BLOCK OF CURRENT THROUGH T-TYPE CALCIUM CHANNELS
BY TRIVALENT METAL CATIONS AND NICKEL IN
NEURAL RAT AND HUMAN CELLS

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SUMMARY

1. The effects of the trivalent cations yttrium (Y³⁺), lanthanum (La³⁺), cerium
   (Ce³⁺), neodymium (Nd³⁺), gadolinium (Gd³⁺), holmium (Ho³⁺), erbium (Er³⁺),
   ytterbium (Yb³⁺) and the divalent cation nickel (Ni²⁺) on the T-type voltage gated
   calcium channel (VGCC) were characterized by the whole-cell patch clamp technique
   using rat and human thyroid C cell lines.

2. All the metal cations (M³⁺) studied, blocked current through T-type VGCC (Iₜ)
   in a concentration-dependent manner. Smaller trivalents were the best T-channel
   antagonists and potency varied inversely with ionic radii for the larger M²⁺ ions.
   Estimation of half-maximal blocking concentrations (IC₅₀) for Iₜ carried by 10 mM
   Ca²⁺ resulted in the following potency sequence: Ho³⁺ (IC₅₀ = 0.107 μM) ≈ Y³⁺
   (0.117) > Yb³⁺ (0.124) > Er³⁺ (0.153) > Gd³⁺ (0.267) > Nd³⁺ (0.429) > Ce³⁺ (0.728) >
   La³⁺ (1.015) ≫ Ni²⁺ (5.65).

3. Tail current measurements and conditioning protocols were used to study the
   influence of membrane voltage on the potency of these antagonists. Block of Iₜ by
   Ni²⁺, Y³⁺, La³⁺ and the lanthanides was voltage independent in the range from -200
   to +80 mV. In addition, the antagonists did not affect macroscopic inactivation
   and deactivation of T-type VGCC.

4. Increasing the extracellular Ca²⁺ concentration reduced the potency of Iₜ
   block by Ho³⁺, indicative of competitive antagonism between this blocker and the
   permeant ion for a binding site.

5. The results suggest that the mechanism of metal cation block of T-type VGCC
   is occlusion of the channel pore by the antagonist binding to a Ca²⁺/M³⁺ binding
   site, located out of the membrane electric field.

6. Block of T-type VGCC by Y³⁺, lanthanides and La³⁺ differ from the inhibition
   of high voltage-activated VGCC block in several respects: smaller cations are more
   potent Iₜ antagonists; block is voltage independent and the antagonists do not
   permeate T-type channels. These differences suggest corresponding structural
   dissimilarities in the permeation pathways of low and high voltage-activated Ca²⁺
   channels.
INTRODUCTION

Trivalent cations including Y$^{3+}$, La$^{3+}$ and the lanthanides (elements 58–71 in the periodic table) share biologically important chemical properties with the divalent calcium (Ca$^{2+}$) cation (Nieboer, 1975; Williams, 1982; Evans, 1990). Their similarity, especially in ionic radii, co-ordination chemistry and preference for the oxygen donor groups, have provided the basis for wide use of Y$^{3+}$, La$^{3+}$ and the lanthanides in studying the function of Ca$^{2+}$ in biological systems (Nieboer, 1975; reviewed in Evans, 1990). Because of their strong interaction with Ca$^{2+}$ binding sites on membrane proteins (dos Remedios, 1981), they have been useful in studying voltage-gated calcium channels, where they potently inhibit Ca$^{2+}$ currents.

Previous studies of Ca$^{2+}$ channel block by trivalent metal cations (M$^{3+}$) have focused primarily on high voltage-activated voltage-gated calcium channels (VGCC) (see Hille, 1992 for VGCC nomenclature and a review). Several of the more extensive studies have provided valuable insights into high voltage-activated VGCC structure and function (Nachsen, 1984; Lansman, Hess & Tsien, 1986; Lansman 1990). In contrast, block of low voltage-activated T-type VGCC by trivalent metal cations has not been systematically studied. Similarly, the blocking properties of Ni$^{2+}$, a widely used T-type channel antagonist, have not been described. Several reports have appeared describing the potency of T-type VGCC block by La$^{3+}$ (Narahashi, Tsunoo & Yoshii, 1987; Akaike, Kostyuk & Osipchuk, 1989; Akaike, Kanaide, Kuga, Nakamura, Sadoshima & Tomoikeye 1989), Gd$^{3+}$ (Biagi & Enyeart, 1990) and Y$^{3+}$ (Biagi & Enyeart, 1991).

Using neural crest-derived rat and human thyroid C cell lines, we found that Y$^{3+}$ and smaller lanthanides are the most potent inorganic antagonists of T-type VGCC. The properties of $I_T$ block by M$^{3+}$ and Ni$^{2+}$ indicate that structural differences exist between T-type and high voltage-activated Ca$^{2+}$ channels.

METHODS

Materials

Tissue culture media, horse serum, and fetal calf serum were obtained from Gibco (Grand Island, NY, USA). Culture dishes were purchased from Corning (Corning, NY, USA). YCl$_3$ and lanthanide chlorides (at least 99.9% purity) were obtained from Aldrich Chemical Co. Ltd (Milwaukee, WI, USA). All other chemicals were purchased from Sigma Chemical Co. Ltd (St Louis, MO, USA).

Cell culture

The rat medullary thyroid carcinoma 6–23 (clone 6) cell line (rat C cells) was purchased from the American Type Culture Collection and grown in 35 mm dishes on poly-D-lysine-coated coverslips in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum at 37 °C in a humidified atmosphere of 95% air and 5% CO$_2$. The human medullary thyroid carcinoma TT cell line (human C cells), kindly provided by André de Bustros (John Hopkins University), was grown on coverslips as described above in DMEM supplemented with 10% fetal bovine serum.

Solutions and bath perfusion

The standard electrode filling solution was (mm): 120 CsCl, 1 CaCl$_2$, 2 MgCl$_2$, 11 1,2-bis(2aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA), 10 Hepes and 1 MgATP, with pH titrated to 7.25 ± 0.05 using CsOH. The standard external solution consisted of (mm): 117 tetraethylammonium chloride (TEACl), 5 CsCl, 10 CaCl$_2$, 2 MgCl$_2$, 5 Hepes, 10 glucose, with
pH adjusted to 7·3 using TEAOH. In experiments with different concentrations of extracellular CaCl₂ isosmolarity was maintained by adjusting the concentration of TEACl. All solutions were filtered through 0·22 μm cellulose acetate filters.

Handling of Y³⁺, La³⁺ and the lanthanides is greatly restricted by their specific chemical properties (reviewed by Evans, 1990). To avoid formation and precipitation of insoluble M(OH)₃ and M₂(CO₃)₃, as well as formation of radiocolloids and loss of M³⁺ ions to the container surface, millimolar aqueous stock solutions of MCl₂ were prepared daily in polyethylene microtubes. Stock solutions were diluted to final concentration directly in the bath perfusion system immediately before use. The perfusion system consisted of polyethylene and polypropylene containers and tubing, since Y³⁺, La³⁺ and the lanthanides strongly bind to negatively charged groups on glass surfaces. The recording chamber (volume ≈ 1 ml) was continuously perfused by gravity at a rate of 5–6 ml/min. Bath solution exchange was done by a manually controlled six-way rotary valve, flushing at least 10 ml of perfusate.

In spite of the relatively constant activity coefficients of trivalent lanthanide chlorides in aqueous solution and precautions taken in experimental procedures, free concentration of unhydrolysed M(H₂O)₅³⁺ ionic species in the extracellular solution cannot be determined with certainty. Partial hydrolysis of M³⁺ results in rapid formation of significant quantities of relatively soluble M(OH)⁺, M(OH)₂⁺ and other species (Biedermann & Ciavatta, 1961; Evans, 1990), the final concentrations of which are largely unknown. It is likely that some of the hydrolysis products contributed to the observed effects in this study.

Recording conditions and electronics

Rat and human C cells were used in patch clamp experiments 1–4 days after plating. Cover slips with cells were transferred from 35 mm culture dishes to the recording chamber. Spherical cells 10–40 μm in diameter and without processes were selected for recording. Patch electrodes with resistances of 1–2 MΩ were fabricated from TM0010 glass (Corning) and R-6 glass (Garner Glass Co., Claremont, CA, USA) using a Brown-Flaming Model P-80 microelectrode puller (Sutter Instruments, Novato, CA, USA). Access resistance during recording was 2–5 MΩ. Whole-cell currents were recorded at room temperature (22–24 °C) following the procedure of Hamill, Marty, Neher, Sakmann & Sigworth (1981), using a List EPC-7 (List-Medical, Darmstadt, Germany) or an Axopatch 1D (Axon Instruments, Inc., Burlingame, CA, USA) patch clamp amplifier. Pulse generation and data acquisition were done using an IBM-AT computer and pCLAMP software with an Axolab interface (Axon Instruments, Inc.). Currents were filtered using an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA, USA) with cut-off frequency (~3 dB) set at 1–22 kHz and digitized at 1–3–100 kHz. Linear leak and capacity currents were subtracted from current records using summed scaled hyperpolarizing steps of 1/2 to 1/6 pulse amplitude.

Data were analysed and plotted using pCLAMP (CLAMPAN and CLAMPFIT) and InPlot 4 (GraphPAD Software, San Diego, CA, USA) programs and an IBM-compatible PC. All fits to single exponential functions were done from the point of maximal decay to the end of current records using the pCLAMP least-squares regression subroutine. Inhibition curves are InPlot 4 least-square regression fits, where current in control saline is normalized to 1 and assuming complete block of current with sufficient concentration of antagonists. All quantitative results are given as the means ± s.e.m. or, in the case of least-square fits, as the estimate ± s.e. (standard error of the estimate).

Identification of \( I_T \) in rat C cells

The majority of experiments were performed on rat C cells, which express prominent T-type Ca²⁺ current (\( I_T \)). Occasionally, experiments as noted in the text were performed using human C cells which express only T-type VGCC, but have less prominent \( I_T \) (Biagi, Mlinar & Enyeart, 1992).

In rat C cells, \( I_T \) can be easily distinguished from other Ca²⁺ current components by its voltage-dependent and kinetic properties (Biagi & Enyeart, 1991). When voltage clamp steps are applied at test potentials between -30 and -15 mV, the low threshold \( I_T \) is selectively activated, and appears as a transient component of Ca²⁺ current which inactivates completely. A distinctive feature of T-type Ca²⁺ channels is their slow rate of closing, which upon repolarization after a short test depolarization is observed as a slowly decaying ‘tail current’. For our studies, \( I_T \) was isolated as an inactivating component of Ca²⁺ current present at test potentials more negative than -15 mV, or as a tail current measured 1·5 to 3 ms after repolarization to -80 mV.
RESULTS

Concentration-dependent block of T-type VGCC by $Y^{3+}$, $La^{3+}$ and lanthanides

$Y^{3+}$, $La^{3+}$ and each of six lanthanides studied were found to block $I_T$ at submicromolar concentrations. Examples of concentration-dependent block by four of these agents are shown in Fig. 1. Block by any of the $M^{3+}$ ions was only partially reversible upon switching to control perfusate, even after 10 min of washing. However, inclusion of 50–100 $\mu$M EGTA (which potently chelates $Y^{3+}$, $La^{3+}$ and lanthanides) in the control solution resulted in the rapid and usually complete reversal of the inhibition (Fig. 1E).

To quantitate the relative potency of the $M^{3+}$ ions as $I_T$ antagonists, inhibition curves were constructed for each of the eight elements, after measuring relative block at a variety of concentrations (Fig. 2A). IC$_{50}$ varied over a tenfold range from $107 \pm 5$ nm (Ho$^{3+}$) to $1015 \pm 1$ nm (La$^{3+}$). Inhibition curves for all of the $M^{3+}$ had Hill coefficients close to $-1$ ($-0.98$ to $-1.33$), a characteristic of 1:1 ligand:receptor binding.

The relationship between ionic radii of the eight $M^{3+}$ ions and their respective potency as antagonists of T-type Ca$^{2+}$ channels is shown in Fig. 2B. The most potent block (smallest IC$_{50}$) was produced by the smaller lanthanides and $Y^{3+}$. For elements with radii above 0.102 nm (Gd$^{3+}$ to La$^{3+}$), potency varied inversely with ionic radius.

To determine whether $M^{3+}$ ions compete with Ca$^{2+}$ for specific binding sites on T-type VGCC, inhibition curves were constructed for Ho$^{3+}$ block of $I_T$ carried by 2, 10 and 50 mM Ca$^{2+}$. As expected for a competitive antagonist, increasing the external Ca$^{2+}$ concentration reduced the potency of Ho$^{3+}$ as evidenced by a nearly parallel shift to the right in the inhibition curve (Fig. 3).

Voltage (in)dependence of block of $I_T$ by $Y^{3+}$, $La^{3+}$ and lanthanides

The potency of ion channel block by charged antagonists often depends on the conducting state of the channel and therefore on the transmembrane potential. Voltage-dependent block of L-type VGCC by di- and trivalent metal cations is well documented (Lansman et al. 1986; Lansman, 1990). Experiments were designed to determine whether block of T-type VGCC is voltage dependent. Transition from open to non-conducting states may be faster in the presence of an antagonist which preferentially blocks open channels. To determine whether such a change in kinetics occurs during the block of $I_T$ with $M^{3+}$, we compared rates of macroscopic inactivation and deactivation with and without $M^{3+}$ in the solution.

All eight $M^{3+}$ ions tested failed to change the macroscopic inactivation rate, which occurred along a single exponential time course. For example, in control saline, the inactivation time constant of $I_T$ was $18.5 \pm 0.2$ ms ($n = 6$) at a test potential of $-20$ mV. After addition of 20, 100 and 500 nM Er$^{3+}$, the corresponding time constants were $18.2 \pm 0.2$ ms ($n = 6$), $18.9 \pm 0.3$ ms ($n = 5$) and $18.4 \pm 0.6$ ms ($n = 5$). Figure 4A shows scaled traces illustrating the absence of any effect of Gd$^{3+}$ on macroscopic $I_T$ inactivation.

The effects of $M^{3+}$ on deactivation kinetics was studied by comparing tail currents upon repolarization to $-80$ mV, after an activating test pulse. Deactivation, like inactivation, occurred by a process which could be fitted with a single exponential
Fig. 1. Concentration-dependent block of $I_T$ by $M^{3+}$ in rat C cells. $A-D$, superimposed current records illustrating block of $I_T$ by four different $M^{3+}$ concentrations. The averaged traces in descending order of amplitude, represent steady-state current in: $A$, control saline (0 Y$^{3+}$, 10 mM Ca$^{2+}$), 50, 250 and 500 nM Y$^{3+}$; $B$, control, 50, 200 and 1000 nM Yb$^{3+}$; $C$, control, 0.1, 0.5 and 2.5 μM Gd$^{3+}$; $D$, control, 0.1, 0.5, 2.5 μM Nd$^{3+}$. Currents were activated by depolarizing steps of either 300 (A and C) or 10-5 (B and D) ms duration, applied at 0.1 Hz from a holding potential of -80 mV. Test potentials were: $A$, -24 mV; $B$, $C$ and $D$, -20 mV. Tail currents were recorded at -80 mV. $E$, time course of $I_T$ block by Gd$^{3+}$ (concentrations in μM) followed by reversal upon superfusion of saline containing 50 μM EGTA. Data from the same cell as shown in $C$.

time course. All $M^{3+}$ ions were tested in at least four cells; each failed to affect deactivation kinetics (data not shown). The tail currents shown in Fig. 4B deactivated with time constants ($\tau_d$) of 2.14 ms (control), 2.12 ms (50 nM Y$^{3+}$) and 2.05 ms (250 nM Y$^{3+}$). In the same cell, $\tau_d$ in the presence of 500 nM Y$^{3+}$ was 2.09 ms.
Fig. 2. Potency of $I_T$ block by $M^{3+}$ and relationship to ionic radius. A, inhibition curves for eight different $M^{3+}$ ions. Data obtained in experiments as described in legend of Fig. 1. Data points are normalized mean values obtained in 5–10 (usually 6) separate measurements after steady-state block was reached. Inhibition curves are best fits of data to an equation of the form:

$$Y = 1/1 + (IC_{50}/X)^h,$$

where $Y$ is the fraction of control current remaining after addition of the antagonist and $X$ is the log of antagonist concentration. $IC_{50}$ and Hill slope ($h$) have been estimated from the fits. $IC_{50}$s and Hill slopes for the $M^{3+}$ ions are: $107 \pm 5\, \text{nm},$ $-1.27 \pm 0.08$ for $Ho^{3+};$ $117 \pm 8\, \text{nm},$ $-1.05 \pm 0.09,$ $Y^{3+};$ $125 \pm 1\, \text{nm},$ $-1.07 \pm 0.01,$ $Yb^{3+};$ $153 \pm 9\, \text{nm},$ $-1.18 \pm 0.08,$ $Er^{3+};$ $267 \pm 7\, \text{nm},$ $-1.33 \pm 0.04,$ $Gd^{3+};$ $429 \pm 81\, \text{nm},$ $-0.98 \pm 0.21,$ $Nd^{3+};$ $728 \pm 43\, \text{nm},$ $-0.98 \pm 0.06,$ $Ce^{3+};$ $1015 \pm 1\, \text{nm},$ $-1.25 \pm 0.001,$ $La^{3+}$. B, potency as a function of ionic radius. $IC_{50}$s of eight $M^{3+}$ ions are plotted against corresponding cationic radius (Shannon, 1976), assuming a co-ordination number of 8. Although 8 is the most likely co-ordination number for $Y^{3+}$, lanthanides and $La^{3+}$ under most biological conditions (Moeller, Martin, Thompson, Ferrus, Feistel & Randal, 1965; Nieboer, 1975), the presence of ionic species with other co-ordination numbers is probable. Symbols representing $IC_{50}$s are for the same $M^{3+}$ as those in A.
METAL CATION BLOCK OF T-TYPE CALCIUM CHANNELS

Using the human C cells, it was possible to study the effects of M3+ on deactivation kinetics over a wide range of potentials (Fig. 4). Neither Y3+ (n = 2) nor La3+ (n = 2) affected τd at repolarization potentials ranging from -200 to -60 mV.

![Diagram](image)

**Fig. 3.** The effect of Ca2+ concentration on potency of Iτ block by Ho3+. A–C, the inhibition of Iτ tail currents by 0.1 μM Ho3+ was measured at three different external Ca2+ concentrations. Tail current records before and after superfusing 0.1 μM Ho3+ in the presence of 2, 10 and 50 mM external Ca2+ as indicated. Voltage protocols were similar to those described in the legend of Fig. 1. Test potentials in A (-25 mV) and C (0 mV) were adjusted to account for shifts in the current–voltage relationship, produced by changing the external Ca2+ concentrations. D, inhibition curves for Ho3+ block of Iτ. Data points (n = 5–7) have been calculated as described in the legend of Fig. 1. IC50s for Ho3+ block and Hill slopes of corresponding curves are: 24.6 ± 2.4 nM, -1.23 ± 0.15, in 2 mM Ca2+ (■); 107 ± 5 nM, -1.27 ± 0.08, in 10 mM Ca2+ (▲); 716 ± 45 nM, -0.97 ± 0.06, in 50 mM Ca2+ (●).

To explore the possibility of slowly developing voltage-dependent block, we tested whether changing the conditioning and/or holding potential affected the potency of the M3+ antagonists. Conditioning pulses of 20 s duration to potentials ranging from -95 to -45 mV were ineffective in changing the potency of 100 nM Ho3+ (n = 1) or 1 μM La3+ (n = 1; Fig. 5) in rat C cells. Changes in holding potential did not affect the block by 750 nM Y3+ (n = 1) and 750 nM La3+ (n = 1) in human C cells (data not shown).

In addition to the lack of voltage dependence, no evidence of frequency-dependent block was observed. Increasing the stimulation frequency to values between 0.008
and 10 Hz did not change the potency of \( \text{La}^{3+} \) in two rat C cells (Fig. 5B), nor that of \( \text{Y}^{3+} \), \( \text{Gd}^{3+} \) and \( \text{La}^{3+} \) in six human C cells (data not shown).

In some preparations, applying extreme transmembrane potentials transiently relieves block of ion channels by charged antagonists (Swandulla & Armstrong, 1989; Thévenod & Jones, 1992). For positively charged pore blockers, extreme hyperpolarization appears to attract the trapped cation into the cell while strong depolarization expels it to the extracellular space. To determine whether block of \( I_T \) in human C cells by \( \text{Y}^{3+} \) and \( \text{La}^{3+} \) may be transiently relieved at positive potentials, we measured tail currents at \(-80 \text{ mV}\) after depolarizing steps to potentials as positive as \(+80 \text{ mV}\). To find if block was relieved at negative voltages, tail currents were measured at potentials between \(-50 \) and \(-200 \text{ mV}\), following an activating test pulse to \(0 \text{ mV}\) (Fig. 6, insets). Since the transient unblocking and/or reblocking upon change in membrane potential may occur rapidly (less than 1 ms), an effort was made to achieve fast voltage clamp. Using small spherical human C cells (cell

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Fig. 4. Effect of \( \text{M}^{3+} \) on T-type VGCC gating. \( A \), inactivation kinetics: scaled current traces recorded at test potentials of \(-20 \text{ mV}\) are shown beginning with the point of fastest inactivation. The vertical scale bar applies to the control record (0 \( \text{Gd}^{3+} \)). Currents recorded after addition of 100 and 500 \( \text{nM} \) \( \text{Gd}^{3+} \) are enlarged 1.25 and 3.73 times, respectively. \( B \), deactivation kinetics: scaled tails, recorded at \(-80 \text{ mV}\), are shown from the point of the fastest deactivation. The vertical scale bar applies to the control record (0 \( \text{Y}^{3+} \)). The scaling factors for tail currents recorded upon addition of 50 and 250 \( \text{nM} \) \( \text{Y}^{3+} \) are 1.33 and 4.03, respectively. \( C \), effect of \( \text{La}^{3+} \) on deactivation kinetics in a human C cell: deactivation time constants \( (\tau_D) \), obtained by fitting of raw data records, have been plotted in respect to the repolarization potential at which tails were recorded. ●, control; ○, 1 \( \mu \text{M} \) \( \text{La}^{3+} \).
METAL CATION BLOCK OF T-TYPE CALCIUM CHANNELS

Capacitance \( \approx 10 \) pF) and low resistance patch electrodes, which during recording resulted in access resistances of \( 2-3 \) M\( \Omega \), useful current measurements were obtained at times of \( \geq 80 \) \( \mu \)s (at least 4 time constants of voltage clamp, \( \tau_{vc} \)) after switching to the repolarization potential. Tail currents were measured at least

90 \( \mu \)s after tail inactivation in control conditions and in the presence of \( Y^{3+} \) or \( La^{3+} \).

Neither extreme depolarization (\( n = 6 \) for 0-2 to 2-5 \( \mu \)M \( Y^{3+} \); \( n = 2 \) for 1 \( \mu \)M \( La^{3+} \)) nor extreme repolarization (\( n = 2 \) for both 0-2 \( \mu \)M \( Y^{3+} \) and 1 \( \mu \)M \( La^{3+} \)) significantly relieved the block by \( Y^{3+} \) or \( La^{3+} \) (Fig. 6).

**Block of \( I_T \) by divalent nickel cation (\( Ni^{2+} \))**

Although \( Ni^{2+} \) is widely used as a preferential antagonist of T-type VGCC, properties of its blocking action apart from concentration dependence have not been described. \( Ni^{2+} \) reversibly blocked \( I_T \) in rat C cells with an \( IC_{50} \) of 5-8 \( \pm 0-5 \) \( \mu \)M and Hill slope (\( N \)) significantly less than 1 (0-69 \( \pm 0-05 \)) (Fig. 7 A), and in human C cells (\( n \geq 5 \), not shown) with \( IC_{50} \) of 5-45 \( \pm 0-5 \) \( \mu \)M and Hill slope of -0-62 \( \pm 0-04 \). In
rat C cells, blocking potency with respect to equipotential control was not affected by changing of conditioning potentials in the range from −95 to −45 mV (n = 3; Fig. 7B). Voltage dependence of block was studied over a wider range of potentials in human C cells, with experimental protocols the same as those used in Fig. 6.

![Diagram](image)

*Fig. 6. Effect of extreme voltages on block of \( I_T \) by \( Y^{3+} \) and \( La^{3+} \). A and B, tail currents were recorded at a repolarization potential of −80 mV after activation by 10−5 ms steps to various potentials between −60 to + 50 mV before and after block by 200 nM \( Y^{3+} \) (A) or 1 µM \( La^{3+} \) (B). Cut-off frequency of analogue filter was 12 kHz and sampling rate was 30 µs. \( \tau_{VC} \) was 35 µs (A), and 31.5 µs (B). Tail current amplitudes are the average of 3 consecutive sample points recorded 120 to 180 µs after repolarization. C and D, tail currents were recorded at potentials between −50 and −200 mV after activation by 10−5 ms depolarizing steps to 0 mV. Data points represent amplitudes of recorded tail currents before and after block by 200 nM \( Y^{3+} \) (C) or 1 µM \( La^{3+} \) (D). Sampling and filtering specifications as in (A). \( \tau_{VC} \) for C and D were 35 and 60 µs respectively. Lag of averaged sample points was 210−270 µs.

Neither depolarization to + 80 mV (n = 6; Fig. 7C), nor repolarization to −200 mV (n = 2; Fig. 7D) influenced the potency of \( I_T \) block by 5 to 200 µM \( Ni^{2+} \).

**DISCUSSION**

*Mechanism of block of T-type VGCC by \( Y^{3+} \), \( La^{3+} \), lanthanides and \( Ni^{2+} \)*

The results presented here show that \( Y^{3+} \), \( La^{3+} \), lanthanides and \( Ni^{2+} \) block current through T-type VGCC in a competitive and concentration-dependent manner. Sufficiency of nanomolar concentration for block, lack of effects on channel
gating and notable antagonism between Ca\(^{2+}\) and the blockers favour ion pore occlusion as the blocking mechanism for each of these cations. Blocking actions through screening of negative surface charges, or by allosteric modulation seems unlikely for the same reasons.

![Graphs and plots](image)

Fig. 7. Properties of \(I_T\) block by Ni\(^{2+}\). A, concentration-dependent block of \(I_T\) by Ni\(^{2+}\) in rat C cells. Inhibition curve with an IC\(_{50}\) of 5.65 ± 0.84 and Hill slope of −0.69 ± 0.05 was obtained using procedures as described in Fig. 2. Experimental replicates, \(n\), are given in parentheses. B, effect of 5 μM Ni\(^{2+}\) on \(I_T\) availability in a rat C cell. Experimental procedure as in Fig. 5 A. \(V_5\) was −69.5 ± 0.1 mV for the control (●) and −71.09 ± 0.2 mV for the inhibition curve in Ni\(^{3+}\) (○). C and D, the effect of 25 μM Ni\(^{2+}\) (△,C; control, ▲) and 5 μM Ni\(^{3+}\) (◇,D; control, ◆) on tail currents in human C cells. Experiments were performed as described in the legend of Fig. 6. In C, \(\tau_{vc}\) was 47 μs, 3 consecutive sample points, beginning 180 to 240 μs after the repolarization step, were averaged to give tail current amplitudes. In D, \(\tau_{vc}\) = 36 μs and the lag was 120 to 180 μs.

In order to pass through an ion channel, a permeant ion must bind to at least one, and probably two or more binding sites located in the permeation pathway (Hess & Tsien, 1984; Hess, Lansman & Tsien, 1986). Inorganic channel blockers and permeant ions appear to compete for common binding sites at the channel. Therefore, inorganic blockers are useful tools for studying properties of binding sites (see Lester, 1991; Hille, 1992 for references and discussion). The voltage-independent block of T-type VGCC by Y\(^{3+}\), La\(^{3+}\) and lanthanides, described in this study, implies that block occurs by their binding to a site which is outside the membrane electric field. However, small changes in potency of the M\(^{3+}\) antagonists
at different potentials cannot be ruled out because of difficulties in measuring small (< 50 pA) tail currents at high cut-off frequencies (e.g. Fig. 6D). The inability of extreme hyperpolarizing pulses to even transiently alleviate block indicates that Y\(^{3+}\), La\(^{3+}\) and lanthanides, as well as Ni\(^{2+}\) cannot pass through T-type VGCC, and thus represent non-permeant T-type VGCC antagonists.

Inability of Y\(^{3+}\), La\(^{3+}\) and Ni\(^{2+}\) to change macroscopic deactivation and inactivation kinetics suggests that T-type VGCC can normally close or inactivate when occupied with one of the antagonists. This, in turn, implies that under physiological conditions, in the absence of antagonists, at least one Ca\(^{2+}\) ion stays trapped in the closed or inactivated channel, respectively. Swandulla & Armstrong (1989) discussed the possibility that binding and unbinding the Ca\(^{2+}\) ion to the channel pore participates in gating of ion channels.

Inferences about structure of the T-type VGCC binding site

Cationic radius is an important variable that determines the affinity and rate of ion interactions with protein-binding sites (Tew, 1977; Tam & Williams, 1985). Lanthanides, La\(^{3+}\) and Y\(^{3+}\) are the most useful metal cations for studying Ca\(^{2+}\)-dependent processes because they share similar chemical properties and ionic radii with Ca\(^{2+}\) (Nieboer, 1975; dos Remedios, 1981). Assuming that the IC\(_{50}\) which we measured provide a good approximation of the lanthanide antagonists’ affinity for the T-type VGCC, our results show a gradual decrease in the affinity of larger lanthanides for the binding site. Such an affinity sequence resembles those of lanthanide complexes with EGTA, EDTA and especially acetylacetonate, as well as those reported for interactions of lanthanides with several proteins. However, different affinity sequences for proteins and various biological preparations, with maximal affinity for larger elements or for those in the middle of the lanthanide series, have more often been observed (for references see Nieboer, 1975; dos Remedios, 1981; Evans, 1992). The interpretation of ionic radius-dependent interactions of lanthanides with Ca\(^{2+}\)/lanthanide-binding sites of proteins according to a scheme proposed by Tew (1977), explains the affinity sequence on the basis of differences between the free energy of hydration of the cation and the energy of interaction between the cation and the negative binding site (see dos Remedios, 1981; Evans, 1990; Lansman, 1990 and references cited therein for more detailed discussion).

Comparison with previous studies of Y\(^{3+}\), La\(^{3+}\) and lanthanide effect on VGCC

Quantitative description of T-type VGCC block by La\(^{3+}\) has been reported for mouse neuroblastoma cells (IC\(_{50}\) = 1·5 µM, in 50 mM Ba\(^{3+}\), Narahashi et al. 1987), rat hypothalamic neurones (IC\(_{50}\) = 0·7 µM, in 10 mM Ca\(^{2+}\), Akaike et al. 1989) and rat aorta smooth muscle cells (IC\(_{50}\) = 0·6 µM, in 20 mM Ca\(^{2+}\), Akaike et al. 1989). IC\(_{50}\) for Y\(^{3+}\) and lanthanide block of T-type VGCC have not been reported.

In comparing our results to Lansman’s study (1990) of lanthanide block of current through L-type VGCC, we have noted the following differences: (1) the potency of steady-state block by lanthanides is voltage independent for T-type VGCC, but shows characteristics of open channel block for L-type VGCC; (2) repolarization of
open channels to negative potentials can force lanthanides through L-type, but not T-type VGCC; (3) larger lanthanides block L-type channels more potently than those with smaller ionic radii. The inverse relationship holds for lanthanide-mediated block of T-type VGCC.

High voltage-activated N- and P-type Ca$^{2+}$ channels are prominent in nerve terminals of the CNS (Turner, Adams & Dunlap, 1992). A series of lanthanides were shown to inhibit voltage-dependent Ca$^{2+}$ influx into rat brain synaptosomes according to a potency sequence resembling that observed for block of L-type VGCC (Nachsen, 1984). Differences between high voltage-activated VGCC (L-, N- and P-type) and low voltage-activated T-type VGCC with respect to sensitivity to lanthanides of different radii suggest corresponding differences in the structure of their respective Ca$^{2+}$/M$^{3+}$ binding sites. Surprisingly, with respect to potency ($IC_{50} \approx 2 \mu M$ in 2 mm Ca$^{2+}$, Rechling & MacDermot, 1992) and lack of voltage dependence, block of T-type Ca$^{2+}$ channels by La$^{3+}$ resembles inhibition of the NMDA ligand-gated ion channel.

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