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Modulation of the α1A Ca^{2+} channel by β subunits at physiological Ca^{2+} concentration

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Abstract The class A Ca^{2+} channel αl subunit (αlA) was expressed in *Xenopus* oocytes alone or in combination with the βlb, β2a, β3, or β4 subunit. Analysis of voltage-dependent activation and inactivation in the presence of 1.8 mM external Ca^{2+} showed an hyperpolarising shift of both relations when compared to similar recordings performed in the presence of 40 mM Ba^{2+}. These shifts, which differed for activation and inactivation, were strongly modulated by the nature of the co-expressed β subunit. On the other hand, for each combination, the kinetics of inactivation were similar in 1.8 mM Ca^{2+} and 40 mM Ba^{2+} (for example co-expression of the β2a subunit reduced inactivation using either 40 mM Ba^{2+} or 1.8 mM Ca^{2+}). Thus, modulation of channel properties by the β subunit is different in physiological Ca^{2+} or high Ba^{2+} concentrations. These results must be taken into consideration to extrapolate the role of the β subunit in native cells.

Key words: P- and Q-type Ca^{2+} channels; αlA subunit; ωConotoxin MVII C; Rat brain

1. Introduction

Taking advantage of the isolation of a new spider-toxin from *Agelenopsis aperta* (ωAga-IV A), two new types of neuronal Ca^{2+} currents have been recently identified. The P-type Ca^{2+} current displays a high-activation threshold (> -40 mV), almost no inactivation, nanomolar sensitivity to ωAga-IV A, and micromolar sensitivity to the cone snail peptide ωCmTx-MVII C. The Q-type Ca^{2+} current, exhibiting the same voltage range for activation, is poorly inhibited by ωAga-IV A but is more sensitive to ωCmTx-MVII C ([1–5], for review [6]). The physiological roles of these P- and Q-type Ca^{2+} channels are not yet fully understood, but it has been suggested that both types play a crucial role in the Ca^{2+}-dependent neurotransmitter release in central synapses and at the mammalian neuromuscular junction [7–9].

Neuronal Ca^{2+} channels are composed of a central pore-forming α1 subunit associated with at least 2 different auxiliary subunits α2-δ and β [10–12]. The αl subunit specifies the gating kinetics and the pharmacological profile of the channel [4,13]. Each of the β subunits (four genes identified to date) modulates specifically the kinetics and the voltage-dependent gating of the αl subunit [3,14–16]. The α2-δ subunit (only one gene identified so far, [11]) seems to have only minor regulatory role on αl current properties.

The molecular components of the P- and Q-type Ca^{2+} channels have not been completely identified. However, in situ hybridisation and immuno-localisation experiments have shown that the class A α1 subunit is expressed in Purkinje cells [17,18] and at the neuromuscular junction [19], where P-type Ca^{2+} currents are known to be predominant [2,6]. This α1A subunit, when expressed in *Xenopus* oocytes, encodes a ωCmTx-MVII C-sensitive Ca^{2+} channel, closely related to the Q-type Ca^{2+} channel described in neurones [3]. Therefore, it has been suggested that both channels might be encoded by the same α1 subunit class A gene: the functional differences being related to either alternative splicing and/or the nature of auxiliary subunits [4,9,10]. The identification of the molecular components of the functional P- and Q-type Ca^{2+} channels might thus be assessed by comparing the biophysical and pharmacological properties of P- and Q-type Ca^{2+} channels recorded in vivo with the α1A channel currents expressed with different combinations of auxiliary subunits, using similar experimental conditions.

However, the biophysical characterisation of αlA-directed current and the modulation by auxiliary β subunits have only been performed, in previous studies, by using Ba^{2+} ions as the charge carrier [3,4,16,18]. Such a modulation may not be simply extrapolated to the physiological situation since the nature and the concentration of the permeating ion are known to regulate voltage-dependent properties of Ca^{2+} channels when recorded in native cells. As a first step towards the understanding of the potential regulatory role in vivo of the β subunit, we have analysed Ca^{2+} channel currents in *Xenopus* oocytes injected with various combinations of α1A and β subunits, in normal saline solution containing 1.8 mM Ca^{2+}.

2. Materials and methods

2.1. Plasmid amplification and preparation

The α1A, α2-δ, β1b [20], β2a [21], β3 [22] and β4 [23] subunit cDNAs were inserted into the pmt2 expression vector under the control of a SV40 promoter [15]. Large-scale plasmid preparations were obtained using standard protocols (Qiagen Inc., Chadworth, CA). Each sample was dissolved in sterile water at a final concentration of 1 ng/nl. Mixtures of cDNAs (α1, α1+β) were made by mixing the α1 cDNA with an equal volume of the appropriate subunit or sterile water. Final α1 cDNA concentration was 0.5 ng/nl.

2.2. Xenopus oocytes isolation and injection

Ovaries were surgically removed from *Xenopus laevis* female (Elevage de Lavalette, Montpellier, France) anaesthetised using a 0.2% MS222 solution (Sigma, Laverpilliere, France). After a first mechanical dissociation and extensive washing using the OR-2 solution, (containing in mM: NaCl, 100; $MgCl_2$, 2; KCl, 2; HEPES, 10), oocytes were isolated by a 2 h enzymatic dissociation using 1 mg/ml collagenase IA (Sigma) dissolved in OR-2. Oocytes were then washed several times with OR-2 and selected in the survival medium (containing in mM: NaCl, 96; $MgCl_2$, 2; KCl, 2; $CaCl_2$, 1.8; HEPES, 10; pyruvic acid, 2.5; gentamycin, 50 μg/ml; neutralised at pH 7.2 using NaOH). Nuclear injection of oocytes was performed by employing a home-made pneumatic injector. Oocytes were then incubated for 2–7 days at

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19°C under gentle agitation before recording. The survival medium was renewed daily.

2.3. Electrophysiological recordings

Whole cell Ca^{2+} currents were recorded under two electrode voltage-clamp by employing the GeneClamp 500 amplifier (Axon Inst., Burlingame, CA). Current and voltage electrodes were filled with a solution containing: CsCl, 2.8 M; BAPTA, 10 mM; pH = 7.2 with CsOH. The bath-clamp headstage was connected to the bath using two agar bridges filled with 2% agar in 3 M KCl. Injection of BAPTA (in mM: BAPTA-free acid (Sigma), 100; CsOH, 10; HEPES, 10; pH 7.2) was performed using a third microelectrode. Under these conditions uncontaminated Ca^{2+} currents can be recorded. Ca^{2+} currents were elicited by series of depolarising steps of 400 ms duration from a holding potential of −100 to −40 mV, and up to +60 mV in 10 mV increment. Voltage-dependent inactivation was assessed by applying preconditioning voltage steps of 2.5 s duration to various test potentials. The standard extracellular recording solutions had the following composition (in mM) **ND96**: NaCl, 96; $MgCl_2$, 2; KCl, 2; $CaCl_2$, 1.8; HEPES, 10; pH 7.2 (using NaOH) and **BA40**: BaOH, 40; TEAOH, 20; NMDG, 30; CsOH, 2; HEPES, 10; pH 7.2 (using methane sulfonate). Current recordings were filtered and digitised using a DMA-Tecmar labmaster, and subsequently stored on an IPC 486 personal computer by using the version 6.02 of the pClamp software (Axon Inst.). Peak current, time-to-peak and inactivation time-constants were measured using Clampfit (pClamp ver. 6.02, Axon Inst.). Normalised current-to-voltage relations were fitted according to a modified Boltzmann distribution using the equation:

$$i/i_{max} = g*(V-E_{rev})/(1+\exp((V-E_{1/2a})/k_a)),$$

where g is a normalised conductance, V is the voltage step, E_{rev} is the current reversal potential, $E_{1/2a}$ is the potential for half-activation, k_a is the slope factor and i_{max} is the current at the peak of the current-to-voltage curve. Inactivation curves were fitted using the equation:

$$i/I_{max} = R+(1-R)/(1+\exp((V-E_{1/2i})/k_i)),$$

where V is the preconditioning voltage, R is the fraction of non-inactivating current, $E_{1/2i}$ is the potential of half-inactivation, k_i is a slope factor and I_{max} is the current recorded after a predepolarising pulse at −80 mV. All values are presented as mean ± standard deviation. ωCmTx-MVII C (RBI, Bioblock Scientific, Illkirch France) was dissolved in distilled water to make a stock solution at 0.1 mM and subsequently stored at −80°C. The final dilution in ND96 (1 μM) was made just before the experiment. The toxin was added at the desired concentration to the bath and the effect was measured as the percentage of inhibition of the Ca^{2+} current recorded during a depolarising pulse from −100 to +10 mV, when the steady-state effect was reached (after ≈2–5 min).

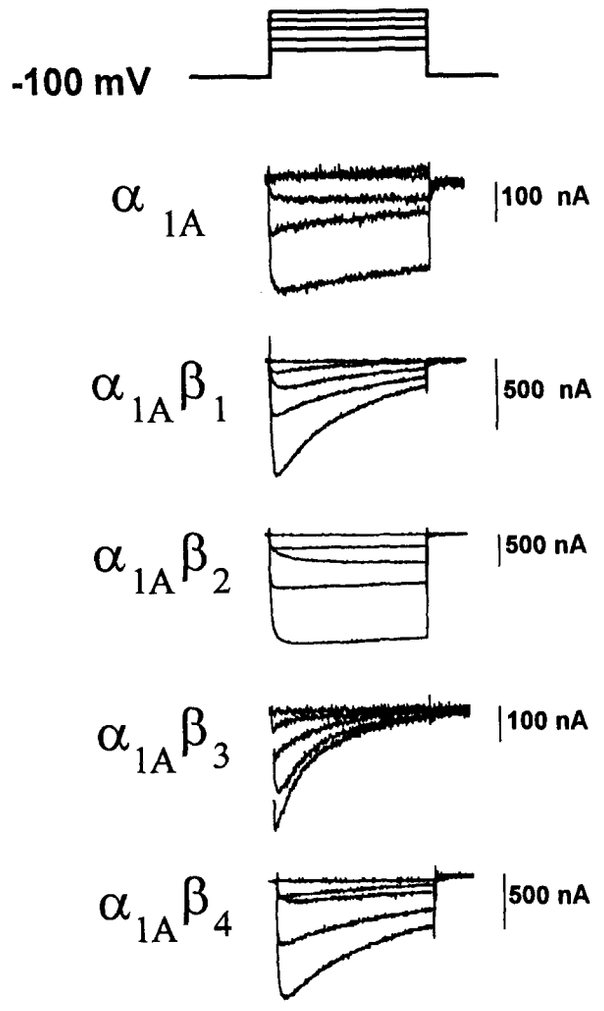

Fig. 1. Typical current traces recorded from oocytes expressing the α1A, the α1A+β1b, the α1A+β2a, the αlA+β3 or the α1A+β4 subunits. Currents were evoked by 400 ms long step depolarisations to −40, −20, 0, 20 or 40 mV from a holding potential of −100 mV. The recording solution contained 1.8 mM extracellular Ca^{2+}.

3. Results

The intracellular injection of BAPTA in *Xenopus* oocytes [24] and the use of the highly efficient pmt2 expression vector, enable recordings of the α1A Ca^{2+} channel activity at a low Ca^{2+} concentration (1.8 mM). In these conditions, the contamination by the endogenous Ca^{2+} channel [25] is negligible, as shown for low concentrations of Ba^{2+} [26].

Fig. 1 shows currents recorded from oocytes expressing different combinations of Ca^{2+} channel subunits: the α1A subunit alone or co-expressed with either the β1b, β2a, β3, or β4 subunit. Voltage pulses of 400 ms duration were applied from a holding potential of −100 mV to −40, −20, 0, 20 and 40 mV every 15 s. The level of expression, as estimated by the amplitude of the Ca^{2+} current, was very variable, but currents in the range of −200 to −1000 nA were routinely recorded from all combinations in the presence of 1.8 mM extracellular Ca^{2+}. As expected, the mean current amplitude was significantly increased upon co-expression of a β subunit (from −235 ± 75 nA for α1A alone, to up to −1220 ± 547 nA for α1A+β2a). Currents recorded from oocytes expressing the α1A subunit alone peaked and slowly decayed during the depolarising pulse. In contrast, co-expression of the β1b, β3, or β4 subunit induced a significant acceleration in the activation and inactivation kinetics. Co-expression of the β2a subunit had opposite effects, and Ca^{2+} currents recorded from oocytes expressing the α1A+β2a subunits had typical slow onset of activation and inactivation decay (see below).

This α1A Ca^{2+} current was high-voltage activated, and no inward current was observed with depolarising pulses below −30 mV. As seen in Fig. 2A, the threshold for activation was not changed by the co-expression of a β subunit in 1.8 mM Ca^{2+}. However, co-expression of the β1b, β3 or β4 subunit induced a leftward shift in the peak of the current-to-voltage curve (peak at +10 mV for α1A alone and 0 mV for α1A+β1b, α1A+β3 or αlA+β4), and currents through the α1A+β2a subunits peaked at even more hyperpolarised potential (−10 mV, see Table 1 for half-activation potentials). When comparing to similar recordings performed in 40 mM Ba^{2+}, the potential for half-activation was shifted in the negative direction for all subunit combinations. The magnitude of this shift, however, was more pronounced for currents re-

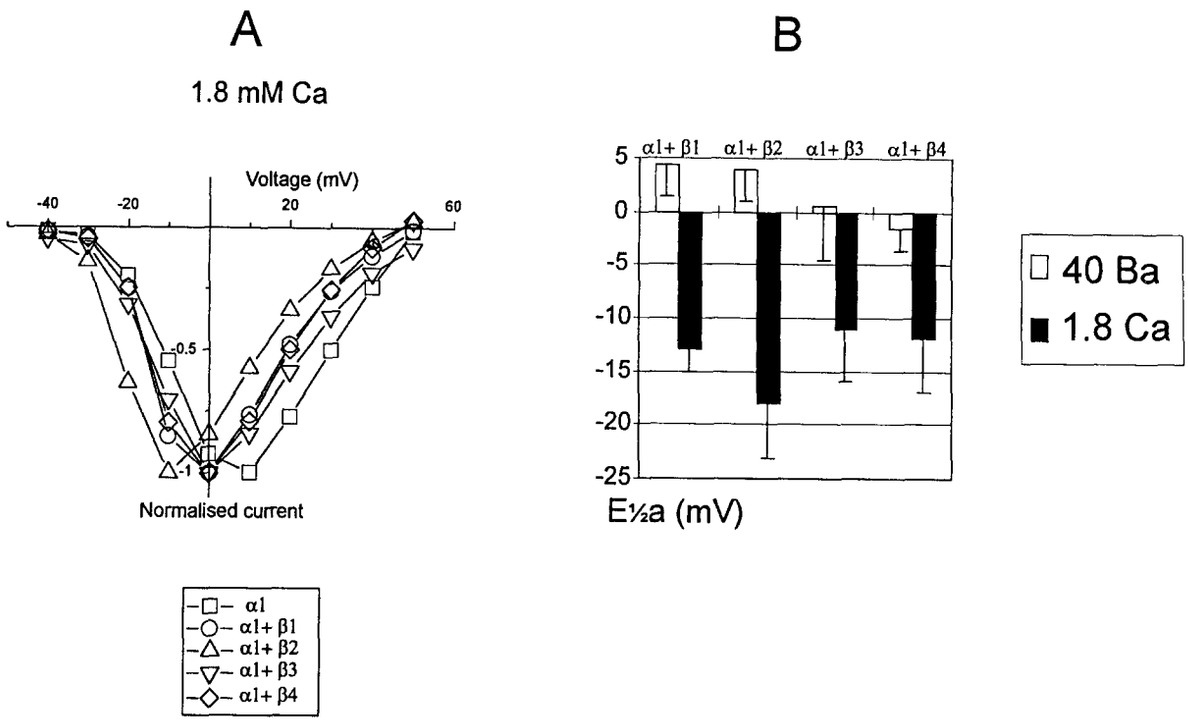

Fig. 2. A: Effect induced by the co-expression of a β subunit on the current–voltage relationship recorded at 1.8 mM Ca^{2+}. Currents were recorded from oocytes injected with the α1A, the α1A+β1b, the α1A+β2a, the α1A+β3 or the α1A+β4 subunit cDNAs at different depolarising potentials (from −40 to +50 mV, 10 mV increment from a holding potential of −100 mV). The peak current was then normalised with respect to the maximum current and plotted against the depolarising potential. Note the differential shift in the current-to-voltage relationship induced by co-expression of the β subunit. B: Comparison of the potential for half-activation in 1.8 mM Ca^{2+} and 40 mM Ba^{2+}. Currents were recorded as described in A in ND96 or Ba40.

corded from oocytes expressing the α1A+β2a subunits, which becomes the combination with the lowest activation range (see Fig. 2B). On the other hand, the modification of the slope of the activation-curve induced by the co-expression of a β subunit in 40 mM Ba^{2+} [16] was also observed in 1.8 mM Ca^{2+} (a change in the slope from $k_a = 7$ mV/e-fold with the αlA subunit to $k_a = 4$ to 5 mV/e-fold when β is present, see Table 1).

By contrast to previous reports using Ba^{2+} as the charge carrier, in 1.8 mM Ca^{2+}, a β subunit-induced hyperpolarising shift of the α1A Ca^{2+} currents inactivation-curve was recorded only when expressed with the β1b, β3, or β4 subunit (24, 22 and 21 mV, respectively, see Fig. 3A and Table 2). In these conditions, co-expression of the β2a subunit had no effect on the voltage-dependence of inactivation (see Fig. 3A). Therefore, and as noted for activation, the rank of order of the β subunit with respect to the potential for half-inactivation was not the same in high Ba^{2+} (β4 = β3 < β1b = β2a, Fig. 3B and [16]) or in 1.8 mM Ca^{2+} (β1b = β3 = β4 < β2a, Fig. 3B, Table 2). This 'late' voltage-dependent inactivation of the α1A channel containing the β2a subunit, together with the reduction in the extent of inactivation (see Fig. 3A) should allow persistant Ca^{2+} entry at depolarised potentials. While injection of α1A, α1A+β1b, α1A+β3 and α1A+β4 subunits produced Ca^{2+} channels that completely inactivated (> 90%) when the holding potential was depolarised (> −10 mV), a 2.5 s long pulse at +20 mV failed to inactivate the Ca^{2+} current by more than 50% on oocytes expressing α1A+β2a subunits (see Table 2 and Fig. 3A). Therefore, at low Ca^{2+} concentrations, while the β2a subunit was the most potent β subunit in shifting the activation curve of the class A α1 subunit, it was the less efficient in displacing the inactivation curve, although it produced important changes in the extent and the kinetics of inactivation.

Table 1
Current-to-voltage curve parameters

Combination	$E_{1/2a}$(mV)	k_a(mV)
α1 (7)	− 4.5 ± 4.3	−7.3 ± 1.7
α1+β1b (20)	−13.2 ± 3.1	−4.5 ± 0.5
α1+β2a (16)	−17.9 ± 3.4	−4.1 ± 0.5
αl+β3 (6)	−11.2 ± 4.8	−5.6 ± 0.8
αl+β4 (9)	−12.0 ± 2.5	−4.9 ± 0.6

See Section 2 for detail; numbers between parenthesis indicate the number of oocytes tested.

As shown in Fig. 4A, the inactivation decay of the class A does not seem to be strongly voltage-dependent (308 ± 75 ms at −10 mV and 241 ± 55 ms at +10 mV for the α1A subunit expressed alone). Although this poor voltage-dependency was also found upon co-expression of the β1b, β3 and β4 subunits, we noted important modifications in the time constant of inactivation. An acceleration was observed when the β1b, β3 and β4 subunits were co-expressed, while co-expression of the β2a subunit induced a marked slowing of the current decay (see Fig. 4A), which appeared to depend on the amplitude of the voltage step (see Figs. 1 and 4A). Similarly, the time-to-peak for activation was also slightly voltage-dependent and was decreased by co-expression of the β1b, β3, and β4 subunits or increased by the β2a subunit (Fig. 4B).

We finally studied the influence of the β subunit on the inhibition of the α1A Ca^{2+} current by the cone snail toxin

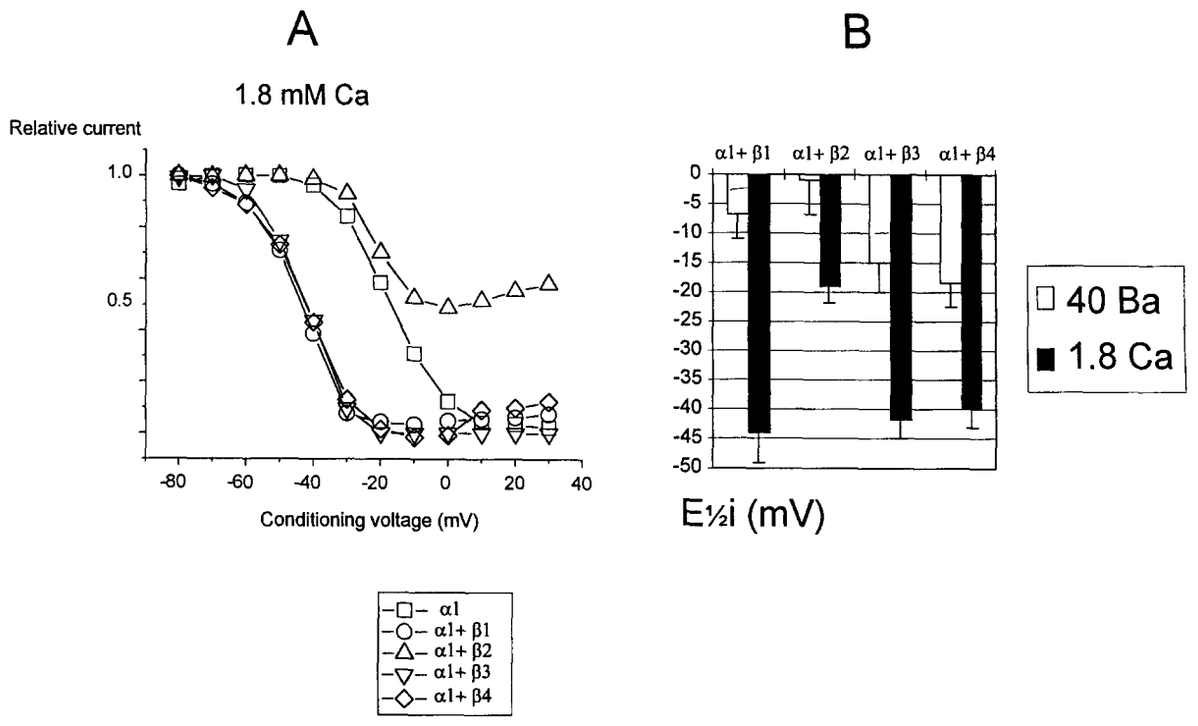

Fig. 3. A: Steady-state inactivation curves recorded from oocytes injected with the α1A, the α1A+β1b, the α1A+β2a, the αlA+β3 or the α1A+β4 subunit cDNAs. B: Comparison of the potential for half-inactivation in 1.8 mM Ca^{2+} and 40 mM Ba^{2+}. Currents were recorded as described in A in ND96 or Ba40.

ωCmTx-MVII C. When 1 μM of this toxin was applied (in 1.8 mM extracellular Ca^{2+}) on oocytes expressing the α1A subunit alone, an almost complete and reversible inhibition of the current was recorded. This inhibition was not significantly different ($P < 0.05$) when either the β1b (77 ± 10%, $n = 4$), the β2a (80 ± 10, $n = 2$), the β3 (79 ± 10%, $n = 7$) or the β4 (85 ± 5%, $n = 2$) subunit was co-expressed with the α1A subunit.

The α2-δ subunit has also been shown to be present in the α1A, αlB and α1C Ca^{2+} channel complexes, and suggested to modulate Ca^{2+} channel activity. We therefore checked for the possible influence of the α2-δ subunit in the presence of 1.8 mM Ca^{2+} by comparing the electrophysiological properties of Ca^{2+} channels expressed in oocytes injected with either the α1A+β1b subunits or the α1A+β1b+α2-δ subunits. As seen in Table 3, the α2-δ subunit had no influence on the kinetics and voltage-dependency of activation and inactivation of the expressed currents. Moreover, no effect was observed on the sensitivity to the toxin ωCmTx-MVII C (Table 3). The expression of the α2-δ subunit induced however, when expressed with the α1A and β1b subunits, the usual increase in the expressed Ca^{2+} current, as already noted for the class C, B and A Ca^{2+} channels using 40 mM Ba^{2+} as the charge carrier (not shown, but see [3,14–16,18]).

4. Discussion

These results constitute the first characterisation of the class A Ca^{2+} channel recorded at physiological concentration of Ca^{2+}. Up to now, most of the studies concerning the class A channel has been conducted in *Xenopus* oocytes, using high divalent cation concentrations (40 mM or 10 Ba^{2+} [3,16,18]). Recently, two groups reported the characterisation of the class A Ca^{2+} channel at 10 and 1 mM Ca^{2+} concentrations after stable expression in Hamster Kidney cells or *mdg* cells [27,28], but the well-known modulation of the α1A channel properties by the auxiliary β subunit has never been studied in such conditions.

The comparison of the β subunit-induced changes in the α1A-subunit properties using either 1.8 mM extracellular Ca^{2+} (this study) or 40 mM Ba^{2+} [3,16,18] evidences similarities but also marked differences, that may be physiologically important. We show here that the modulation of the activation and inactivation kinetics upon co-expression of the β1b, β3, β4 or even the β2a subunits, are similar either in 1.8 mM Ca^{2+} (this work), 2 mM Ba^{2+} [3] or 40 mM Ba^{2+} concentrations [16,18], suggesting that this modulation of the class A Ca^{2+} channel kinetics is affected neither by the nature of the permeating ions nor by its concentration. Similar conclusions can be drawn on the role of the α2-δ subunit (this work in 1.8 mM Ca^{2+}, and [16–18]). The regulation of the voltage-dependent gating by β subunits is, however, clearly influenced by the permeating ion.

Table 2
Inactivation curve parameters

Combination	$E_{1/2i}$(mV)	k_i(mV)	R
α1 (5)	−19.6 ± 2.2	−8.0 ± 0.8	0.08 ± 0.08
α1+β1b (l6)	−43.8 ± 3.9	−7.2 ± 1	0.05 ± 0.09
α1+β2a (3)	−19.2 ± 6.2	−4.7 ± 0.4	0.53 ± 0.04
αl+β3 (6)	−41.8 ± 4.8	−7 ± 1.0	0.11 ± 0.13
αl+β4 (3)	−40.6 ± 3.9	−7.9 ± 0.9	0.02 ± 0.02

See Section 2 for detail; numbers between parenthesis indicate the number of oocytes tested.

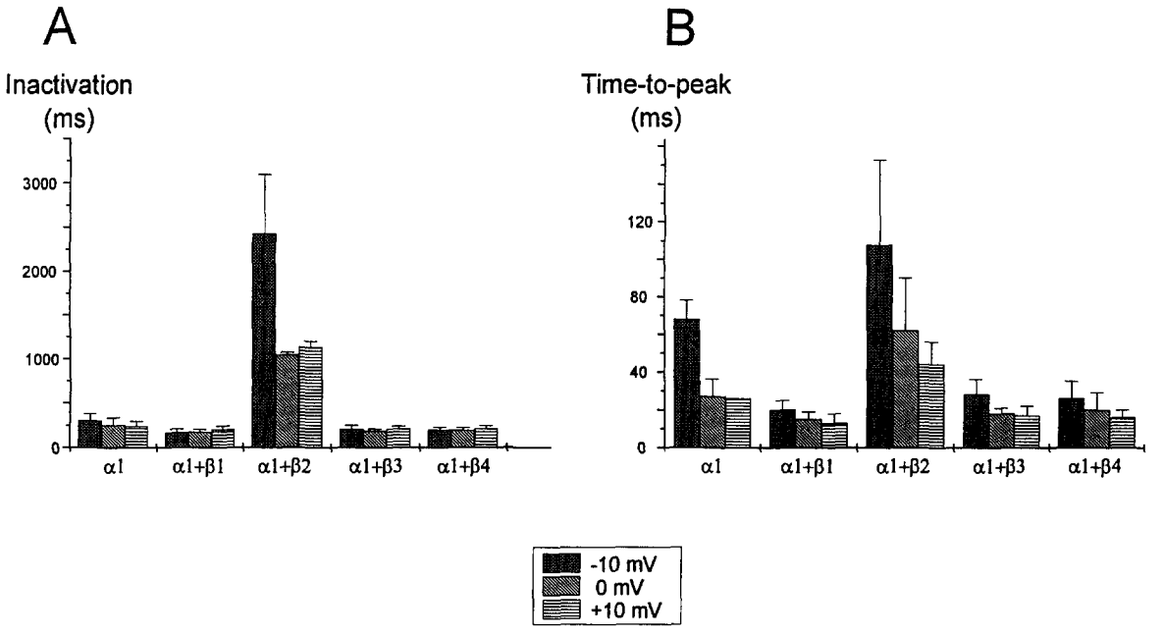

Fig. 4. Inactivation time constant (A) and current time-to-peak (B) recorded from oocytes injected with the indicated combination of subunits at 3 different voltages (−10, 0 and 10 mV).

Using 40 mM extracellular Ba^{2+}, the different β subunits have been classified according to their ability to shift the activation curve [16,18]. In these conditions, expression of the β4 subunit produced α1A Ca^{2+} channels with the lowest activation range (see Fig. 2B and [16,18]). At a physiological Ca^{2+} concentration, this subunit induced one of the smallest hyperpolarising shift of the current-to-voltage curve, and the β2a subunit-containing channel became the channel with the lowest activation potential (see Fig. 2B). Similar differences have been noted in the voltage-dependence of the inactivation. In a first study, using a 40 mM $BaCl_2$-containing Na-free solution, Stea and coworkers [18] reported that co-expression of a β subunit induced either a leftward (with the β1b, the β3 or the β4 subunit) or a rightward (with the β2a subunit) shift of the inactivation curve of the α1A Ca^{2+} channel. In another recent study ([16], using 40 mM Ba^{2+}-methane sulfonate), all four β subunits were found to induce an hyperpolarising shift of the inactivation curve when compare to α1A expressed alone. Our work, in 1.8 mM Ca^{2+}, shows that, while the β1b, β3 and β4 subunits induced a 20–25 mV hyperpolarising shift in the inactivation curve, the β2a subunit had no effect on the voltage dependence of inactivation (Fig. 2A). We believe that the discrepancy between these values (obtained under different ionic conditions) can be explained by modifications in the sensitivity of α1A Ca^{2+} channel inactivation to the external surface potential. These results would therefore suggest that (1) the activation and inactivation parameters are differentially affected by the external surface potential and (2) co-expression of the β1b, β2a, β3 or β4 subunit can modify the sensitivity of the activation and inactivation processes to external surface potential.

From a physiological point of view, one important consequence is the fact that at an holding potential of −40 mV, more than 50% of the channels containing the β1b, β3 or β4 will be inactivated. At this potential, however, 100% of the β2a-containing channels are still available for opening. These differences, together with the changes in the kinetics of inactivation, suggest that the β2a-containing channels are more resistant to high bursting activity where cumulative inactivation can occur.

All combinations of subunits tested in the presence of 1.8 mM extracellular Ca^{2+} showed a high sensitivity to the snail-toxin ωCmTx-MVII C, since a block of about 80% was recorded in 2–5 min upon perfusion of 1 μM of the toxin. This block is characteristic of the α1A Ca^{2+} channel, irrespective to the β subunit expressed, and might therefore help, together with the insensitivity to dihydropyridine and ωCgTx-GVI A, to the identification of functional α1A Ca^{2+} channels expressed in vivo (P- or Q-type Ca^{2+} channels, [4,5,18]). The use of *Xenopus* oocytes to record Ca^{2+} channels activity under

Table 3
Functional effects of the expression of the α2-δ subunit

Combination	Activation		Inactivation		Tau inac. at 0 mV	Time-to-peak at 0 mV	% Block ω-MVIIC
	$E_{1/2a}$ (mV)	k_a (mV)	$E_{1/2i}$ (mV)	k_i (mV)	(ms)	(ms)	
α1+β1b	−13.2 ± 3.1 (20)	−4.5 ± 0.5 (20)	−43.8 ± 3.9 (16)	7.2 ± 1 (16)	174 ± 31 (17)	14.7 ± 3.2 (20)	77 ± 10 (4)
α1+α2-δ+β1b	−10.9 ± 3.1 (6)	−4.6 ± 0.3 (6)	−40.4 ± 5.8 (7)	7.3 ± 0.5 (7)	175 ± 28 (6)	17.2 ± 4.4 (6)	88 ± 11 (4)

Values for the α1A+β1b combination are taken from Tables 1 and 2. Numbers between parenthesis indicate the number of oocytes tested.

conditions similar to those employed for isolated cells (or tissue slices) may help in the future to identify the subunit composition of native channels.

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