Extracellular Polyvalent Cation Block of Slow Na^+ Channels in *Xenopus laevis* Oocytes

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Abstract. Sustained depolarization of the *Xenopus* oocyte membrane elicits a slowly activating Na^+ current, thought to be due to the opening of sodium se lective channels These channels are induced to become voltage gated by the de polarization They show unconventional gating properties and are insensitive to tetrodotoxin (TTX) The present study was undertaken to evaluate the effect of extracellular multivalent cations (Ca^{2+}, Co^{2+}, Cd^{2+}, La^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Sr^{2+} and Zn^{2+}) on these TTX-resistant channels, either on membrane potential responses or on current responses Our data show that all the polyvalent cations used blocked Na^+ channels in a concentration-dependent manner The order of potency of the most efficient cations was $Co^{2+} < Ni^{2+} < Cd^{2+} < Zn^{2+}$, the re spective concentration required to cause a 50% decrease of Na^+ current was 0 9 ± 0 29, 0 47 ± 0 15, 0 36 ± 0 09 and 0 06 ± 0 02 mmol/l The comparison of the activation curves from controls and after treatment with the cations indicated a shift towards more positive voltages These results can be interpreted as due to the screening effect of divalent cations together with an alteration of the Na^+ channel gating properties We also show that divalent cations blocked Na^+ channels in an open state without interfering with the induction mechanism of the channels The possibility that cation block was due to a possible interaction between cations and SH groups was investigated, but a sulphydryl alkylating reagent failed to abolish Zn^{2+} block

Key words: *Xenopus* oocytes — Sodium channel — Divalent cations — Electrophysiology — Pharmacology

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Introduction

Voltage dependent sodium channels from brain, heart and skeletal muscles share sequence identity as well as structural and functional similarities However, they also exhibit marked differences in current kinetics, gating properties and pharmacological affinities (for review, see Fozzard and Hanck 1996) Their distinct pharmacological profiles allow classifying the sodium channels into three main classes according to their sensitivity to tetrodotoxin (TTX), saxitoxin and μ-conotoxin peptides More detailed studies have shown that Na^+ channels display different affinity for TTX the cardiac Na^+ channels require a much higher concentration for equivalent block than the brain and adult skeletal muscle (μ1) isoforms (Lombet et al 1982) In addition to their distinct sensitivity to various toxins, Na^+ channels have also been shown to be blocked to a different extent by divalent cations Cd^{2+} and Zn^{2+} have been reported to preferentially block TTX-resistant channels such as the cardiac isoforms (Frelin et al 1986)

Xenopus oocytes are known to possess different types of Na^+ selective channels (Kado et al 1979, Parker and Miledi 1987, Arellano et al 1995, Silberberg and Magleby 1997) Among these channels, two types are voltage-dependent However, they display distinct electrophysiological and pharmacological properties One type is a fast, transient channel, that is sensitive to TTX (Parker and Miledi 1987, Bourinet et al 1992, Krafte and Volberg 1992), the other type is a slow activating, non-inactivating, TTX-resistant channel requiring millimolar concentration of TTX to be completely blocked (Baud et al 1982) The experiments described in this paper were undertaken to investigate the possible sensitivity of these TTX-resistant sodium channels to polyvalent cations

The TTX-resistant Na^+ channels of *Xenopus* oocytes were first described by Kado et al (1979) These channels display rather unusual electrophysiological properties they slowly activate and appear to be incapable of a normal inactivation so that, under voltage clamp conditions, inward currents are maintained for many minutes (Kado and Baud 1981) Subsequent studies showed that in oocytes clamped to positive voltages for many seconds, the clamping current which initially is outward directed slowly becomes inward due to the slow activation of voltage-dependent channels highly selective for sodium ions (Baud et al 1982) These results were interpreted to mean that Na^+ channels are not excitable in the basal state but are activated by a long depolarization, the process by which the channels become potentially gatable has been described as the induction (Baud and Kado 1984) More recent reports have indicated that the induction of Na^+ channels involved an increased activity of a protein kinase C (Charpentier et al 1993) activated by the production of phospholipid metabolites by a phospholipase C (Charpentier et al 1995) and increased intracellular calcium concentration (Bossi et al 1998, Charpentier and Kado 1999) due to calcium release from $InsP_3$-sensitive stores (Charpentier and Kado 1999) In this paper, the slow sodium channels of the *Xenopus* oocytes are designated by using the term "sNa^+ channels"

The aim of the present work was to study the interaction of some polyvalent

cations with the sNa^+ channels. We showed that multivalent cations depressed Na^+ currents with different efficacy. The sNa^+ channels which are TTX-resistant were found to be more sensitive to Cd^{2+} and Zn^{2+} cations than to other divalent cations, like the TTX-resistant cardiac channels. We also showed that divalent cations exerted a voltage-independent block of open sNa^+ channels without alteration of the induction mechanism.

Materials and Methods

Oocyte preparation

Oocytes were obtained from mature, non-hormonally treated *Xenopus laevis* females (from Centre de Recherches de Biochimie Macromoléculaire, Montpellier, France). Pieces of ovary were excised from tricaine methane sulphonate (0.2%) anaesthetized females. Oocytes were treated with collagenase (type IA, 1 mg/ml for 2 h) or dispase II (0.5 mg/ml for 2 h) to remove the follicular envelopes and cells. Stage V oocytes were then chosen for the experiments and maintained in OR2 medium for up to 6 days at 16 ± 1°C. The composition of OR2 medium was in mmol/l: NaCl 82.5; KCl 2.5; $MgCl_2$ 1; $CaCl_2$ 1; Na_2HPO_4 1; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 5; pH adjusted to 7.4 with NaOH (Wallace et al. 1973), no antibiotics were added. Incubation medium was renewed daily.

Oocyte treatments

Polyvalent cations tested were chosen in the group IIA (Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}), in the transition group (Co^{2+}, La^{3+}, Mn^{2+} and Ni^{2+}) or in the group IIB (Cd^{2+} and Zn^{2+}). The cations were added to the superfusate, as their chloride salts, at different concentrations (from 10 μmol/l to 10 mmol/l) by aliquoting from a stock solution. Chemicals were purchased from Sigma (St. Quentin Fallavier, France).

Electrophysiological measurements

Electrophysiological recordings were performed using the conventional two-electrode voltage clamp amplifier (Dagan Instruments, Minneapolis, MN, USA). Generation of clamp control voltages, data acquisition and analysis were done using the pCLAMP software (vers. 5.5, Axon Instruments, Burlingame, CA, USA). Oocytes were placed in a recording chamber (250 μl, impaled with 3 mol/l KCl filled electrodes (1–1.5 MΩ for current electrode and 5–15 MΩ for voltage electrode) and superfused with ND medium. Agar bridges were used to isolate the bath electrode from the ionic changes of the superfusate. The medium flow and the replacement of medium in the recording chamber were estimated by recording the membrane potential changes of an oocyte superfused alternatively with OR2 medium and OR2 in which 51 mmol/l of Na^+ were substituted by K^+. Depolarization of the oocytes occurred in 33 ± 13 s ($n = 6$). Oocytes were tested at room temperature, 21–23°C.

To avoid combination and precipitation of phosphate with cations, oocytes were impaled in ND medium (composition in mmol/l: NaCl 96; KCl 2; $MgCl_2$ 1;

$CaCl_2$ 1 8, HEPES 5, pH adjusted to 7 4 with NaOH) (Leonard et al 1987) Nevertheless, control experiments showed that divalent cations in OR2 medium exerted similar effects on the oocytes After a stable resting potential was achieved, input resistance (R) of the oocyte membrane was routinely measured by injecting a hyperpolarizing current (5 nA for 5 to 7 s) (Kado 1989) ($R = 1\,4 \pm 0\,1$ MΩ, $n = 45$) Induction of sNa^+ channels was obtained either in current clamp or in voltage clamp conditions (Kado and Baud 1981) In current clamp experiments, sNa^+ channels were induced by repetitive injections of depolarizing currents (up to 200 nA for 5 s) In voltage clamp experiments, sNa^+ channels were induced by a single depolarization step to +50 mV of 120 or 90 s duration from a holding potential at −60 mV Peak Na^+ currents were obtained at membrane potentials at +50 mV and measured as the peak value from the level of the holding current Each oocyte was used only for one induction, because the time courses for successive inductions were not identical, unless more than 1 h has elapsed between two inductions (Baud and Kado 1984) However, once sNa^+ channels were induced, repetitive depolarization test steps applied to the same oocyte, elicited reproducible currents

In the course of our current clamp experiments, a few batches of oocytes were found to spontaneously respond to the addition of divalent cations in the superfusion medium The membrane potential oscillated and decreased to about −20 mV This membrane potential decrease was accompanied by a dramatic drop of membrane resistance These effects lasted as long as the divalent cations were maintained in the bath They have been shown to be due to the increase of intracellular calcium concentrations which in turn activated the calcium-dependent chloride channels (Miledi et al 1989) When the divalent cations were washed, the membrane potential and the resistance returned to their initial values in 20 to 45 min These batches of oocytes were not used for the experiments described in this paper

The current/voltage (I/V) relationship was performed on the same oocytes first without induced sNa^+ channels and then with induced sNa^+ channels Investigated potentials ranged from −90 to +90 mV, from a holding potential at −60 mV, with 10 mV increments In the non-induced oocytes, currents were elicited by 500 ms test pulses at 30 s intervals to avoid inducing the sodium channels With induced sNa^+ channels, the I/V relations were obtained with test pulse duration of 2 s at 500 ms intervals Elicited currents were measured at the end of the test pulses from the level of the holding current The activation curves were drawn by using the following procedure the leak current was measured in response to 10 mV steps between −90 and −20 mV and extrapolated The sodium equilibrium potential (E_{Na}) was estimated at the intercept between the extrapolated leak current and the I/V curve (see Fig 2) The sodium current I_{Na} was calculated by subtracting the extrapolated leak current from all values The activation curve was constructed after normalization of chord conductance measurements obtained from equation (1)

$$g = \frac{I_{Na}}{V_m - E_{Na}} \tag{1}$$

in which g is the conductance and V_m is the membrane potential. The theoretical curve was determined with a Boltzmann equation (2):

$$g = \frac{g_{max}}{1 + \exp \frac{V_m - V_{0\,5}}{k}} \tag{2}$$

where g_{max} is the maximum conductance, $V_{0\,5}$ is the potential corresponding to the half maximal conductance and k is the steepness factor (see Hille 1992). Leak subtraction was done only on data used to plot activation curves.

To study the concentration-dependent block of divalent cations, sNa^+ channels were induced by a 2-min depolarization step to +50 mV from a holding potential at −60 mV. Afterward, a 3-s depolarization step to +50 mV was applied in ND medium to determine Na^+ current amplitude. Then, various concentrations of a divalent cation in ND medium were successively superfused on the same oocyte and Na^+ current was elicited with a 3-s depolarization test to +50 mV. During the washout of each concentration of a blocking cation, the state of sNa^+ channel induction was explored by a depolarization in current clamp condition to avoid a too large sodium entry. Measured currents of a large number of oocytes were pooled and expressed as means ± S.E.M. The dose-response curves were fitted with equation (3), where y is the fractional receptor occupancy at equilibrium, K_d is the dissociation constant, C the divalent cation concentration and n_H the Hill coefficient (see Hille 1992):

$$1 - y = \frac{1}{1 + \left(\frac{C}{K_d}\right)^{n_H}} \tag{3}$$

Results

Current clamp membrane responses in the presence of multivalent cations

The sNa^+ channels were induced by injection of successive depolarizing current pulses. With repeated depolarizations, the potential responses began to show a delayed return to the resting potential at the end of the current pulses, until a large, long-lasting depolarization was obtained (see Fig. 1A). Thus, sNa^+ channels were open and the membrane potential remained depolarized at +66 to +82 mV (n = 45, 6 donors). In oocytes superfused with ND medium, the membrane potential remained depolarized for many minutes and slowly decreased to about +50 mV (Fig. 1A). From this value, it rapidly declined to about −10 mV indicating a fast closure of the sNa^+ channels. Thereafter, the membrane potential slowly decreased to reach the initial resting potential (−55 ± 1 mV, n = 45, 6 donors).

Multivalent cations were added to the medium to assay the extent of the ionic block on open sNa^+ channels, induced as described above. The addition of

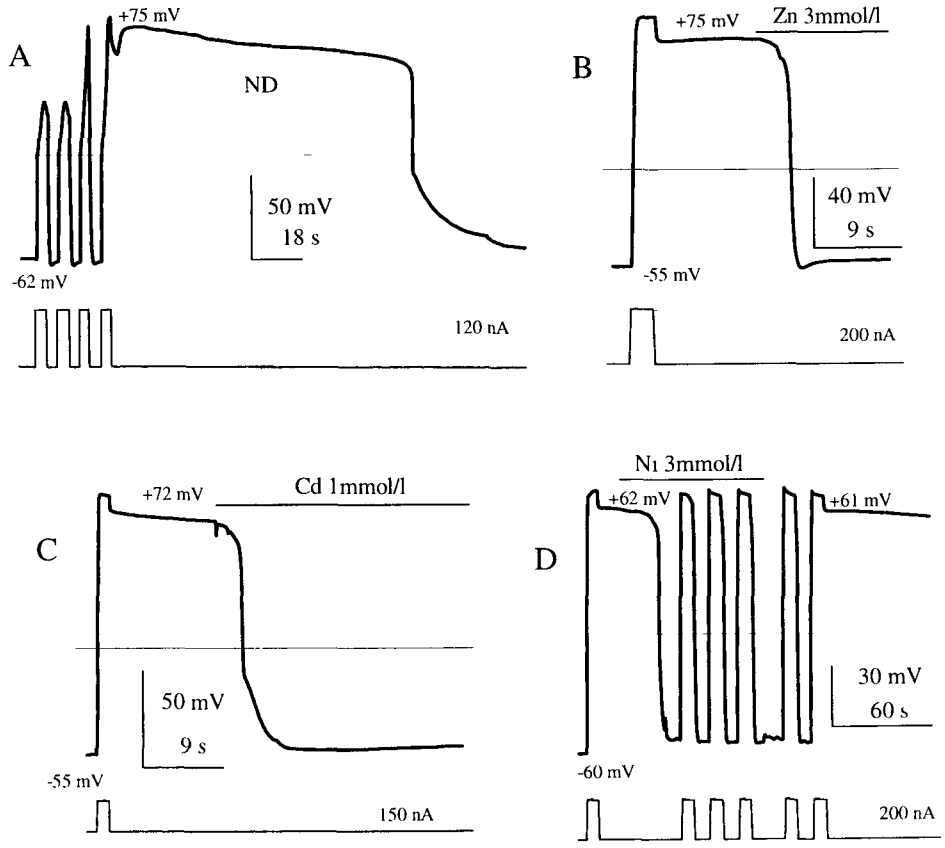


Figure 1. Effects of divalent cations on membrane potential.
(A) Successive depolarizing currents (120 nA for 5 s), which are shown below the potential traces, were delivered every 5 s into an oocyte in ND medium. In response to the fourth pulse of current, sNa^+ channels opened and the membrane potential remained depolarized at +75 mV. From this value, it declined slowly to reach about +50 mV within 100 s and then, it rapidly dropped to −10 mV. Afterwards, it decreased to its initial resting value (−62 mV). (B, C) In another oocyte, a similar procedure was used until sNa^+ channels were induced. Then, large depolarizing currents were injected to open the sNa^+ channels. As the membrane potential stabilized at a positive value, a cation-supplemented ND medium was added with Zn^{2+} (3 mmol/l) (B) and Cd^{2+} (1 mmol/l) (C) The addition of divalent cations in the bathing medium resulted in a rapid drop of the membrane potential (D) In another oocyte, sNa^+ channels were induced and opened then a ND medium supplemented with Ni^{2+} (3 mmol/l) was superfused. In 22 s, the membrane potential decreased to a value close to the initial level and the injection of large depolarizing current pulses of 200 nA produced a purely passive membrane potential change. The divalent cation-supplemented medium was washed out, and 37 s after the beginning of the wash, the membrane potential depolarized to +61 mV in response to the injection of depolarizing currents.

extracellular Ba^{2+}, Mg^{2+} or Ca^{2+} (5 or 10 mmol/l) had little or no effect on the depolarized membrane potential in 15 oocytes from 3 donors (data not shown). Sr^{2+} (10 mmol/l) caused a drop of membrane potential in 2 oocytes out of 5. Mn^{2+} (10 mmol/l) produced a rapid drop of membrane potential, whereas at a reduced concentration Mn^{2+} (1 mmol/l) was inefficient. In contrast, the addition of extracellular Co^{2+}, Cd^{2+}, La^{3+}, Ni^{2+} or Zn^{2+} at 1, 3 or 5 mmol/l concentrations resulted in an immediate decrease in the membrane potential to a value close to its initial level ($n = 30$, 6 donors) (see for example Fig. 1B,C and D the effect of Zn^{2+}, Cd^{2+} and Ni^{2+}). As shown by Fig. 1D, when Ni^{2+} was washed from the recording chamber, the injection of depolarizing currents resulted in the opening of the sNa^+ channels and the membrane potential stabilized at a positive value. This indicates that the effect of the cations was reversible. The following sections will focus mainly on the effect of the most efficient divalent cations (Co^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+}) on the sNa^+ channels.

Voltage clamp membrane responses in the presence of divalent cations

The current/voltage (I/V) relationships were determined on oocytes with and without induced sNa^+ channels. The I/V curves on Fig. 2 illustrate the changes of membrane electrical properties before and after induction of the channels. When the cell was in its basal state of inexcitability, two currents appeared: one activated at about −20 mV and peaked around +20 mV. It was dependent on the external calcium concentration and was blocked by chloride channel inhibitors such as SITS (Charpentier and Kado 1999). It was due to calcium-dependent chloride channels that activated in response to calcium influx through voltage-dependent calcium channels (Miledi 1982; Barish 1983). The other one was a cationic current, mainly carried by potassium ions and slowly activated by depolarization above +50 mV, due to the opening of non-selective cationic channels (Arellano et al. 1995). When the electrical membrane properties of the cell were changed by a long-lasting depolarization, an inwardly directed current was elicited. It activated at about −10 mV and peaked around +50 mV. The potential at which the current reversed was greater than +70 mV and was dependent on the presence of sodium ions in the superfusate.

Various concentrations of divalent cations were tested on pre-induced sNa^+ channels (Fig. 3, left panels). The divalent cations of the group IIA Mg^{2+} ($n = 13$, 2 donors) (Fig. 3A) and Ca^{2+} ($n = 10$, 1 donor) at large concentrations (10 mmol/l) were able to reduce Na^+ current. Nevertheless, the effect of large Ca^{2+} concentrations were difficult to interpret (data not shown), because Ca^{2+} entering the oocyte through voltage-dependent Ca^{2+} channels evoked a large Cl^- current (peak of the outward current at +20 mV), which obscured the onset of the Na^+ current (see above).

In contrast, the divalent cations of the transition group and of the group IIB reduced the sodium current at all voltage ranges investigated. The relative efficacy for cation block was: $Co^{2+} < Ni^{2+} < Cd^{2+} < Zn^{2+}$. The effect of Zn^{2+} and Co^{2+} are illustrated on Fig. 3B and C. The outwardly directed current elicited

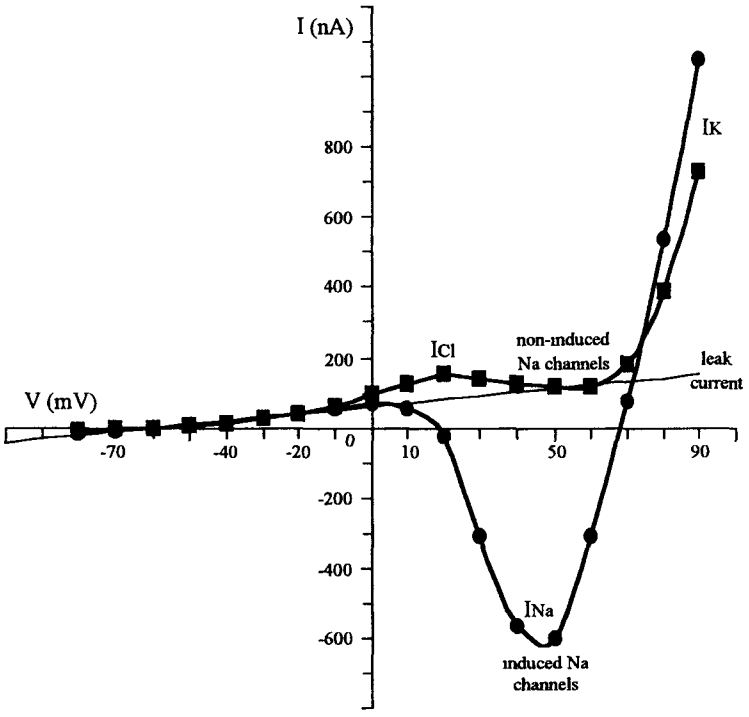
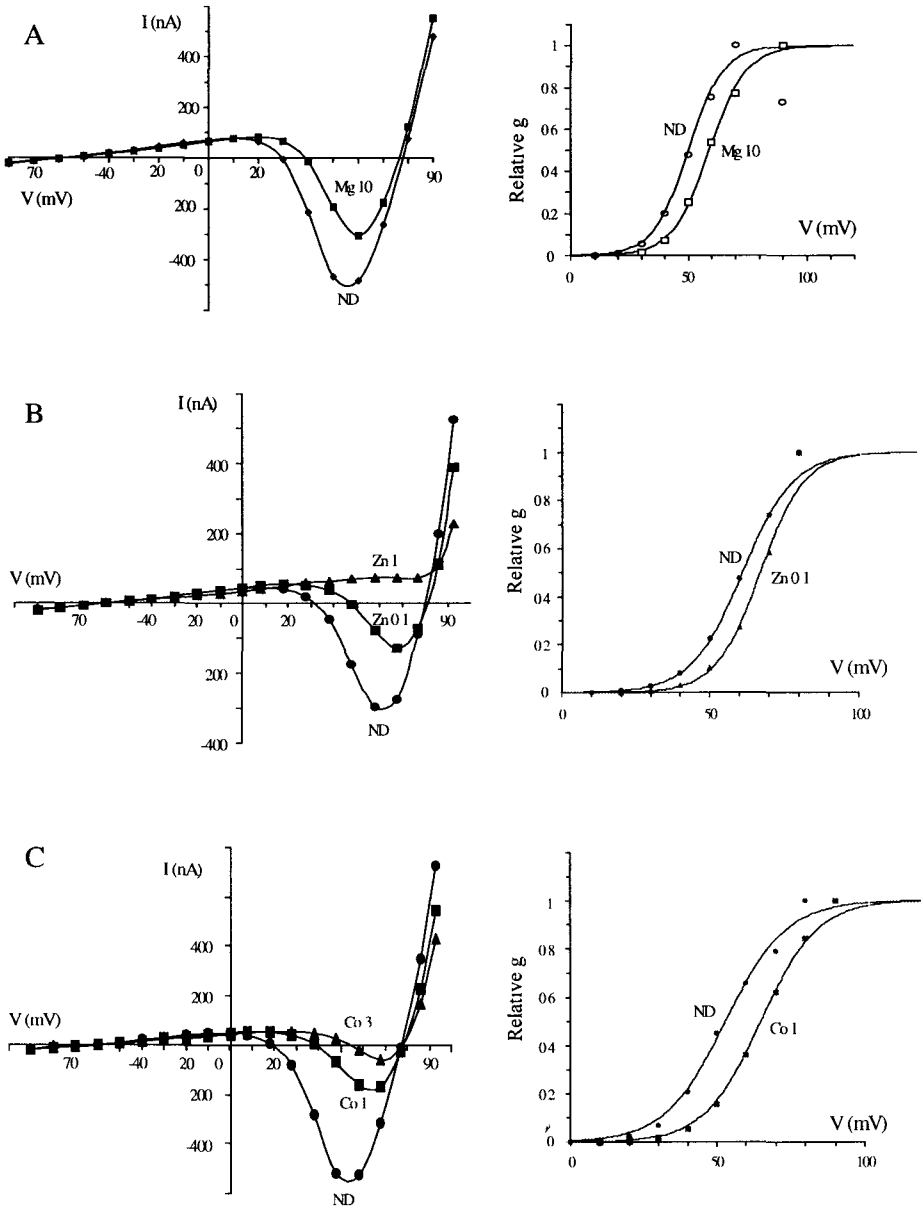


Figure 2. Oocyte voltage-clamped currents before and after sNa^+ channel induction The current/voltage relations were obtained on the same cell Currents were almost identical up to −10 mV At more depolarized potentials, in non-induced oocytes, two currents were elicited a chloride current (I_{Cl-}) activating at about −20 mV and peaking at +20 mV, and a potassium current (I_{K+}) activating at about +60 mV After the induction of sNa^+ channels, a large inward current (I_{Na+}) activated at about −10 mV, the maximum was obtained at +50 mV The leak current estimated in the range of potentials from −80 to −20 mV was extrapolated (thin line) The intercept with the I/V curve yielded the *equilibrium potential* for sodium ions

Figure 3. Effect of divalent cations current/voltage relationships
Typical current/voltage relations were obtained in different bathing media as indicated in each panel The same oocyte was used for the experiments shown in each of these panels Oocytes were voltage clamped at −60 mV and sNa^+ channels were induced with a long lasting depolarization I/V curves obtained in control medium (ND) were compared to those obtained after the addition of cations from the group IIA (Mg^{2+}, 10 mmol/l) (A), from the group IIB (Zn^{2+}, 1 and 0 1 mmol/l) (B), or from the transition group (Co^{2+}, 1 and 3 mmol/l) (C) The activation curves (right panels) were constructed as described in Materials and Methods The lines are the theoretical curve drawn using the Boltzmann equation The addition of divalent cations in the superfusion medium caused a shift of



the activation curve towards more positive voltages In the presence of ND and Mg^{2+}-supplemented medium (A), the values for $V_{0\,5}$ were 50 and 59 mV, and those for k were $-7\,5$ and $-7\,9$ respectively In the presence of ND and Zn^{2+}-supplemented medium (B), the values for $V_{0\,5}$ were 61 and 67 mV, and those for k were $-8\,6$ and $-7\,4$ respectively In the presence of ND and Co^{2+}-supplemented medium (C), the values for $V_{0\,5}$ were 53 and 65 mV, and those for k were $-10\,3$ and $-9\,5$ respectively

by depolarizing test pulses more positive than the equilibrium potential of sodium ions was decreased in the presence of the divalent cations. This suggests that the binding of the cations also reduced Na^+ efflux. However, one can not exclude a possible effect of the divalent cations on the K^+ current.

In the presence of divalent cations, the activation curves of the sodium current were significantly shifted towards more positive voltages (Fig 3, right panels). The shift was approximately 5 mV for Cd^{2+}, Zn^{2+} and Ni^{2+} (0.1 mmol/l), and it was about 10 mV for Cd^{2+}, Co^{2+}, Ni^{2+} (1 mmol/l) and Mg^{2+} (10 mmol/l). On the other hand, neither the steepness factor (see legend of Fig. 3) nor the equilibrium potential for sodium ions were significantly changed.

To test further the blocking effect of divalent cations, various cation concentrations ranging from 0.01 to 10 mmol/l were used and the degree of Na^+ peak current reduction was determined while the membrane was clamped to +50 mV. Na^+ peak currents were measured at the end of the test pulse (see for example Fig. 4A and C). With the values obtained, dose-response curves were drawn (Fig. 4B and D). Our data showed that the blocking effect of divalent cations was concentration-dependent. It also appeared that the extent of current block varied with the cation species: the relative efficacy of the divalent cations tested was $Co^{2+} < Ni^{2+} < Cd^{2+} < Zn^{2+}$, with respective concentration required to cause 50% inhibition of Na^+ current of 0.9 ± 0.29; 0.47 ± 0.15; 0.36 ± 0.09 and 0.06 ± 0.02 mmol/l. Nonetheless, part of the block can be attributed to the shift of the activation curve of the sodium channels towards more positive potentials.

Voltage-independent block by divalent cations

In the following experiments, the possible voltage-dependence of the cation block was addressed. Na^+ current was elicited and measured at the end of a test potential at +50 mV after a conditioning pre-pulse (Fig. 5A and B). Conditioning voltages investigated ranged from +90 to −90 mV (with 20 mV decrements). As illustrated on Fig. 5C, no inward current could be recorded in the presence of a blocking concentration of divalent cations (5 mmol/l) ($n = 13$, 3 donors). The current elicited by the depolarization test remained constant and identical after the entire range of conditioning pre-pulse potentials. No significant difference could be observed with the cation species used. These results showed that divalent cations could bind to their blocking site at positive as well as at negative potentials suggesting a voltage-independent block. In addition, the outwardly directed current elicited by the conditioning pre-pulse at +90 mV was decreased in the presence of the divalent cations. This indicates that the binding of the cations reduced either Na^+ efflux through sNa^+ channels or K^+ influx through the non-selective cationic channels.

Effect of divalent cations on the induction of Na^+ channels

We studied the possible effect of divalent cations on the induction of the sNa^+ channels (Fig. 6). In voltage clamp experiments, oocytes superfused with ND medium supplemented with Zn^{2+} (3 mmol/l) were depolarized by a conditioning step to +50 mV of 80-s duration to induce sNa^+ channels. At the end of the conditioning

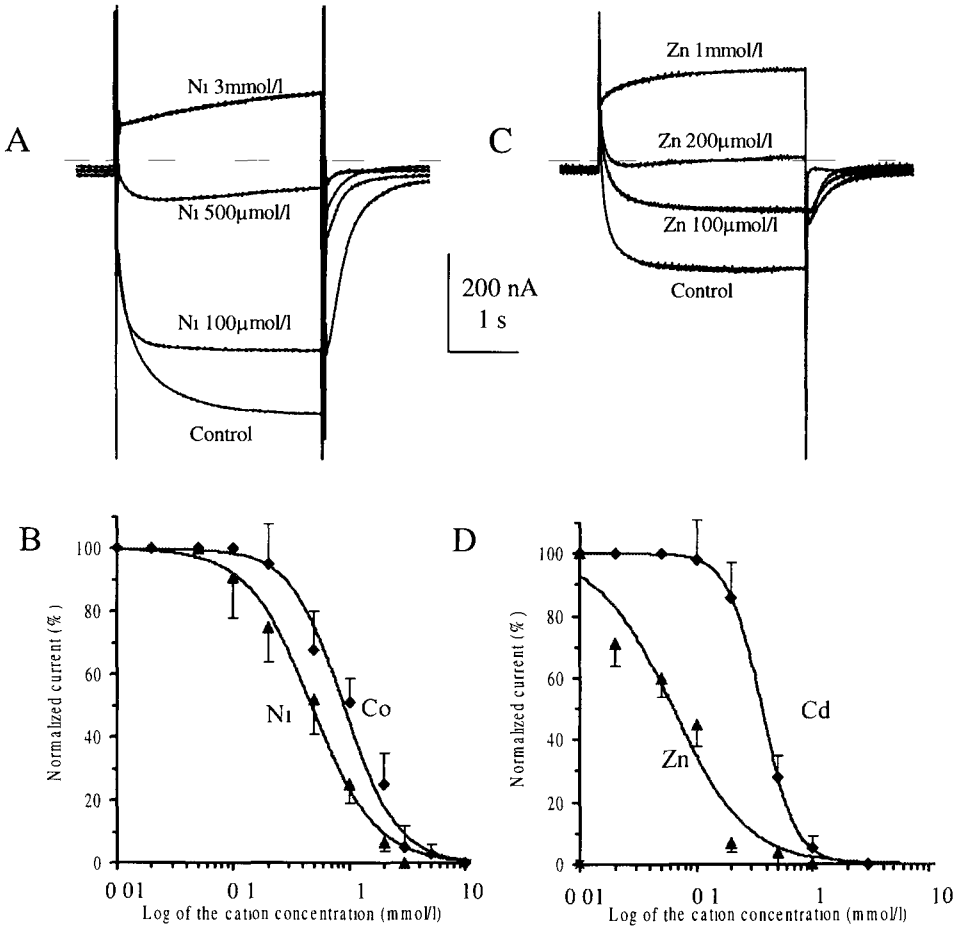


Figure 4. Concentration dependence of external divalent cation block
The sNa^+ channels were induced in ND medium by a 2 min depolarization step (conditioning pulse) Then a test pulse to +50 mV of 3 s duration was applied as the oocyte was superfused with a cation-supplemented ND medium (A) In the presence of Ni^{2+} 3 mmol/l, no inward current was recorded With reduced concentrations (500 and 100 μmol/l), partial blocks were obtained (C) No inward current could be recorded in the presence of Zn^{2+} 1 mmol/l With reduced Zn^{2+} concentrations, Na^+ current increased (B and D) concentration-dependent block of Na^+ current by divalent cation of the transition group (Ni^{2+} and Co^{2+}) and of the group IIB (Zn^{2+} and Cd^{2+}) Each point is the average of at least 5 oocytes The curves were drawn using equation (3) (in Materials and Methods) Among all tested divalent cations, Zn^{2+} was the most potent blocker of sNa^+ channels

pulse, the oocyte was superfused with a ND Zn^{2+}-free medium. The potential was stepped for 5 s to +50 mV from the holding potential, 30 s after wash began. At the end of the conditioning step, no inward current could be recorded (n = 10, 2

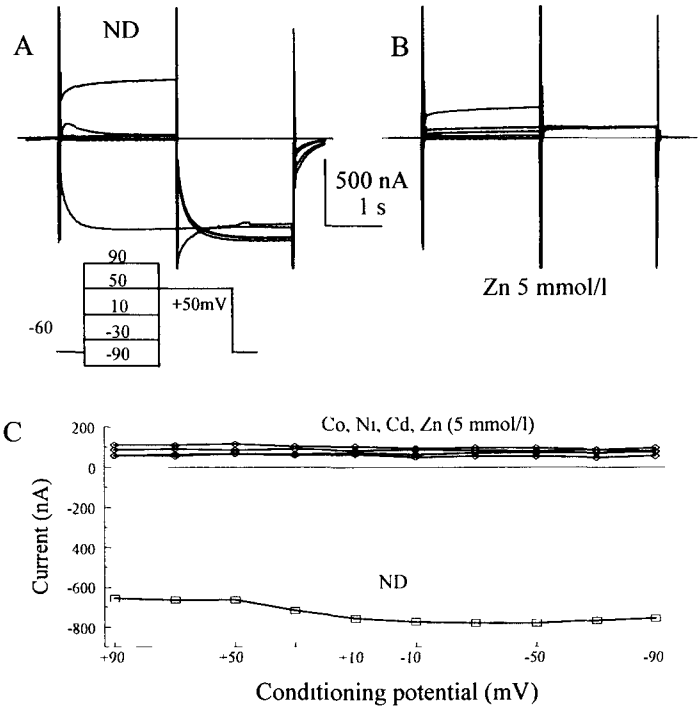


Figure 5. Effect of various pre-pulses voltages on cation block
The voltage dependence was studied by determining the cation block of Na^+ current after a conditioning step to various potentials Oocytes were voltage clamped at −60 mV, conditioning pre-pulses of 2 s duration ranging from +90 to −90 mV (with 20 mV decrements) were applied Na^+ currents were measured at the end of a 2 s test pulse at +50 mV The stimulation protocol is shown below the current traces in A The panels A and B show the currents evoked in response to the conditioning pre-pulses (−90, −30, 10, 50 and 90 mV) either in control medium (ND) or in ND supplemented with Zn^{2+} (5 mmol/l) Note that the amplitude of the outwardly directed current elicited by the conditioning pre-pulse at +90 mV was reduced in the presence of the divalent cation suggesting a block of Na^+ efflux (C) Na^+ current elicited at +50 mV was plotted versus the potentials of the conditioning pre-pulses No inward current could be recorded in the presence of divalent cations

donors). After washout of the blocking cation, inward current was recorded. The divalent cation did not interfere with the cytoplasmic process of sNa^+ channel induction since inward currents were elicited by the first test step This indicates that the effect of the divalent cation occurred as channels were induced and opened and that sNa^+ channels could recover from Zn^{2+} block. Sodium current could be recorded as Zn^{2+} unbound and was washed away. Other experiments with Ni^{2+} or Cd^{2+} (5 mmol/l) ($n = 7$; 1 donor) provided similar results.

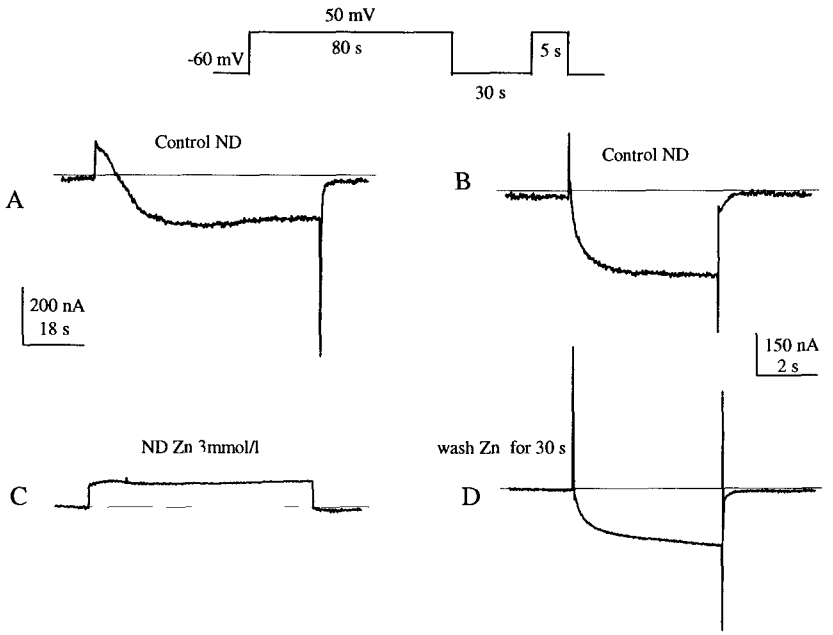


Figure 6. Effect of Zn^{2+} on sNa^+ channel induction
Oocytes were voltage-clamped at −60 mV and depolarized to +50 mV for about 80 s to induce the sNa^+ channels (A and C) 30 s after this conditioning prepulse, another depolarization to +50 mV of 5 s duration was delivered (B and D) The stimulation protocol is shown above the current trace in A In ND medium, the prepulse caused the induction of the sNa^+ channels and Na^+ current was evoked (A) Once induced, sNa^+ channels were re-opened by a subsequent test pulse (B) In the presence of Zn^{2+} (3 mmol/l), no inward current could be recorded during the conditioning prepulse (C) The oocyte was then washed for 30 s with Zn^{2+}-free ND medium In response to the test pulse, the sodium current was elicited

Effect of an alkylating reagent on Zn^{2+} binding

In a series of experiments, the possible involvement of SH-groups in the binding of the divalent cations was investigated by testing the ability of a sulphydryl alkylating agent to abolish Zn^{2+} block of sNa^+ channels. Oocytes were incubated for 20 to 70 min in ND medium supplemented with a sulphydryl alkylating agent (iodoacetamide, 5 mmol/l). The sNa^+ channels were induced either in current clamp or in voltage clamp conditions, in ND medium supplemented with iodoacetamide In current clamp experiments, once membrane potential stabilized at the equilibrium potential of Na^+, oocytes were superfused with ND medium containing both iodoacetamide (5 mmol/l) and Zn^{2+} (3 mmol/l). In spite of the presence of iodoacetamide, Zn^{2+} blocked the sNa^+ channels resulting in a fast drop of the membrane potential (data not shown) (n = 7, 2 donors). In voltage clamp experiments, Na^+

currents were first elicited in ND medium supplemented with iodoacetamide. Then, the oocytes were superfused with ND medium containing both iodoacetamide (5 mmol/l) and Zn^{2+} (3 mmol/l). In the presence of Zn^{2+}, no inward current could be recorded ($n = 9$, 1 donor). The addition of sulphydryl alkylating agent failed to prevent zinc binding on the sNa^+ channels.

Discussion

Our data show that divalent cations blocked *Xenopus* slow Na^+ channels in a concentration-dependent fashion. The extent of Na^+ current block depended upon the concentration of divalent cations tested which displayed an efficacy sequence of group IIA < transition < group IIB. All cations tested exerted a reversible block. Among the cations of group IIA, no significant difference was observed in the effect of Ba^{2+} or Ca^{2+} or Mg^{2+} on Na^+ conductance. In contrast, the potency of the cations inside each of the two other groups was very different: $Co^{2+} < Ni^{2+}$ and $Cd^{2+} < Zn^{2+}$; Zn^{2+} was the most potent inhibitor of Na^+ current. More than a ten-fold higher concentration of Co^{2+} was required to cause approximately the same block compared to Zn^{2+}. A similar sequence of affinity has been described on sodium current from canine cardiac Purkinje cells (Sheets and Hanck 1992). In their extensive work on voltage-dependent Na^+ channels prolonged by batrachotoxin, Ravindran et al. (1991) suggested that divalent cations bound to the same site but the location varied for the different metal ions. The cations with the smallest ionic radius have the highest affinity and enter the channel more deeply than the larger cations. Our data do not show any evidence suggesting a different binding site for each of the tested cations and neither clear positive nor negative correlation between the potency of ionic block and ionic radius. All cations may bind to the same site but with different affinities.

As first described by Frelin and coworkers (1986), the sensitivity of Na^+ channels to group IIB divalent cations is inversely related to their affinity for tetrodotoxin (TTX). TTX-resistant Na^+ channels are preferentially blocked by divalent cations from group IIB such as Zn^{2+} (cardiomyocytes or Purkinje cells from heart: Frelin et al. 1986; Visentin et al. 1990; Sheets and Hanck 1992). In contrast, TTX-sensitive channels from neuronal cells require at least millimolar concentration of Zn^{2+} to be half inhibited (*Xenopus*: Århem 1980; *Rana*: Hille et al. 1975; chick: Frelin et al. 1986). The sNa^+ channels which are known to be highly resistant to the effect of TTX (Baud et al. 1982), since complete block occurred with millimolar concentration of the toxin, were also very sensitive to group IIB cation block. This observation adds evidence to the finding that TTX-resistance and group IIB cation sensitivity are strongly correlated.

Under our experimental conditions, divalent cations from the transition and IIB groups exerted a voltage-independent block of sNa^+ channels. Other studies on heart cells have reported voltage-independent block (Visentin et al. 1990). However, other investigators demonstrated the existence of two distinct binding sites for divalent cations: one producing a voltage-dependent block and the other a voltage-

independent block of cardiac Na^+ channels (Sheets and Hanck 1992) According to Woodhull (1973), voltage-dependent block arises when binding to the site occurs into the membrane electric field Inversely, voltage-independent block is obtained when binding occurs outside the membrane field, as shown in cardiac cells for Ni^{2+} and group IIB cations (Sheets and Hanck 1992) This suggests that the binding site for divalent cations on the sNa^+ channels could likely be located outside the membrane electric field One possible site is the guanidinium-binding site Indeed, there is a striking competitive interaction between the binding of Zn^{2+} and the binding of saxitoxin (STX) (Green et al 1987) In addition, the guanidinium toxin binding has been reported to lack voltage-dependence (reviewed in Hille 1992) Previous experiments indicated that the sNa^+ channels were insensitive to micromolar concentration of TTX (Baud et al 1982) Therefore, the possible blockade of STX on open sNa^+ channels was investigated In current clamp experiments, STX (1 μmol/l) had no effect on membrane potential that remained depolarized ($n = 4$, 1 donor, data not shown) The fact that sNa^+ channels are insensitive to the guanidinium toxins, indicate that they could miss this specific site or that this site could not be easily accessible

Divalent cations are known to form complexes with membrane ligands and/or to bind to sulphydryl groups such as on cysteine residues, some cations can bind to SH-groups with extremely high affinity (Århem 1980) These data point out the critical role that cysteine residues could play on the binding of Zn^{2+} Indeed, treatment of calf heart cells with a sulphydryl reagent (iodoacetamide) abolishes Zn^{2+} block on Na^+ channels (Schild and Moczydlowski 1991) In our hands, iodoacetamide failed to inhibit Zn^{2+} blocking effect One hypothesis is that a sulphydryl group might not be involved in cation binding Alternatively, the lack of effect of iodoacetamide could be due to the inaccessibility of SH groups from the sNa^+ channel protein Indeed, several mechanisms are involved in the reactivity of SH groups to modifying agents, such as accessibility, pH, temperature or the presence of negatively charged groups of amino acids in the immediate vicinity of the SH moiety (Strauss 1984) Our electrophysiological data do not provide any explanation for the molecular origin of the divalent cation block of sNa^+ channels

As indicated by the current and voltage clamp experiments, the binding of divalent cations on pre induced sNa^+ channels resulted in a decrease of the Na^+ flux But these data did not provide any indication on the effect of the divalent cations on the induction process Divalent cations, such as Ni^{2+} and Cd^{2+}, act as Ca^{2+} channel blockers and can reduce Ca^{2+} influx (see Hagiwara and Byerly 1981) Ca^{2+} could be involved in the induction or in the activation of Na^+ channels One possible mechanism is that Ca^{2+} influx could activate calcium-dependent enzymes, such as a phospholipase C and a protein kinase C (Kikkawa and Nishizuka 1986), since both enzymes are required in the induction of voltage sensitivity of the sNa^+ channels (Charpentier et al 1993, 1995) As shown in Fig 5, the addition of Zn^{2+} during the time when oocytes were depolarized to induce the channels did not interfere with the cellular mechanism of induction, since inward currents were recorded after washout of the blocking cation Similar results were obtained with

other cation species. In addition, induction has been shown to proceed in Ca^{2+}-free medium (Baud and Kado 1984; Bossi et al. 1998; Charpentier and Kado 1999) Therefore, divalent cations exerted their inhibiting activity by binding to the sNa^+ channels and not by interfering with Ca^{2+} influx.

Divalent cations shifted the potential-dependent parameters along the voltage axis. The shift was found to differ in magnitude for the different cations. In order to compare the data, normalized activation curves were calculated from the current/voltage relationships. Our data are interpreted to mean that the positive shift obtained by divalent cations of the transition group and of the group IIB does not play a major role in the reduction of the sodium current. All the divalent cations caused a shift of the activation curve, whereas not all the divalent cations were found to significantly decrease the sodium conductance. For example (Fig 3A and B), Mg^{2+} (10 mmol/l) caused a 33% decrease of Na^+ current and a positive shift of about 10 mV. These effects can be attributed in part to both the screening and the binding of the cation to fixed negative surface charges of the membrane (McLaughlin 1989), but also to a significant change in the gating properties of the sNa^+ channels following the binding of the divalent cation. Whereas in the presence of Zn^{2+} (0.1 mmol/l, the shift was 6 mV and the decrease 42%. In addition, transition and group IIB cations also reduced the outward currents elicited by depolarization steps to more positive potentials than the equilibrium potential for sodium. Visentin and coworkers (1990) have also reported a shift of the activation curve with divalent cations (Cd^{2+} and Mn^{2+}), the displacement was moderate (+4 mV) for Cd^{2+} (0.07 mmol/l) and more substantial (+17 mV) for Mn^{2+} (2 mmol/l).

In this paper, we have shown that the TTX-resistant sNa^+ channels of the *Xenopus* oocyte share some similarities with the cardiac Na^+ channels, at least at the pharmacological level, even though gating properties and current kinetics are strikingly different.

Acknowledgements. The authors are greatly indebted to Dr J M Dubois for critical reading of the manuscript They also wish to thank Dr R T Kado for valuable discussion and gratefully acknowledge Dr E Benoit for her unwavering support

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Final version accepted May 22, 2001