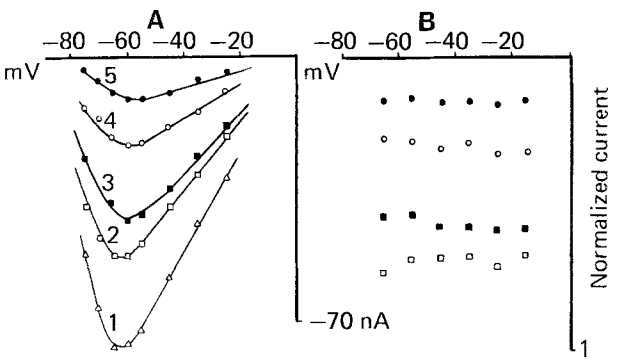


Fig. 7. Induced sodium currents at different concentrations of extracellular Ca^{2+} ions. *A*. Current-voltage curves of the induced sodium current obtained at $[Ca^{2+}]_{out}$ 3×10^{-8} M (1), 1.2×10^{-7} M (2), 2.4×10^{-7} M (3), 4×10^{-7} M (4) and 1.2×10^{-6} M (5). External solution contained 30 mM NaCl. Intracellular solution: 120 mM Tris-aspartate + 30 mM NaCl. Holding potential −100 mV. *B*. Relative reduction of the induced sodium current with the increase of external Ca^{2+} concentration depending on the potential. Current value at $[Ca^{2+}]_{out}=3\times10^{-8}$ M is taken as unity

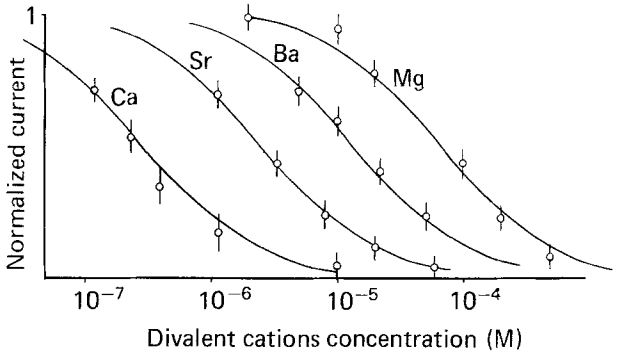


Fig. 8. Dose-effect dependences for the blocking effect of different divalent cations on the induced sodium current. Circles – experimental values. Vertical bars – SD of values for five cells. Solid lines – Langmuir's isotherms. Corresponding dissociation constants are indicated in the text

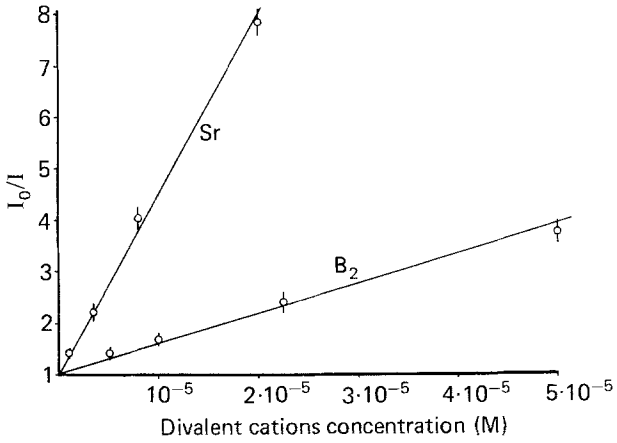


Fig. 9. Example of the determination of dissociation constants during block of the induced sodium current by Sr^{2+} and Ba^{2+} ions (*see* text). Circles – mean experimental values; vertical bars – SD of values for five cells

of EDTA with the ion M^{2+}, which depends on the pH of the solution. The latter is related to the true dissociation constant in the following way (Butler, 1964)

$$K'_{M\text{-EDTA}}=K_{M\text{-EDTA}}(1+[H^+]/K_{H^+}) \qquad (3)$$

where K_{H^+} is the first dissociation constant of the acid, i.e. EDTA. As the pH values of the extracellular solutions were 7.6 to 7.7, according to Eq. (2) the following effective dissociation constants of the complex *M*-EDTA were used for calculations: $Ca^{2+}-10^{-8}$ M; $Mg^{2+}-10^{-6}$ M; $Sr^{2+}-1.7\times10^{-6}$ M; $Ba^{2+}-10^{-5}$ M. The values of K_{H^+} and $K_{M\text{-EDTA}}$ were taken from Butler (1964).

Figure 7*A* presents the current-voltage characteristics of the induced sodium current obtained at different extracellular concentrations of free Ca^{2+} ions. They show that the amplitude of the current decreased with the increase in $[Ca^{2+}]_{ext}$. This decrease did not depend on the applied potential (*see* Fig. 7*B*) indicating that the suggested active center of the calcium channel responsible for its modification in the absence of extracellular calcium is located near the external surface of the membrane.

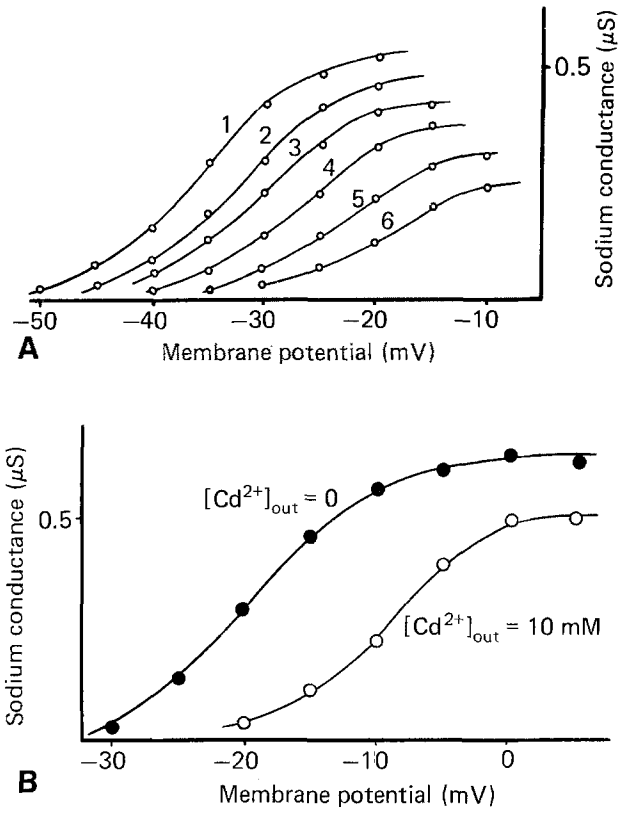
A similar effect was also produced by other divalent cations; however, in this case the suppression of the induced sodium current occurred at higher ionic concentrations. If one suggests that the modification of the calcium channel is evoked by removal of one divalent cation from the active center, then the observed concentration dependence of the induced sodium current should be described by Langmuir's isotherm

$$I/I_o=K_M/(K_M+[M]) \qquad (4)$$

where $I_o=I$ at $[M]=0$, and K_M is the dissociation constant for the complex of the divalent cation with the active center considered. Data presented in Fig. 8 confirm this suggestion. For each cation the value K_M was determined by plotting the experimental data according to equation

$$I_o/I=1+[M]/K_M. \qquad (5)$$

Examples of such determinations for Ba^{2+} and Sr^{2+} are shown in Fig. 9. The calculated mean values of K_M (data from five cells) were $K_{Ca}=2\times10^{-7}$ M, $K_{Sr}=3.5\times10^{-6}$ M, $K_{Ba}=1.4\times10^{-5}$ M, $K_{Mg}=6\times10^{-5}$ M.



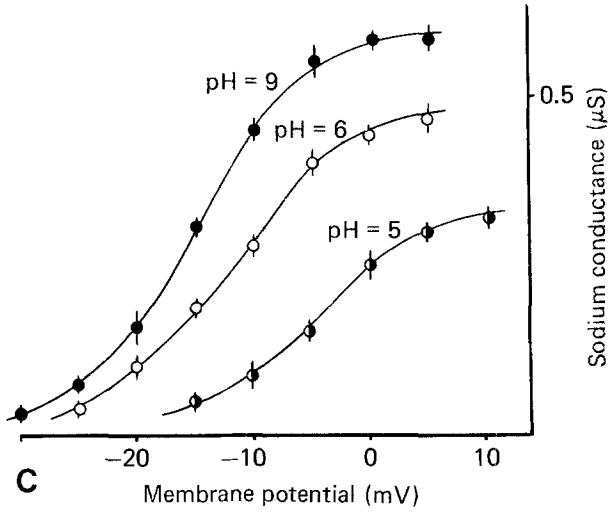


Fig. 10. Effect of Ca^{2+}, Cd^{2+} and H^+ ions on sodium conductance. All data represent the averages on five cells. *A*. Potential-dependence of the sodium conductance at following $[Ca^{2+}]_{out}$: 2 mM (1), 5 mM (2), 10 mM (3), 20 mM (4), 50 mM (5) and 100 mM (6). Extracellular solution contained 60 mM NaCl, intracellular −190 mM Tris-F + 30 mM NaF. Tonicity of the intra- and extracellular solutions was increased by 1.5 times. Holding potential −60 mV. *B*. The same after addition of 10 mM Cd^{2+}, being calculated from the data of Krishtal (1976). *C*. The same at different pH of the external solution, being calculated from the data of Kostyuk et al. (1982*b*)

The Effect of Divalent Cations on the "Fast" Sodium Channel

To compare the functional structure of the modified calcium and common sodium channels in more detail, we have also investigated the effect of extracellular Ca^{2+} on the "fast" sodium currents. Contrary to the modified calcium channel, the effect of Ca^{2+} ions on the fast sodium current starts to develop not in the micromolar but in the millimolar range of their concentration. To increase the range of possible changes in $[Ca^{2+}]_{ext}$ in the presence of 60 mM Na^+, the tonicity of both extracellular and intracellular solutions (the latter contains also 30 mM Na^+) was increased 1.5 times by addition of $Tris^+$. The reversal potential for the fast sodium current did not alter during the changes of the extracellular Ca^{2+} concentration. Its mean value was 15 ± 3 mV, which is close to the Nernst potential for an equilibrium sodium electrode. This value was used for the calculation of sodium conductance, and Fig. 10*A* presents the dependence of the latter on the membrane potential at different $[Ca^{2+}]_{ext}$. This Figure shows that both a shift of the activation curve in depolarizing direction and a decrease in limiting conductance with an increase in extracellular calcium occur. The former effect is well known and represents the change of the surface charge of the membrane due to a binding of Ca^{2+} to its fixed anionic groups. The latter effect may be explained by either surface charge changes leading to a decrease in the near-membrane concentration of Na^+ because of the decrease in negative surface potential or by direct blocking of the sodium channels by Ca^{2+}. The evaluation of the first possibility can be made assuming a linear dependence of the amplitude of the sodium current on the concentration of carrier ions near the membrane; in this case the limiting sodium conductance should decrease exp $(\Delta\varphi F/RT)$ times when the surface potential decreases by the value $\Delta\varphi$ (*see also* Mironov, 1983).

When the concentration of Ca^{2+} ions in the extracellular solution was equal to 2, 5, 10, 20, 50 and 100 mM, the limiting conductances g_{Na} were related as 1:0.86:0.79:0.72:0.61:0.50, respectively (Fig. 10*A* averaged on five cells). If we determine the values of surface potential shift from the given g_{Na} curves and use them for the determination of the change of near-membrane concentration of Na^+ ions, we obtain an almost identical sequence of g_{Na}: 1:0.89:0.81:0.77:0.63:0.53.

Similar effects were observed for Cd^{2+} ions: the addition of 10 mM Cd^{2+} to the external solu-

tion leads to the shift of g_{Na} curve in depolarizing direction by 10 mV (Fig. 10*B*). This change of the surface potential value should decrease the limiting conductance 1.5 times. From Fig. 10*B* we see that just this decrease in g_{Na} is actually observed. It is important to emphasize that the decrease of the sodium conductance due to lowering of pH of the extracellular solution (Fig. 10*C*) could be interpreted in a similar way: the observed sequence of g_{Na} values was 1:0.80:0.55 for pH values 9.0, 6.0 and 5.0, respectively, and that calculated on the basis of surface potential shift was 1:0.88:0.63.

These observations show that contrary to the calcium channel and the modified calcium channel, Ca^{2+}, Cd^{2+} and H^+ ions may affect normal sodium conductance in an indirect way – through the corresponding change in the surface membrane potential and in the near-membrane concentration of permeant ions.

Discussion

The present paper is mainly devoted to the investigation of the molecular organization of those structures that control the selectivity of sodium and calcium channels in mollusc neurons. In normal physiological conditions the sodium channel is mainly permeable to sodium or other monovalent cations (*cf.* Hille, 1975) and the calcium channel transports exclusively divalent cations (Kostyuk, 1981). However, in the present work it was shown that calcium channels can be transformed in such a state in which they act as a sodium transporting system. This effect is achieved by using Ca-free extracellular solution containing EDTA or other Ca-binding substances. The transformation is intimately connected with the removal of a single Ca^{2+} ion from some highly specific Ca-binding site of the calcium channel. Experimentally determined pK value of the corresponding dissociation constant equals 6.6. This binding is not potential dependent indicating the location of this site near the external surface of the membrane. The decrease of $[Ca^{2+}]_{ext}$ below 0.1 μM makes this site free, and the channel acquires the ability to pass monovalent ions demonstrating selectivity towards them similar to that of common sodium channels. As the function of this Ca-binding site is to regulate the selectivity of the calcium channel (possibly through some conformational changes) towards mono- and divalent cations, it can be designated as "the external ion-selecting filter" of the calcium channel.

Experimentally obtained dissociation constants for complexes of alkaline-earth cations with this external binding site are related as $pK_{Ca}:pK_{Sr}:pK_{Ba}:pK_{Mg}=6.6:5.5:4.8:4.2$. This sequence is similar to a series of the corresponding EDTA-complexes which is $pK_{Ca}:pK_{Sr}:pK_{Ba}:pK_{Mg}=10.6:8.6:7.4:8.7$ (Butler, 1964). It should be noted that the complexes of divalent cations with EDTA have the structure of octahedron with the cation in its center. Six ligands – four carboxylic groups and two tertial nitrogen atoms (acting as donors of a lone electron pair) are situated in its apices. The obtained value for pK_{Ca} strongly resembles pK_{Ca} values for different Ca-binding proteins such as parvalbumin, troponin C, and calmodulin. The high affinity of these proteins to Ca^{2+} is also thought (Kretzinger & Nelson, 1976) to be determined by the presence in their active center of several carboxylic groups belonging to aspartate and glutamate amino acid residues. Therefore, we suggest that the high affinity of the external Ca-binding site is also due to the presence of serveral carboxylic groups.

When all divalent ions are removed from the extracellular solution, the potential-dependent characteristics of the calcium channels shift in the hyperpolarizing direction by 60 to 70 mV. The same effect is also observed for the fast sodium channels but in this case they shift only by 25 to 30 mV. It is clear that this difference cannot be explained only on the basis of the surface potential changes caused by complete desorption of Ca^{2+} as earlier we have shown that the surface charge density near calcium and sodium channels is practically identical (Kostyuk et al., 1982*b*). It seems probable that the removal of Ca^{2+} from the external binding site of the calcium channel strengthens the negative local electric field near its external mouth. This suggestion is in agreement with the above conclusion about the presence of several anionic groups in the external ion-selecting filter of the calcium channel.

In our previous paper (Kostyuk et al., 1980*a*) it was shown that the calcium channel has binding sites located inside it. One of them is lo-cated about 1/3 of the way down the channel and another one may be present near the internal mouth of the channel. The presence of the latter site is still speculative; however, the characteristics of the former are now well defined. This site seems to contain only one carboxylic group and it controls channel permeability for different divalent cations. The stronger the binding of the corresponding ion to the carboxylic group, the lower is the permeability of the channel. For example, transition metal ions form strong complexes with the carboxylic group ($pK > 3$) and they are blockers of the calcium conductance.

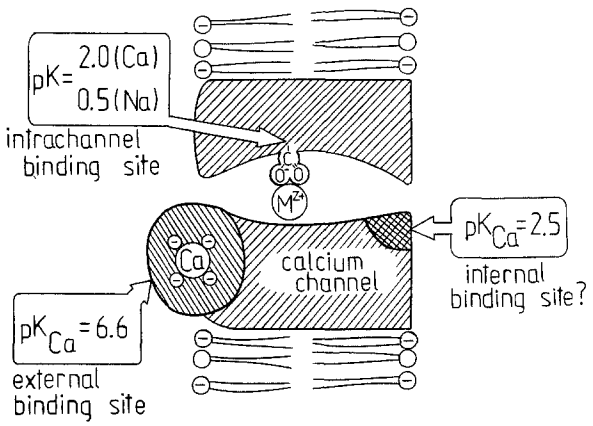


Fig. 11. Schematic description of the calcium channel

In the present paper we shall call it the intrachannel binding site or intrachannel ion-selecting filter. This intrachannel binding site has much lower affinity towards divalent cations than the external one and the corresponding dissociation constants differ 3 to 4 orders in magnitude. Thus, these sites cannot lie in series in the channel, otherwise (using the similar arguments as in the previous work, Kostyuk et al., 1982*a*) it can be shown that the single calcium channel currents would be 2 to 3 times lower than measured (*cf.* Kostyuk, 1981).

As to the sodium channel binding site, Hille (1975) proposed a model of ion-selecting filter consisting of six carbonyl (—C=O) groups. He suggested that its capability to pass monovalent ions depends on their size and possibility to from hydrogen bonds with carbonyl groups. To account for his experimental observation on the decrease of the sodium conductance in external solutions of low pH, Hille (1975) had to postulate that one of these carbonyl groups belongs to the charged carboxylic group $\left(-C\begin{matrix}O^-\\ \diagdown\!\!\!=\\ O\end{matrix}\right)$.

This model seems to fit also the calcium channel ion-selecting filter because the selectivity properties of the modified calcium channel of mollusc neurons and of many other excitable membranes towards the monovalent cations are quite similar. Moreover, the presence in Hille's model of a single carboxylic group would ensure the necessary selectivity properties of the calcium channel towards divalent cations.

However, in the case of sodium channels of the somatic membrane of mollusc neurons our experimental data contradict Hille's model. In fact, if we suppose the presence of the carboxylic group in the ion-selecting filter of the sodium channel, then H^+, Ca^{2+} and Cd^{2+} ions must be the most effective blockers of sodium conductance, because they strongly bind to the carboxylic group (Martell & Smith, 1977; Kostyuk et al., 1982*a*). As shown above, they actually decrease the sodium conductance. We have convincingly shown that this effect can be explained without the assumption of their binding inside the sodium channel. The observed decrease of the sodium conductance is solely determined by lowering of the near-membrane concentration of Na^+ ions caused by a decrease of a negative surface potential (*see* Results). In addition, it can be theoretically shown (Mironov, 1983) that the effect of the surface potential change is also potential dependent and the "block" of H^+, Ca^{2+} and Cd^{2+} ions must be less efficient for more positive values of the membrane potential, as it is actually observed in experiments (*cf.* Hille, 1975).

Thus, contrary to the calcium channel, the assumption about the existence of an acidic binding site in the sodium channel does not seem to be necessary for the description of the experimental data now available. Therefore, it seems reasonable to modify the model proposed by Hille (1975) for the ion-selecting filter of the sodium channel replacing the ionized carboxylic group $\left(-C\begin{matrix}O^-\\ \diagdown\!\!\!=\\ O\end{matrix}\right)$ by a neutral carbonyl one (—C=0) which possesses similar donor properties. Recollecting the fact about small differences between the relative permeabilities of the fast sodium channel and the modified calcium channel in mollusc neurons (the permeability of sodium channel towards Li^+ is higher and towards organic cations is lower for the modified calcium channel), we can suggest that this effect might probably reflect the absence of a carboxylic group in the sodium channel.

Thus, the presented hypothesis about the molecular organization of the calcium channel can be summarized as follows (Fig. 11). The channel is a pore with a binding site inside it (the intrachannel ion-selecting filter). This filter can pass monovalent ions on the basis of their steric correspondence to its section and divalent ions on the basis of their relative binding capacity to the carboxylic group present in this filter. At the external mouth of the channel another ion-selecting filter is present. This site does not bind those ions which pass the calcium channel. Its function, instead, is to prevent monovalent ions from entering the channel in normal physiological conditions. This effect is achieved by the presence of a small amount of divalent ions in the external medium and probably is switched by some conformational transformation.

Finally, the problem of calcium channel blocking by D-600 and niphedipine should be raised. Our data indicate that the increase of the effectiveness of their blocking action after modification is only apparent and caused by a change in the near-membrane concentration of the drug. This finding excludes the possible competitive interaction between Ca^{2+} and these drugs at the intrachannel binding site. The potential dependence of their block indicates that they act also inside the channel. It seems that these drugs block the calcium channel in a more complex and specific way acting, probably, on a lipid part of the channel.

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