Extracellular Ca2+ ions induced a decrease in an inward current in thalamic neurons

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Publisher's version / Version de l'éditeur:

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Canada

Changes in extracellular Ca^{2+} can affect the pattern of discharge in rat thalamic neurons

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1. The aim of this study was to investigate some of the cellular mechanisms involved in the effects caused by changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$).

2. Current- and voltage-clamp experiments were carried out on acutely isolated thalamic neurons of rats.

3. Increasing $[Ca^{2+}]_o$ alone induced a transition of the discharge from single spike to burst mode in isolated current-clamped neurons.

4. Increasing $[Ca^{2+}]_o$ caused the voltage-dependent characteristics of the low voltage-activated (LVA) transient Ca^{2+} currents to shift towards positive values on the voltage axis. Changing $[Ca^{2+}]_o$ from 0.5 to 5 mM caused the inactivation curve to shift by 21 mV.

5. Extracellular Ca^{2+} blocked a steady cationic current. This current reversed at -35 mV, was scarcely affected by Mg^{2+} and was completely blocked by the non-selective cation channel inhibitor gadolinium (10 μM). The effect of $[Ca^{2+}]_o$ was mimicked by 500 μM spermine, a polyamine which acts as an agonist for the Ca^{2+}-sensing receptor, and was modulated by intracellular GTP-γ-S.

6. At the resting potential, both the voltage shift and the block of the inward current removed the inactivation of LVA calcium channels and, together with the increase in the Ca^{2+} driving force, favoured a rise in the low threshold Ca^{2+} spikes, causing the thalamic firing to change to the oscillatory mode.

7. Our data indicate that $[Ca^{2+}]_o$ is involved in multiple mechanisms of control of the thalamic relay and pacemaker activity. These findings shed light on the correlation between hypercalcaemia, low frequency EEG activity and symptoms such as sleepiness and lethargy described in many clinical papers.

The role of internal Ca^{2+} in neuronal processing has been widely studied but less is known about Ca^{2+} from an extracellular point of view. In pathological conditions involving persistent hypercalcaemia, various neuro-psychiatric symptoms can be present, including a state ranging from drowsiness to lethargy, and there is low frequency electroencephalographic (EEG) activity (Evaldsson *et al.* 1969; Allen *et al.* 1970; Guisado *et al.* 1975; Joborn *et al.* 1991). The thalamic neurons play an important role in the electro-cortical rhythms. They fire with single spikes during wakefulness (relay mode), and oscillate and synchronise their firing, producing bursts of action potentials, during slow-wave sleep (oscillatory mode). The rhythms arise as a consequence of the intrinsic pacemaker property of the neurons and the network of excitatory and inhibitory synaptic connections within

the thalamus and between the thalamus and the cortex (Steriade & Llinas, 1988; McCormick & Bal, 1997).

The transition from relay mode to oscillatory mode is caused by a resting membrane hyperpolarisation of the thalamo-cortical neurons (Jahnsen & Llinas, 1984; Steriade & Llinas, 1988; Von Krosigk *et al.* 1993; McCormick & Bal, 1997). The hyperpolarisation de-inactivates the LVA Ca^{2+} currents. Activation of the LVA Ca^{2+} current depolarises the membrane towards the threshold for a burst of Na^+- and K^+-dependent action potentials. Ca^{2+} also enters the cell during the burst through a high voltage-activated (HVA) Ca^{2+} conductance, leading to the activation of a Ca^{2+}-dependent K^+ current. Combined with the inactivation of LVA Ca^{2+} channels this causes membrane hyperpolarisation, which subsequently de-inactivates LVA Ca^{2+} channels and activates a

Table 1. Solutions used for whole-cell patch-clamp recordings

	$CaCl_2$	$MgCl_2$	NaCl	KCl	NaH_2PO_4	$NaHCO_3$	Glucose	pH
Ext 1	0.5–5	2	125–117.5	3	1.2	26	10	7.4
Ext 2	0.5–4.5	4.5–0.5	122	3	1.2	26	10	7.4

	$CaCl_2$	$MgCl_2$	NaCl	TEA	Hepes	Choline chloride	Glucose	pH
Ext 3	0.5–5	1	—	20	10	100.5–105	10	7.4

	$CaCl_2$	$MgCl_2$	NaCl	KCl	Hepes	Choline chloride	Glucose	pH
Ext 4	4.5–0	0–4.5	141	3	10	—	10	7.4
Ext 5	0.01–0.1	—	141	3	10	—	10	7.4
	0.5–1–5							
	10							
Ext 6	4.5–0	0–4.5	—	—	10	144	10	7.4
Ext 7	4.5–0	0–4.5	—	20	10	124	10	7.4
Ext 8	4.5–0	0–4.5	—	144	10	—	10	7.4
Ext 9	4.5–0	0–4.5	20	—	10	124	10	7.4
Ext 10	4.5–0	0–4.5	144	—	10	—	10	7.4
Ext 11	0.5–4.5	—	141	3	10	—	10	7.4
Ext 12	—	0.5–4.5	141	3	10	—	10	7.4
Ext 13	0.5–4.5	4.5–0.5	141	3	10	—	10	7.4

	$CaCl_2$	$MgCl_2$	Potassium gluconate	KCl	Hepes	EGTA	—	pH
Int 1	1	1	135	—	10	11	—	7.3
Int 2	1	2	—	140	10	11	—	7.3

	CsCl	$MgCl_2$	TEA	—	Hepes	EGTA	Glucose	pH
Int 3	110	0.8	30	—	10	10	8	7.3

Ext, extracellular solution; Int, intracellular solution. Concentrations are millimolar. Solutions Ext 3, 6, 7 and 8 and Int 3 were titrated with CsOH.

hyperpolarisation-activated cation current (I_h), which slowly depolarises the membrane towards the threshold for another Ca^{2+} spike (McCormick & Bal, 1997). Resting membrane hyperpolarisation therefore leads to the interplay between the low threshold Ca^{2+} spike and the I_h that results in low frequency oscillations.

According to the surface potential theory, Ca^{2+} ions should modify the cellular transmembrane potential, masking the negative fixed charges on the surface, hyperpolarising the membrane (for review see Green & Andersen, 1991; Hille, 1991) and causing the thalamic pacemaker and cortical neurons to shift from the relay to the oscillatory mode.

Here we show that in current-clamped isolated thalamic neurons an increase in extracellular Ca^{2+} alone causes the neuronal firing to change from single spikes to the bursting oscillatory mode. Whole-cell patch-clamp recordings indicate that this transition is caused by a voltage shift to positive values of the voltage-dependent characteristics of the channels, and by an increase in the Ca^{2+} driving force, but unexpectedly also by membrane hyperpolarisation due to a decrease in a novel membrane cationic inward current coupled through G-proteins to a Ca^{2+}-sensing receptor. Preliminary reports of our results have already been presented (Formenti et al. 1996, 1998a; De Simoni et al. 2000).

METHODS

Cell preparation

The experiments were done on primary rat thalamic cell cultures. The neurons were prepared as previously described (Formenti *et al.* 1995; Formenti & De Simoni, 2000). Briefly, 14- to 18-day-old Wistar rats were anaesthetised with ether, decapitated and the brain was removed and placed in cold Pipes-buffered saline (4 °C) containing (mM): NaCl 120, KCl 5, $MgCl_2$ 4, glucose 25 and Pipes 20; pH was adjusted to 7.4 with NaOH. The brain was then dissected in the frontal plane using a vibroslice (Campden Instruments Ltd, UK). Frontal slices (500 μm thick) containing the thalamus were incubated at room temperature for 75 min in Pipes-buffered saline containing 0.8 mg ml^{-1} trypsin (Sigma type XI), 1 mM kynurenic acid and 50 μM 2-amino-5-phosphonovalerate (APV), saturated with O_2. After the enzymatic treatment, the thalamus was identified, carefully isolated, and gently minced in Pipes-buffered solution using a Pasteur pipette. The isolated neurons were plated on Petri dishes and stored at room temperature. All procedures were in accordance with local and national laws and ethical guidelines (municipal act no. 143650.400/16754/90; decree 36/94-A Ministry of Health).

Isolated cell recordings

Current- and voltage-clamp recordings were carried out a few hours after plating at 22 °C, using the whole-cell patch-clamp technique (Hamill *et al.* 1981). The methods and instrumentation used in this study were similar to those previously described (Formenti *et al.* 1998b). Patch pipettes of about 6 MΩ resistance were used, with a NPI Sec1L switching amplifier (Tamm, Germany; sampling rate 15–30 kHz), interfaced with an IBM-compatible computer. Stimulation and data acquisition were done using pCLAMP programs

(Axon Instruments, Foster City, CA, USA) and the data were digitised at 50 μs sampling times using a 12-bit A/D Tecmar Master board. Statistical data are presented as means ± S.E.M.

Solutions for recordings

The solutions used for whole-cell patch-clamp recordings are summarised in Table 1. To reduce the run-down of calcium current, an ATP regenerating system (Forscher & Oxford, 1985) consisting of 20 mM creatine phosphate, 4 mM Na$_2$-ATP and 50 IU ml^{-1} creatine phosphokinase (bovine heart, Sigma Type III) was added to the patch pipette solution. All the drugs were supplied by Sigma (St Louis, MO, USA).

RESULTS

An increase of [Ca^{2+}]$_o$ caused the discharge to change to burst mode in current-clamped isolated thalamic neurons

To study the effects of [Ca^{2+}]$_o$ on the neuronal firing mode, current-clamp experiments were carried out on isolated thalamic neurons. Figure 1*A* shows the discharge induced by a depolarising current stimulus at different extracellular calcium concentrations. Extracellular and

intracellular solutions Ext 1 and Int 1 (Table 1) were used in these experiments. Cellular firing could change from the single spike mode (relay mode) to the burst mode (oscillatory mode), usually with variations of about 1 mM in the [Ca^{2+}]$_o$, especially in the concentration range of 1 to 2 mM, and the effect was completely reversible. Figure 1*B* shows the appearance of a low threshold calcium spike after an 'anode break' stimulus, when changing the [Ca^{2+}]$_o$ from 1.5 to 2 mM. The increase in [Ca^{2+}]$_o$ was accompanied by measurable membrane hyperpolarisation in all neurons ($n = 22$; Figs 1*A* and 3).

In a preliminary study we used the cell-attached configuration of the patch-clamp technique to quantify the effects of changes in [Ca^{2+}]$_o$ on seal resistance, in order to exclude the possibility that the calcium-induced hyperpolarisation was an artifact caused by increased seal resistance (Formenti & De Simoni, 2000). The seal resistance rose significantly as a function of [Ca^{2+}]$_o$ only for seal resistances lower than 1 GΩ; with better seal resistances changes in [Ca^{2+}]$_o$ had a negligible effect on seal resistance.
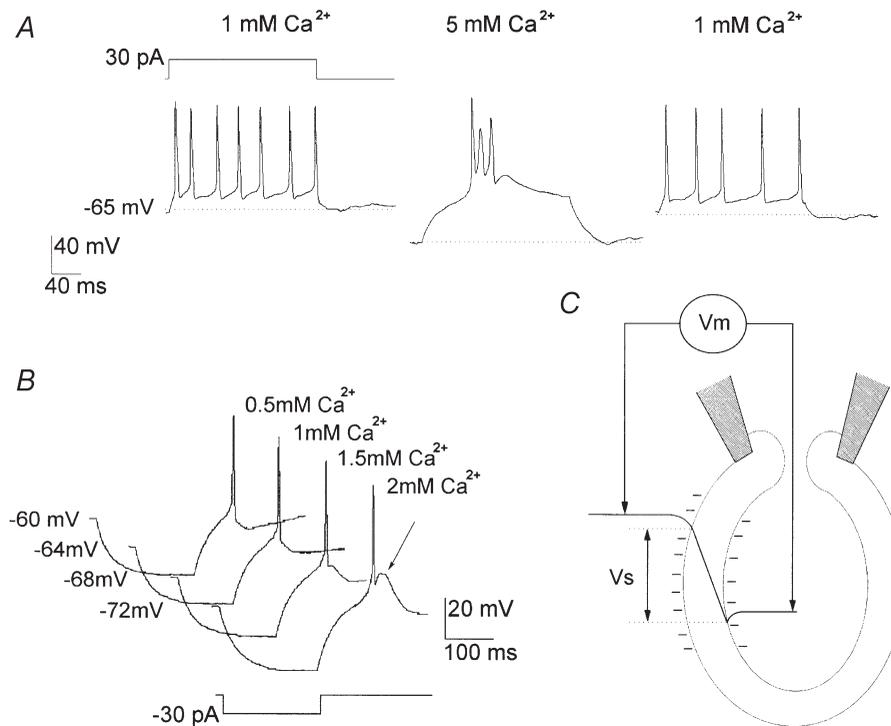

Figure 1. Single spikes and burst mode discharge in isolated thalamic neurons *in vitro* as a function of [Ca^{2+}]$_o$

A, in a current-clamped cell, a depolarising current stimulus (upper trace) evoked cell firing. Altering [Ca^{2+}]$_o$ from 1 to 5 mM caused the discharge to turn into burst mode (oscillatory mode), which returned to the single spike discharge (relay mode) when the [Ca^{2+}]$_o$ was returned to 1 mM. Note the hyperpolarisation of the resting potential induced by the increase in [Ca^{2+}]$_o$. *B*, scaled current traces from a neuron with an 'anode-break' stimulus (duration, 200 ms; amplitude, −30 pA) from a resting potential near −60 mV, during superfusion with [Ca^{2+}]$_o$ at the values indicated beside each trace. Note the appearance of a low threshold calcium spike on changing from 1.5 to 2 mM Ca^{2+} (arrow). *C*, the profile of the electrical potential across the cell membrane. According to the surface potential hypothesis, the potential difference between the bulk solutions (V_m) recorded in the whole-cell patch-clamp configuration is different from the potential between the two membrane surfaces (V_s), which is sensed by the channels.

According to the surface potential theory, Ca^{2+} ions should modify the cellular transmembrane potential (V_s), masking the negative fixed charges on the surface and hyperpolarising the membrane. This effect is not directly measurable, since in our experimental conditions the electric potential (V_m) is measured not between the inner and outer membrane surfaces, but between the intracellular and extracellular bulk solutions (Fig. 1C); however, the transmembrane voltage change should be sensed by the voltage-activated channels in the membrane, and it may appear as a shift of the voltage-dependent characteristics along the voltage axis. On the other hand, this theory can hardly account for the hyperpolarisation observed directly in current-clamp recordings. $[Ca^{2+}]_o$ may have some effect on membrane conductance.

External changes in $[Ca^{2+}]$ shifted the voltage-dependent characteristics of LVA Ca^{2+} channels

To evaluate the voltage shift expected when the $[Ca^{2+}]_o$ increased, whole-cell voltage-clamp recordings were made on cultured thalamic neurons. The effects were studied on LVA calcium channels, because this membrane conductance is involved in generating the low threshold spikes and oscillatory behaviour.

LVA Ca^{2+} currents were evoked by a 200 ms voltage ramp from a holding potential of -140 mV to $+20$ mV. These experiments were done using solutions Ext 3 and Int 2 in Table 1. Increasing $[Ca^{2+}]_o$ from 0.5 to 5 mM raised

the Ca^{2+} current peak amplitude, with a shift of the LVA Ca^{2+} current peak and of the relative activation curve of 18.7 ± 1.6 mV, $n = 8$; Fig. 2A). The voltage-dependent characteristics of HVA Ca^{2+} currents showed a shift that did not significantly differ from the LVA currents (22.5 ± 2.3 mV, $n = 6$; data not shown). Figure 2B shows the voltage shift of the inactivation curve of the LVA Ca^{2+} currents. As the $[Ca^{2+}]_o$ increased, the inactivation curve shifted towards positive potentials, and the channels were de-inactivated.

An increase in $[Ca^{2+}]_o$ hyperpolarised the isolated thalamic neurons, blocking an inward current

The possibility that the Ca^{2+}-induced hyperpolarisation was caused by a voltage shift in the voltage-dependent characteristics of the membrane was considered. Testing different divalent cations in the extracellular solution, we noted that Ba^{2+} mimicked the hyperpolarising effects of Ca^{2+} (data not shown), whereas Mg^{2+} caused only a small negative change in membrane potential in current-clamped thalamic neurons ($n = 11$, Fig. 3A). The screening effect of Mg^{2+} on the negative fixed charges of the membrane was similar to that induced by Ca^{2+} ions since switching from 4.5 mM Ca^{2+} to 4.5 mM Mg^{2+} (solution Ext 13 in Table 1) did not change the voltage at the peak of the Na^+ current evoked in voltage-clamped neurons (Fig. 4, $n = 15$). The voltage-dependent Na^+ current was measured in place of the LVA Ca^{2+} current because the change in LVA Ca^{2+} current intensity, due to the variation in $[Ca^{2+}]_o$,
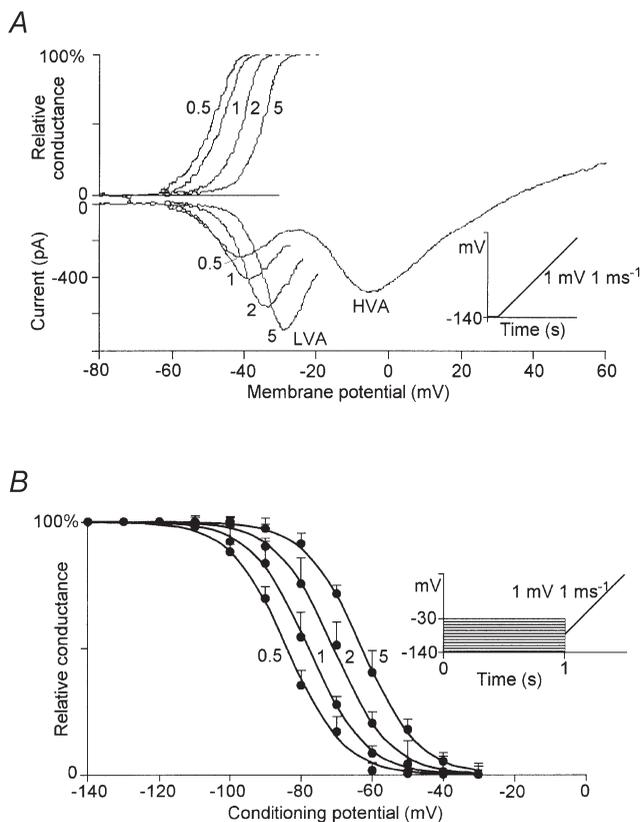
Figure 2. Voltage-dependent characteristics of LVA Ca^{2+} currents measured at different $[Ca^{2+}]_o$

The effects were tested by whole-cell voltage-clamp recordings in acutely dissociated thalamic neurons. *A*, lower panel, the current–voltage relationship (*I–V*) of LVA Ca^{2+} currents was obtained using voltage ramp stimuli from a holding potential of -140 mV to $+20$ mV (inset) at different $[Ca^{2+}]_o$; the concentration (mM) is indicated on each curve. The activation curves (upper panel) were calculated from the experimental *I–V* traces: relative conductance $= I_{Ca}/(V_m - E_{Ca})$, normalised to 100 % (where E_{Ca} is the Ca^{2+} equilibrium potential). An increase in $[Ca^{2+}]_o$ caused an augmentation of the peak Ca^{2+} current amplitude and shifted the current activation and current peak towards positive values. *B*, the inactivation curve measured at different $[Ca^{2+}]_o$. LVA Ca^{2+} currents (not shown) were obtained using a voltage ramp stimulus repeated after conditioning potentials scaled between -140 and -30 mV (inset). The ramp stimulus was adopted in order to identify and measure the peak LVA calcium current and to distinguish it clearly from other currents during the voltage changes induced by different $[Ca^{2+}]_o$. For each concentration and each cell recorded, peak currents were normalised as a percentage of the maximum current. The means $\pm$ s.e.m. from six cells were plotted in the figure as a function of the conditioning potential. The values were fitted with Boltzmann curves: $1/[1 + \exp^{(V - V_{0.5})/k}]$ where $V_{0.5}$, the midpoint, changed from -84 to -78, -71 and -63 mV, when the $[Ca^{2+}]_o$ was 0.5, 1, 2 and 5 mM, respectively; k (8 mV) is the slope factor.

tended to modify the slope of the voltage ramp to which the neuron was subjected, even in well-clamped cells. This would cause a small artefact, shifting the current peak on the voltage axis. In view of its properties, Mg^{2+} was used to set the divalent cations of the extracellular solutions to a constant concentration, to minimise the induction of a voltage shift while changing from one Ca^{2+} concentration to another. In these experimental conditions too, Ca^{2+} had a similar but weaker hyperpolarising effect (Fig. 3B). An increase in $[Ca^{2+}]_o$ raised membrane resistance. This was observed in current-clamp experiments using hyper-polarising current steps (Fig. 3B). The increase in membrane resistance was accompanied by persistent hyperpolarisation when the resting membrane potential was below -35 mV ($n = 11$; Fig. 3C). The hyperpolarisation increased at more negative resting potentials (Fig. 3D). These data suggest that an inward current is blocked or modulated by Ca^{2+}.

A novel Ca^{2+}-sensitive conductance in thalamic neurons

Whole-cell voltage-clamp experiments were performed to test the blocking effect of Ca^{2+} on steady-state total currents at different test potentials. Solutions containing divalent cations (Ca^{2+} plus Mg^{2+}) at a constant concentration were used. The voltage-clamp protocol used to study how $[Ca^{2+}]_o$ affected the whole-cell persistent ionic currents consisted of a positive depolarising step or ramp, followed by scaled steps of 10 s duration from -20 to -80 mV. The first depolarisation was intended to check and to inactivate the transient current components. During the next set of steps, changing $[Ca^{2+}]_o$ from 4.5 mM to 0 mM caused the ionic currents to increase (Fig. 5A). The current amplitude was determined as the mean during the period from 2 s after the beginning of the superfusion till the end. The difference at 0 and 4.5 mM $[Ca^{2+}]_o$ plotted as a function of the potential represents
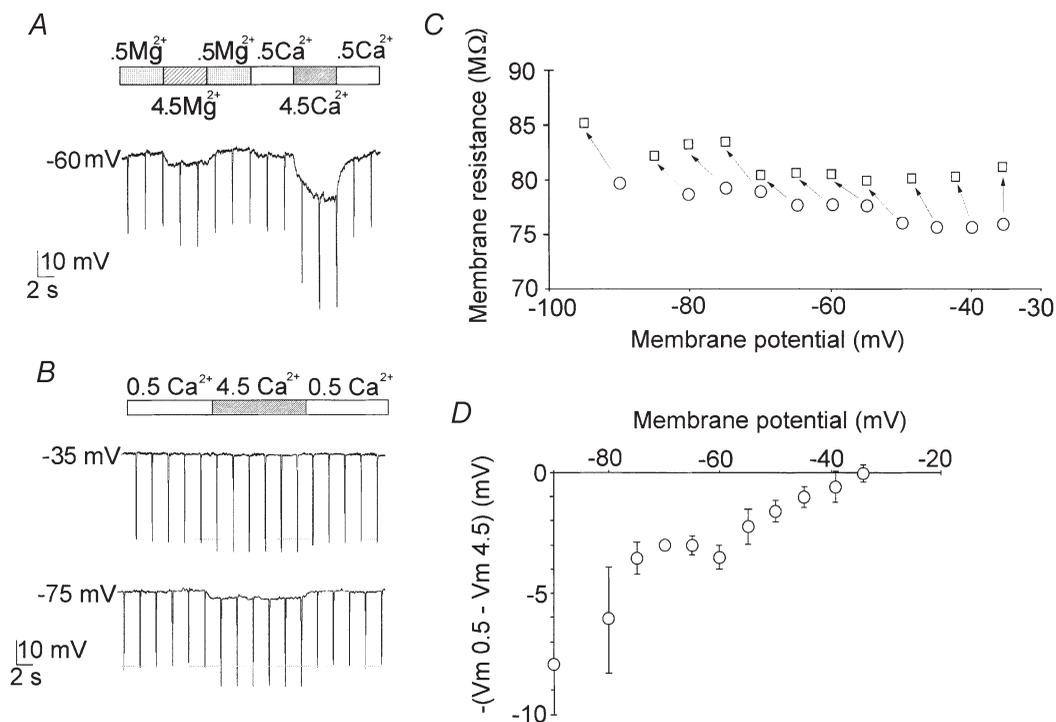

Figure 3. Effects of changes in [Ca^{2+}]$_o$ on the membrane resistance and on the resting potential in whole cell current-clamp experiments

A, increasing extracellular Mg^{2+} from 0.5 to 4.5 mM caused a weak hyperpolarisation compared to that caused by the same change in Ca^{2+} (Mg^{2+}, -6.6 ± 0.7 mV; Ca^{2+}, -22.8 ± 2.5 mV; $n = 4$; Int 2 and Ext 11/12 in Table 1). The membrane hyperpolarisation was accompanied by a proportional increase in membrane resistance, tested by hyperpolarising current steps (amplitude, 500 pA; duration, 50 ms). B, the divalent cations in all the extracellular solutions were balanced to 5 mM by adding Mg^{2+}, to minimise the induction of a voltage shift of the voltage-dependent characteristics of the channels. Raising $[Ca^{2+}]_o$ from 0.5 to 4.5 mM increased membrane resistance from 78.5 ± 0.3 to 82 ± 0.7 MΩ, $n = 11$, when evaluated between -80 and -60 mV. This effect was accompanied by persistent hyperpolarisation when the resting membrane potential was maintained below -35 mV (Int 1 and Ext 13 in Table 1). In A and B the bars at the top indicate the external solution changes. C, the resistance and voltage changes seen on raising $[Ca^{2+}]_o$ from 0.5 mM ($\bigcirc$) to 4.5 mM ($\square$). Same cell as shown in B; each arrow represents one trial. D, the amplitude of the membrane hyperpolarisation on raising $[Ca^{2+}]$ from 0.5 to 4.5 mM as a function of the resting potential in 0.5 mM Ca^{2+}. Each point represents the mean of 11 recordings from different cells.
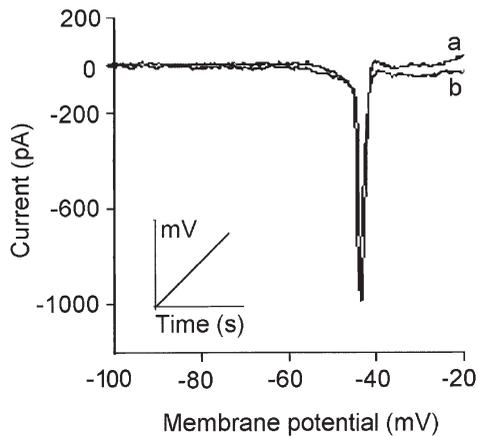
Figure 4. External Ca^{2+} and Mg^{2+} had similar effects on the surface potential

To study the effect of changes in $[Ca^{2+}]_o$ on membrane current, avoiding the voltage shift due to the non-specific screening effect of Ca^{2+} ions, the solutions were balanced with Mg^{2+}. This figure shows that the voltage at the peak of the Na^+ current evoked by a voltage ramp (0.66 mV ms^{-1}; inset) was not affected by changing the external solution from 4.5 mM Ca^{2+} and 0.5 mM Mg^{2+} (trace *a*) to 0.5 mM Ca^{2+} and 4.5 mM Mg^{2+} (trace *b*; Int 1 and Ext 13 in Table 1). The voltage at the peak was -43 ± 0.5 and -45 ± 3.4 mV with 4.5 mM Ca^{2+} and 4.5 mM Mg^{2+}, respectively ($n = 15$). The difference was not significant ($P > 0.05$). This indicates that Ca^{2+} and Mg^{2+} ions exert the same screening effect on the negative fixed charges at the mouth of the Na^+ channels.

the current–voltage relationship of the Ca^{2+}-sensitive current. The reversal potential was about -35 mV ($n = 17$; Fig. 5*B*). The *I–V* relationship was almost linear over the whole potential range tested, suggesting that this conductance is not voltage dependent.

Various $[Ca^{2+}]_o$ were tested and the dose–response curve is shown in Fig. 5*C*. The current was recorded at -80 mV at different Ca^{2+} concentrations (0.01, 0.1, 0.5, 1, 5 and 10 mM), then it was normalised and plotted as a percentage of the total current. The data points were
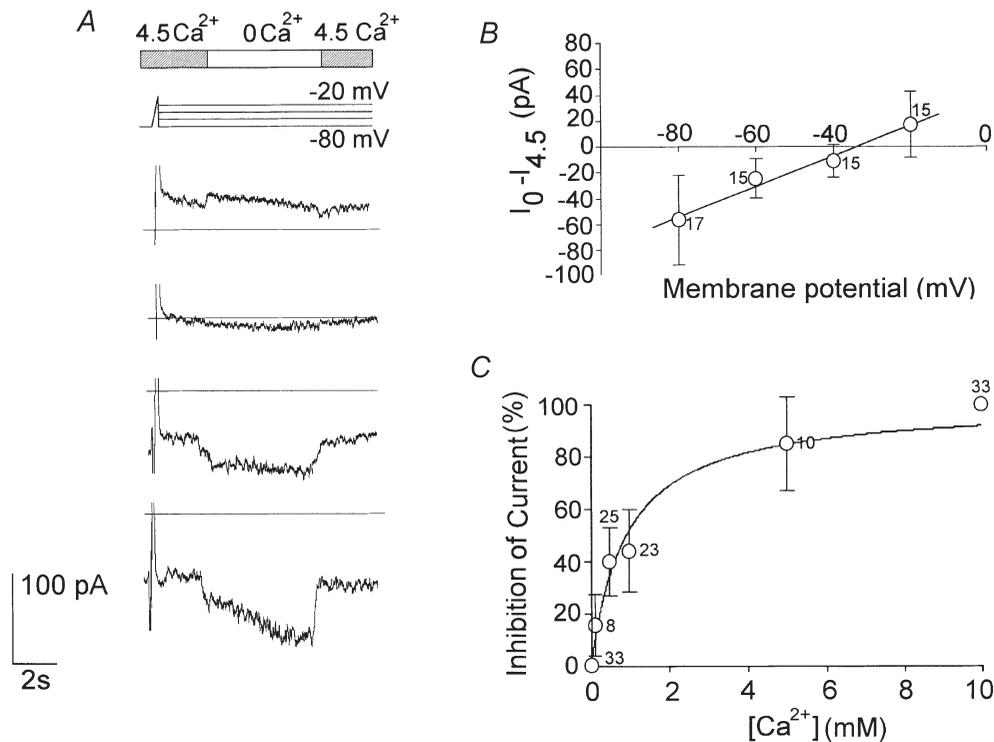

Figure 5. Effects of external Ca^{2+} on the total persistent ionic currents

The effects of different $[Ca^{2+}]_o$ on the total persistent ionic currents were investigated in whole-cell voltage-clamp experiments using solutions balanced with Mg^{2+} (Ext 4, Int 2 in Table 1). *A*, to study the effects of $[Ca^{2+}]_o$ (top bar) on the whole-cell currents (traces below) a voltage-clamp protocol was used, consisting of a 100 ms depolarising ramp from -80 to -20 mV (to check and inactivate the transient current components), followed by 10 s scaled step potentials from -20 to -80 mV. Removing the extracellular Ca^{2+} increased the ionic currents. *B*, the current–voltage relationship shows the fraction of the currents activated by changing from 4.5 to 0 mM Ca^{2+}, as a function of the test potential ($\bigcirc$, $\pm$ S.E.M.). Note that the reversal potential is nearly the same as in current-clamp recordings (Fig. 3*D*). *C*, the percentage inhibition of the current as a function of $[Ca^{2+}]_o$ (Int 2 and Ext 5 in Table 1). Data were fitted with a saturation binding curve: $y = [Ca^{2+}]/K_d + [Ca^{2+}]$, where K_d, the equilibrium dissociation constant, is 0.91 ± 0.15 mM ($n = 19$). All measurements were taken at -80 mV. The number of observations is indicated beside each data point in *B* and *C*.
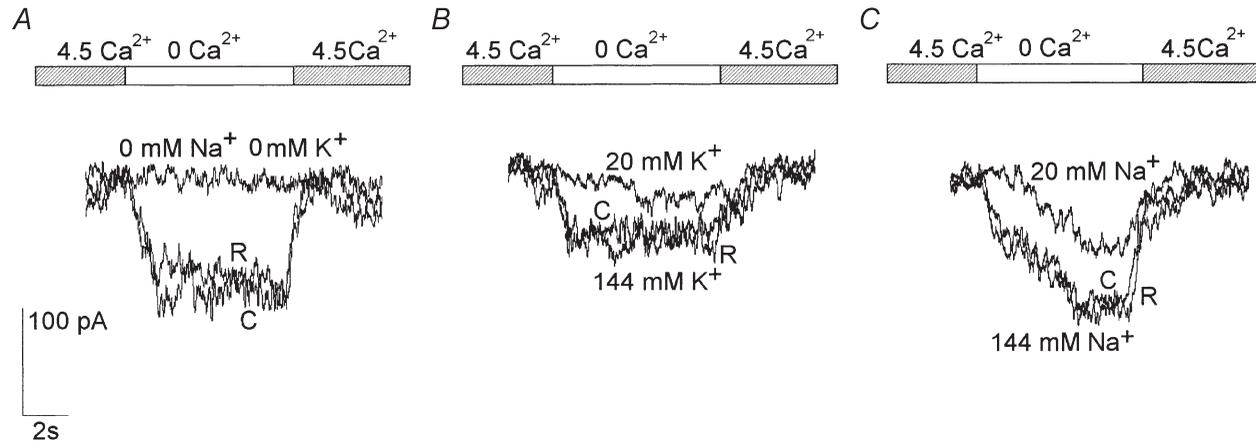
Figure 6. Effects of the external concentration of Na⁺ and K⁺ on the selectivity of the Ca²⁺-sensitive channels

The current activated by decreasing the $[Ca^{2+}]_o$ to zero (top bar) was completely abolished when the external Na⁺ and K⁺ (Ext 4 in Table 1) were replaced with choline (*A*, Ext 6 in Table 1). The Ca²⁺-sensitive current was partially preserved in the presence of external K⁺ at 144 and 20 mM (*B*, Ext 8 and 7 in Table 1) or 144 and 20 mM Na⁺ (*C*, Ext 10 and 9 in Table 1). Note that Na⁺ is more permeant than K⁺. All the recordings were taken at −80 mV. C, control before changing to 0 Ca²⁺; R, recovery on return to 4.5 mM Ca²⁺.

fitted using a saturation binding curve. The thalamic current was maximal at $[Ca^{2+}]_o$ below 10 μM. The concentration that blocked the current by 50% was 0.9 ± 0.15 mM ($n = 19$), and above 5 mM the current was reduced to less than 20% of its maximum amplitude.

The Ca²⁺-sensitive current is carried by sodium and potassium ions and blocked by gadolinium

The current evoked by lowering $[Ca^{2+}]_o$ from 4.5 to 0 mM was completely abolished in a Na⁺-free, K⁺-free solution in all neurons ($n = 4$; holding potential, −80 mV). This therefore excludes any permeability to Cl⁻ since this species was present in the Na⁺-free, K⁺-free solution with a reversal potential of about −2 mV (solutions Ext 6 and Int 3 in Table 1; Fig. 6*A*). We tested the relative permeability of the two ions in 13 neurons. The Ca²⁺-sensitive current was present in Na⁺-free solutions, its intensity proportional to the external K⁺ concentration. The amplitude of the current in the presence of 144 and 20 mM $[K^+]_o$ was 49.6 ± 3.2 and 17.7 ± 1.2 pA ($n = 6$),

respectively (solutions Ext 7/8 and Int 3 in Table 1; Fig. 6*B*). Similarly, the amplitude of the currents in the presence of 144 and 20 mM $[Na^+]_o$ as a charge carrier, using K⁺-free solutions, was 114.1 ± 9.2 and 35.6 ± 7 pA ($n = 7$), respectively (solutions Ext 9/10 and Int 3; Fig. 6*C*).

We used blockers with the aim of pharmacologically identifying the Ca²⁺-sensitive conductance. Gadolinium added to the external solution had a powerful blocking effect: at 10 μM it completely blocked the current evoked by lowering $[Ca^{2+}]_o$ from 4.5 to 0 mM, and also reduced the residual current at 4.5 mM Ca²⁺ ($n = 14$; Fig. 7). External Cs⁺ (1–3 mM) or the Na⁺ channel blocker TTX (1 μM), and internal Cs⁺ (110 mM) or TEA (30 mM) did not affect the Ca²⁺-sensitive current in the neurons ($n = 25$).

The Ca²⁺-sensitive current is modulated by GTP-γ-S

We tested whether these channels were controlled by G-proteins, utilising the non-hydrolysable GTP analogue guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S; 500 μM) in

Figure 7. Gadolinium was a powerful blocker of the Ca²⁺-sensitive cation current

Gd³⁺ (10 μM) added to the external solutions completely abolished the current activated by lowering $[Ca^{2+}]_o$ from 4.5 to 0 mM (top bar), recorded at −80 mV (Int 2 and Ext 4 in Table 1). The effect persisted even when the $[Ca^{2+}]_o$ was raised again to 4.5 mM. This suggests that it blocks a residual Ca²⁺-sensitive current present at that Ca²⁺ concentration. C, control before changing to 0 Ca²⁺; R, recovery on return to 4.5 mM Ca²⁺.
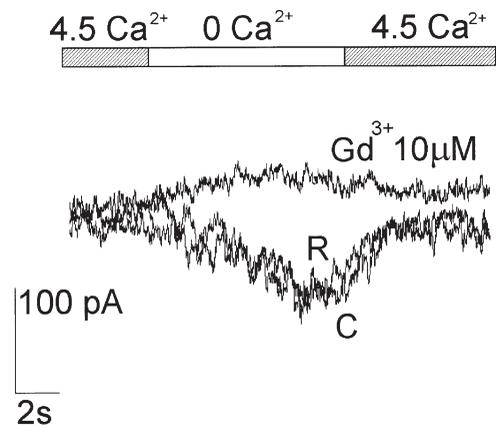
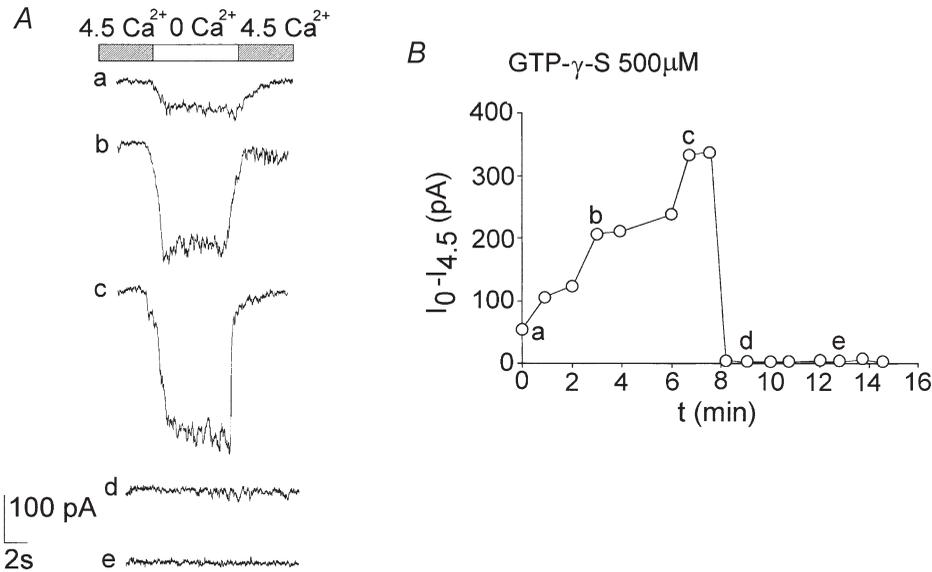**Figure 8. The Ca^{2+}-sensitive current is modulated by GTP-γ-S**

GTP-γ-S (500 μM) added to the intracellular solution in the recording pipette had a biphasic effect. It increased the Ca^{2+}-sensitive current, which reached a peak about 6–7 min after the establishment of the whole-cell configuration. The Ca^{2+}-sensitive current then showed rapid decay until it was almost completely abolished in all the neurons (*B*). The current traces shown in *A* relate to the labelled points in *B*. All the recordings were taken at -80 mV (Int 2 and Ext 4 in Table 1).

the recording pipette. GTP-γ-S caused a marked transient increase in the conductance followed by a complete block in about 8 min of whole-cell current recorded in all the neurons tested ($n = 15$; Fig. 8). This biphasic action suggests that multiple G-protein mechanisms are involved.

Spermine mimics Ca^{2+} in blocking the cationic current

Since the Ca^{2+}-sensitive current found in thalamic neurons was modulated by GTP-γ-S, the non-selective cationic channels may have been G-protein coupled to a Ca^{2+}-sensing receptor. To test this, spermine, a Ca^{2+}-sensing receptor agonist, was added to the extracellular solution (Ext 4, with 0.5 mM Ca^{2+} and 50 μM APV) at a concentration of 500 μM. This drug partially and reversibly inhibited the whole-cell persistent ionic currents evoked at scaled step potentials from -80 to -20 mV. The current–voltage relationship of the inhibited component showed a reversal potential at about -28 mV (Fig. 9) very close to that for the Ca^{2+}-sensitive current component (Fig. 5*B*).
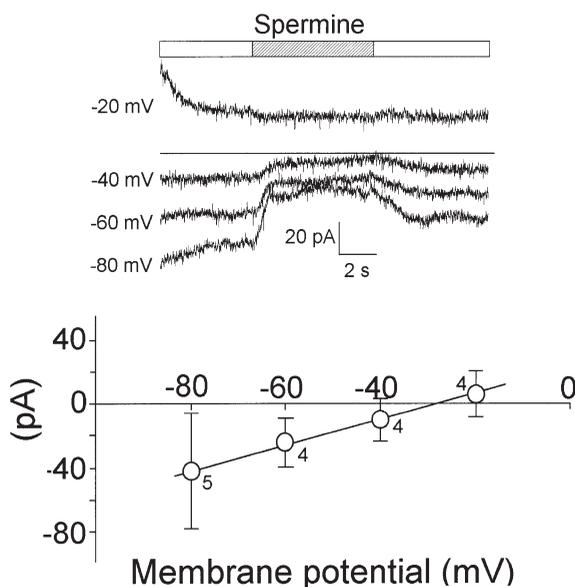
Figure 9. Spermine mimics Ca^{2+} in blocking the cationic current

The effects of spermine (500 μM; top bar) on the total persistent ionic currents (traces below) were tested in whole-cell voltage-clamp experiments. The stimulus protocol was similar to that shown in Fig. 5. The potential at which each current was recorded is indicated (Ext 4 with 0.5 mM Ca^{2+}, Int 2 in Table 1). Spermine perfused extracellularly partially inhibited the persistent ionic currents. The effect was reversible. The graph below shows the spermine-sensitive current plotted against the membrane potential (O, $\pm$ S.E.M.). The current reversed at about -28 mV.

DISCUSSION

A relatively small increase in $[Ca^{2+}]_o$ alone is enough to induce the transition of neuronal firing from single spikes to burst mode in current-clamped isolated thalamic neurons. The potential sensed by the ionic channels is what controls the firing mode of thalamic and other central neurons. We investigated, in whole-cell patch-clamp experiments, the mechanisms by which calcium affects the neuronal discharge.

The rise in $[Ca^{2+}]_o$ hyperpolarises the neuronal membrane, shifting the conductance–voltage relationship of LVA Ca^{2+} channels to the right (see Fig. 2). The block of or changes in the Ca^{2+}-sensitive cation current also seem to be important in controlling the transmembrane potential.

The membrane hyperpolarisation induced by a rise in $[Ca^{2+}]_o$ through the above-mentioned effects, and the increase in the Ca^{2+} driving force at the resting potential, which favours the appearance of low threshold Ca^{2+} spikes, cause the neuronal firing to shift from the relay to the oscillatory mode.

Changes in external Ca²⁺ concentration affect the voltage-dependent properties of ionic channels

As predicted by the surface potential theory (Green & Andersen, 1991; Hille, 1991), there was a shift in the voltage-dependent characteristics of LVA Ca^{2+} channels. On increasing the $[Ca^{2+}]_o$, the inactivation curve of the LVA Ca^{2+} currents shifted to positive values on the voltage axis. This was consistent with de-inactivation of the LVA Ca^{2+} channels, which are necessary to give a low threshold Ca^{2+} spike; in our experimental conditions, it is likely that the LVA Ca^{2+} current was less than *in vivo* since some of these channels are presumably lost with the distal dendrites. However, the effects of Ca^{2+} are not only based on this cellular mechanism. This is particularly evident if we compare the effects of Ca^{2+} and Mg^{2+}. Mg^{2+} shared with Ca^{2+} similar screening effects on the negative fixed charges of the membrane (Fig. 4), but induced a smaller hyperpolarisation at the resting potential in current-clamped neurons (Fig. 3*A*). Mg^{2+}, in place of Ca^{2+}, prevented the generation of low threshold spikes in guinea-pig nucleus reticularis thalami neurons (Bal & McCormick, 1993). Similarly, experiments from our laboratory indicated that Mg^{2+} did not cause the neuronal discharge to shift to the burst mode (authors' unpublished results).

External Ca²⁺ blocks an inward non-selective cation current in isolated thalamic neurons

We constantly observed a directly measurable hyperpolarising effect of the increase in $[Ca^{2+}]_o$. Since this effect persists during the perfusion, and is accompanied by a steady increase in the membrane resistance at all potentials tested, we assume that extracellular Ca^{2+} blocks channels that underlie a steady depolarising current. This block could hardly be attributed to a side effect of the voltage shift, since Mg^{2+} was less effective in inducing hyperpolarisation (Fig. 3*A*) though, as discussed above, its screening properties were comparable to Ca^{2+}. However, in order to completely rule out the effect of the voltage shift, Mg^{2+} was added to the external solutions to a final concentration of 5 mM divalent cations (Ca^{2+} and Mg^{2+} together). The hyperpolarising effect was seen in these experimental conditions too, confirming that it was due to a specific block by extracellular Ca^{2+} of an inward current. The effect of Ca^{2+} was mimicked by Ba^{2+}. The reversal potential of -35 mV implies that more than one ion is involved as a charge carrier.

Various currents, such as the I_h (McCormick & Pape, 1990; Pape, 1996), the persistent Na^+ current ($I_{Na,P}$) (Jahnsen & Llinas, 1984; Crill, 1996), and the Ca^{2+}-activated non-specific cation currents (McCormick & Bal, 1997), were observed in these thalamic cells, and they can all be considered as possible targets for the Ca^{2+} ions.

Although a weak blocking effect of extracellular Ca^{2+} on the inwardly rectifying cation current (I_f, present in heart and similar to I_h) has been reported (Ono *et al.* 1994), we can exclude the possibility that a block of I_h could account for the hyperpolarisation we observed *in vitro*, since in these acutely dissociated thalamic neurons I_h is hardly detectable in the hours that follow cell dissociation (data not shown), in agreement with a previous observation (Budde *et al.* 1994). In addition, 1–3 mM Cs^+ added to the external solution, which is supposed to block I_f (Pape, 1996), did not prevent the blocking effect of an increase in $[Ca^{2+}]_o$, and Ca^{2+} exerts its blocking effect even at potentials of -20 mV, at which I_h should not be activated (Pape, 1996). Nevertheless, *in vivo* there is likely to be a partial block of I_h by extracellular Ca^{2+}, and this should result in further hyperpolarisation of the thalamic neurons in the negative voltage range at which this current is activated.

Non-specific cation currents with a different sensitivity to Ca^{2+} ions have been described in various cells. Calcium-activated non-specific cation (CAN) channels are found in the reticular thalamic neurons and it is proposed that they induce a slow depolarising afterpotential and the generation of a tonic discharge at the end of the oscillatory burst (Bal & McCormick, 1993). The probability of their opening increases as the cytoplasmic Ca^{2+} concentration rises (Partridge *et al.* 1994), whereas, to our knowledge, no effects of the $[Ca^{2+}]_o$ on these channels have been described. Furthermore, Ca^{2+} had a hyperpolarising effect in almost all the cells tested, and in our cell preparations there are reticular neurons, but also relay neurons, whereas I_{CAN} was found only in the former (McCormick & Bal, 1997).

A non-selective cation conductance has been observed in the midbrain dopaminergic neurons of the rat, which is

blocked by extracellular Ca^{2+} (Farkas *et al.* 1996). However, in that study, unlike in ours, Mg^{2+} applied externally had the same blocking effect as Ca^{2+}.

The $I_{Na,P}$ presents some interesting features (Crill, 1996). It is non-inactivating and is affected by $[Ca^{2+}]_o$. High $[Ca^{2+}]_o$ shifted the activation curve to positive potentials, and reduced the current amplitude (Li & Hatton, 1996); however, this cannot account for the effects at very negative potentials, where $I_{Na,P}$ is deactivated. In addition, 1 μM TTX added to the external solution did not prevent the blocking effect induced by increasing $[Ca^{2+}]_o$, and this channel cannot account for the permeability to K^+ (Fig. 6*B*).

Recently, the inhibitory effect of gadolinium on the Na^+–Ca^{2+} exchanger in guinea-pig ventricular myocytes has been described (Zhang & Hancox 2000). The effect observed differs from that described here, since it is necessary to use a 10-fold higher concentration of Gd^{3+} to fully block the Na^+–Ca^{2+} exchanger and the time from Gd^{3+} application to steady-state effect was very slow in their experiments.

The current described here has some characteristics similar to those observed in *Xenopus* oocytes (Arellano *et al.* 1995), in mouse hyppocampal neurons (Xiong *et al.* 1997), and in rat cardiac muscle (Mubagwa *et al.* 1997). All these currents are activated by lowering the $[Ca^{2+}]_o$, are permeable to Na^+ and K^+, and are blocked by Gd^{3+} at micromolar concentrations. However, the current described by Xiong *et al.* (1997) in hippocampal neurons was not affected by non-hydrolysable GTP analogues, the current observed by Arellano *et al.* (1995) had very slow activation kinetics and limited permeability to calcium ions, and that observed by Mubagwa *et al.* (1997) was also blocked by Mg^{2+} and Ba^{2+}. The thalamic current was half-maximal at 0.9 mM $[Ca^{2+}]_o$. This concentration is higher than other Ca^{2+}-sensitive currents and closer to the physiological Ca^{2+} concentration range in the intraventricular fluid.

Ca^{2+}-sensitive cation conductance is G-protein linked to a Ca^{2+}-sensing receptor

The extracellular, G-protein-linked Ca^{2+}-sensing receptor, first identified in the parathyroid gland (Brown *et al.* 1993), has been found in various regions of the brain (Ruat *et al.* 1995; Rogers *et al.* 1997). In some cases this receptor is functionally coupled to ionic channels (Ye *et al.* 1996*a,b*; McGehee *et al.* 1997; Chattopadhyay *et al.* 1999; Washburn *et al.* 2000). Since the Ca^{2+}-sensitive current described here was modulated by GTP-γ-S, the possibility that non-selective cationic channels are G-protein-coupled to a Ca^{2+}-sensing receptor, as observed in pancreatic β cells (Straub *et al.* 2000) and monocytes (Yamaguchi *et al.* 2000), was taken into account. Spermine, an endogenous polyamine that agonises the Ca^{2+}-sensing receptor (Quinn *et al.* 1997), mimicked the effects of external Ca^{2+} when added to the extracellular solution.

This confirms that the Ca^{2+}-sensing receptor is G-protein coupled to a non-selective cationic channel at the postsynaptic level in thalamic neurons too.

Physiopathological considerations

Calcium is exchanged between blood and cerebrospinal fluid (CSF) through the blood–brain barrier and the Ca^{2+} influx to the CSF and brain seems to be linearly related to the plasma ionised calcium concentration (Tai *et al.* 1986). Effects on EEG rhythms are seen in many pathological states primarily involving calcium metabolism, such as hyperparathyroidism and vitamin D poisoning (Evaldsson *et al.* 1969). They may also occur in other conditions in which the $[Ca^{2+}]_o$ is secondarily influenced, such as malignancies (Moure & Houston, 1967; Allen *et al.* 1970), renal failure (Guisado *et al.* 1975), and some disorders of the endocrine system (Evaldsson *et al.* 1969; Allen *et al.* 1970). As mentioned in the Introduction, during persistent hypercalcaemia a state ranging from drowsiness to lethargy can be present, and there is low frequency EEG activity (Evaldsson *et al.* 1969; Allen *et al.* 1970; Guisado *et al.* 1975; Joborn *et al.* 1991).

In vivo experiments from our laboratory showed that intraventricular $CaCl_2$ injections in freely moving adult rats tripled the EEG amplitude and increased the relative power of the low EEG frequencies, with a decrease of the fast activity (Formenti *et al.* 1996). It is reported in the literature that intracerebral injections of $CaCl_2$ induced EEG slow wave electrical activity, synchronisation and a sleep-like state in cats, rabbits and other animals (Cloetta & Fischer, 1930; Stern & Chvoles, 1933).

There is a strong correlation between the thalamic mode of discharge and the EEG rhythms. Some mechanisms have been proposed to account for this correlation (Jahnsen & Llinas, 1984; Steriade & Llinas, 1988; Von Krosigk *et al.* 1993; McCormick & Bal, 1997); however, it is likely that other parts of the nervous system are involved in the effects of Ca^{2+}. *In vivo* $[Ca^{2+}]_o$ may also affect other neurons, for instance neocortical cells exhibit LVA Ca^{2+} currents and can produce a burst discharge.

Although highly synchronous EEG and epileptic seizures may appear at the highest calcium concentrations both *in vivo* and *in vitro* (Bragdon *et al.* 1992), seizures arising from different structures in the brain are mainly observed at low $[Ca^{2+}]_o$ (Heinemann *et al.* 1986; Dichter & Ayala, 1987; Jefferys, 1995) and tend to disappear when the $[Ca^{2+}]_o$ returns to normal. Here again, the electrical potential sensed by the channels presumably controls the mode of discharge. This is corroborated by the observation that seizures are also induced by an increase in the external K^+ concentration or other agents that can also induce depolarisation (for review see Dichter & Ayala, 1987). Many anti-epileptic drugs, on the other hand, have inhibitory and hyperpolarising effects, and can induce drowsiness (Macdonald & Kelly, 1995). Thus, whereas the

hyperpolarising effects lead to EEG synchronisation induced by a neuronal burst discharge dependent on the LVA Ca^{2+} channel, the depolarising effects of low $[Ca^{2+}]_o$, or an increase in external K^+, lead to a lower frequency pathological burst discharge and epileptic seizures. This latter activity may, at least in some cases, involve enhanced Na^+ influx through $I_{Na,P}$ rather than LVA Ca^{2+} currents (Li & Hatton, 1996). In addition, the reduction in $[Ca^{2+}]_o$ that may occur during intense neuronal activity can act as a positive feedback, activating the Ca^{2+}-sensitive depolarising current, leading to further depolarisation and hyperexcitability.

$[Ca^{2+}]_o$ influences many behavioural aspects, depending on the brain structures involved. By increasing $[Ca^{2+}]_o$ in the caudal portion of the hypothalamus, Veale & Myers (1971) induced sleep and a catatonic-like state, while changing $[Ca^{2+}]_o$ within the posterior hypothalamus caused changes in body temperature (Myers & Veale, 1970), and changes in body temperature were accompanied by changes in the ventricular Ca^{2+} concentration (Myers & Tytell, 1972). Cytokines are involved in the immune response (Krueger *et al.* 1995) and drowsiness is correlated with their presence in some organic diseases. It is worth recalling that cytokines stimulate bone resorption (Gowen *et al.* 1983; Greenfield *et al.* 1995). $[Ca^{2+}]_o$ within the ventro-medial or lateral areas of the hypothalamus modifies the set-point for the hunger drive (Myers & Veale, 1971). These observations have led to the interesting suggestion that the mechanism of the set-point for various functions, such as body temperature, feeding behaviour and steady-state level of arousal, may be controlled by the inherent ratio of Na^+ to Ca^{2+} ions in specific brain regions (Veale & Myers, 1971; Myers & Tytell, 1972).

Our data reinforce the idea that functions dependent on $[Ca^{2+}]_o$ are not confined to communication through the Ca^{2+} receptor in the parathyroid, kidney and other peripheral cells. $[Ca^{2+}]_o$ is also an important extracellular factor in the central nervous system.

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