Eur Biophys J (1994) 23:189–195

European Biophysics Journal

Temperature dependence of multiple high voltage activated Ca^{2+} channels in chick sensory neurones

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Received: 15 November 1993 / Accepted in revised form: 20 April 1994

Abstract. The temperature dependence of high voltage activated Ca^{2+} channels has been investigated in cultured dorsal root ganglion neurones from chick embryos, using the cell-attached patch-clamp technique. The dihydropyridine sensitive L-type Ca^{2+} channel had a conductance of 23 pS, with 110 mM Ba^{2+} as charge carrier and in the presence of 3 μM Bay K 8644. When the temperature was raised from 15 to 30 °C, the unitary channel current amplitude increased, with Q_{10} value equal to 1.4. The rising phase of the averaged single-channel current became faster, with Q_{10} value 2.7, whereas the decay phase showed a lower temperature sensitivity. Channel open probability decreased according to an exponential distribution of open and closed times. A second type of Ca^{2+} channel was identified, which was DHP-insensitive and had a lower conductance with a mean value equal to 13 pS. For the current amplitude, the Q_{10} value was 1.3. Both activation and inactivation kinetics were strongly accelerated by an increase in temperature. The corresponding time constants gave Q_{10} values equal to 5.9 for activation, and 2.0 for inactivation. Peak channel open probability was highly sensitive to a change in temperature, with a Q_{10} value of 1.6. Finally, in ω-conotoxin GVIA pre-treated neurones, a non-inactivating DHP-insensitive Ca^{2+} channel with the lowest unitary conductance (10 pS) and a much lower temperature dependence was recorded. Single-channel current was increased by heating, with Q_{10} value 1.3, whereas the channel kinetics were almost unaffected by temperature. Our data are consistent with the assumption that the different temperature dependence of the Ca^{2+} channel behaviours may be explained by separate gating processes of three types of Ca^{2+} channels.

Key words: Neurones – Ca^{2+} channels – Temperature – Patch-clamp

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1. Introduction

Temperature has been shown to induce remarkable alterations in Ca^{2+} channel behaviour (Brown et al. 1983; Byerly et al. 1984; Cavalié et al. 1985; Kostyuk et al. 1981; Lux and Brown 1984; Narahashi et al. 1987; Nobile et al. 1990; Taylor 1988). Although it is well-known that heating causes a sharp increase in ion permeation through Ca^{2+} channels and fast changes in activation-inactivation Ca^{2+} currents, much less is known about changes in single-channel gating kinetics. Furthermore, sufficient data on temperature effects on multiple neuronal Ca^{2+} channel types (Swandulla et al. 1991) are not yet available. The existence of multiple types of Ca^{2+} channels has been suggested by recent studies using pharmacological agents. The two classs of agents most effective in blocking Ca^{2+} channels in neurones are dihydropyridines (DHP), which appear to block selectively a class of High Voltage Activated (HVA) L-type channels (Hess et al. 1984), and ω-conotoxin GVIA (ω-CgTx), which blocks the distinct class of N-type Ca^{2+} channels (Aosaki and Kasai 1989; Plummer et al. 1989). Low Voltage Activated (LVA) T-type channels are resistant to DHP and ω-CgTx (Fox et al. 1987a, b; McCleskey et al. 1987). Most types of neurones also exhibit a significant fraction of HVA current that is resistant to both types of blockers (Regan et al. 1991). Recently, particular attention has been focused on a component of *Agelenopsis aperta* venom, the ω-agatoxin IVA (ω-Aga-IVA), which strongly blocks the predominant Ca^{2+} channels, named P-type, in Purkinje neurones. P-type channels are resistant to blockers of L-type and N-type channels (Mints et al. 1992a). Moreover, in central and peripheral neurones, a fraction of HVA current blocked by ω-Aga-IVA was assumed to be due to Ca^{2+} channels similar to P-type ones in Purkinje neurones (see, for a review, Llinàs et al. 1992; Mintz et al. 1992b). Other studies suggest that the criterion used to distinguish neuronal HVA N-type Ca^{2+} channels from HVA L-type channels (i.e., assuming inactivating activity for N-type Ca^{2+} and maintained, non-inactivating activity for L-type channels) is not adequate. In fact, N-type Ca^{2+}

channels show more than one mode of gating. Single channels display at least two kinetically distinct gating patterns, characterised by spontaneous transitions from a rapidly inactivating behaviour to a sustained, non-inactivating channel activity (Delcour et al. 1993; Plummer and Hess 1991).

In an attempt to discriminate among the possibilities, we have analysed the influenced of temperature on single HVA Ca^{2+} channel types in dorsal root ganglion (DRG) neurones, using the patch-clamp technique in the cell-attached configuration (Hamill et al. 1981). We worked on DRG sensory neurones in order to study, at the single-channel level, the temperature effects that have already been characterised for macroscopic Ca^{2+} currents in the same preparation (Nobile et al. 1990). Furthermore, DRG sensory neurones exhibit different Ca^{2+} channel types, as previously found (Aosaki and Kasai 1989; Regan et al. 1991; Mintz et al. 1992b). The data reported here show that DRG neurones are characterised by at least three HVA Ca^{2+} channel types, identified by differences in biophysical properties, in pharmacological sensitivity, and in temperature dependence.

2. Materials and methods

Experiments were performed on DRG neurones, dissociated from 10–12 day old chick embryos and grown in culture (Barde et al. 1980). The single-channel recording pipette solution contained (mM): 110 $BaCl_2$, 10 N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) (pH 7.3 with $Ba(OH)_2$). The cell resting potential outside the patch was zeroed by an external solution containing (mM): 140 K-Asp, 10 ethylene glycol-bis(β-amino-ethyl-ether)N,N,N′,N′-tetraacetic acid (EGTA), 1 $MgCl_2$, 10 HEPES (pH 7.3 with KOH). DHP agonist Bay K 8644 and ω-CgTx were supplied by SIGMA Co., St. Louis, MO. Bay K 8644 was applied by bath perfusion, whereas ω-CgTx was applied directly to the bath by hand. The experimental set-up and the temperature regulation system were similar to those previously described (Nobile et al. 1990).

Unitary channel currents were recorded using the patch-clamp technique in the cell-attached configuration (Hamill et al. 1981). Borosylicate glass electrodes were pulled so that they had a tip resistance of 4–5 MΩ when filled with the aforementioned solution. Single-channel records were filtered at 3 kHz with an 8-pole low-pass Bessel filter, for a better resolution of open and closed transitions, and digitised with a sampling time of 120 μs. Fast capacitance transient compensation was performed during the experimental procedure. Voltage pulses (normalised values) were 150 ms long and ranged between −40 and 20 mV, by steps of 20 mV at 5 s intervals. For each patch, 150 unitary current traces at 20 mV at each temperature value were recorded. In order to separate the LVA Ca^{2+} channels from the HVA ones, the cell-attached patches were held at −40 mV. LVA (T-type) Ca^{2+} channels were entirely inactivated at this holding potential (HP) value. Furthermore, a −40 mV HP value was chosen in the temperature experiments, as the preparation showed a better stability and a longer life. Data acquisition and the subsequent analysis were performed with an Atari MEGA4 system connected to an analog/digital interface (Instrutech Corp., Elmont, N.Y.). Acquire, Review and Tac programs were used according to the methods described by Sigworth and Sine (1987). Single-channel records were corrected for leakage by subtracting the average of null sweeps from all sweeps displaying channels, and recorded using the same patch parameters. Qualitative analysis was restricted to experiments performed at three temperature values.

3. Results

DHP Ca^{2+} channel agonist Bay K 8644, known to enhance the Ca^{2+} current by increasing the mean open time of the HVA (L-type) channel, was normally used as a pharmacological tool for identifying L-type Ca^{2+} channels in our preparation (Fox et al. 1987b). Experiments in neurones chronically (5 min) treated with 3 μM ω-CgTx were also performed to prevent N-type channel contribution.

Temperature dependence of L-type Ca^{2+} channels

Figure 1A illustrates the DHP sensitivity and temperature dependence of the L-type Ca^{2+} channel. Panels (*a*, *b*) show representative leak-subtracted unitary current traces in control conditions and in the presence of 3 μM Bay K 8644 at 22°C. Bay K 8644 increased the open duration of the channel. In panels (*c*, *d*), currents sweeps with Bay K 8644 are plotted vs. different temperatures: 15 and 30°C. Traces were recorded consecutively, during a series of 150 ms long depolarising pulses to 20 mV from an HP of −40 mV. The reconstituted whole currents shown at the bottoms of panels (*c*, *d*) were given by the averages of 50 single channel traces. We decided to study temperature effects in the presence of Bay K 8644, as L-type channel activity was scant and difficult to separate from those of other Ca^{2+} channels. In Fig. 1B, single channel current values are plotted, which were induced by depolarising pulses between −40 and 20 mV from an HP of −40 mV, with 110 mM Ba^{2+} ions as charge carriers. The unitary slope conductance was 23 pS at 22°C. Bay K 8644 at a concentration of 3 μM did not affect the current amplitude. Several changes in the Ca^{2+} channel activity at different temperatures were observed: 1) heating from 15 to 30°C caused an increase in the single-channel current amplitude; 2) the latency time of the first opening decreased with increasing temperature; 3) channel open probability decayed at temperature values higher than 22°C; in 6 patches, where only one L-type Ca^{2+} channel was active, the open probability (P_0) was around 85% at 22°C and dropped to 60% at 30°C; 4) partially because of this fact, the average current inactivation was very slow below 30°C, whereas at 30°C the time course was monoexponential, with a time constant of about 900 ms. In Fig. 1C, an Arrhenius plot shows the temperature sensitivity of the single-channel current amplitude at a 20 mV

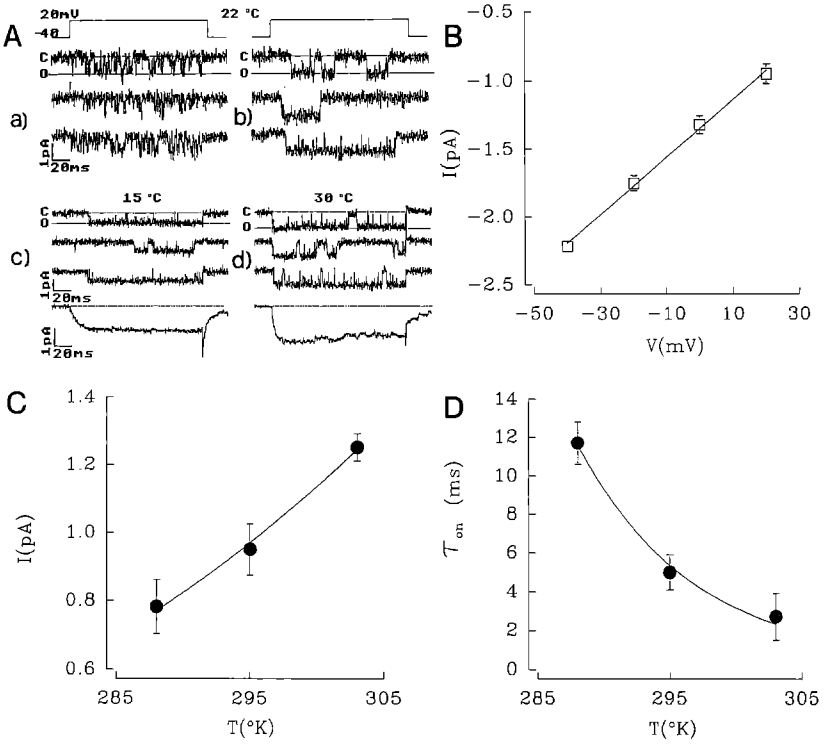

Fig. 1 A–D. Effects of temperature on DHP-sensitive Ca^{2+} channels. **A** Consecutive unitary current records before (*a*) and after addition of 3 μM Bay K 8644 to the bath at 22°C (*b*), and representative traces of unitary current at 15 (*c*) and 30°C (*d*). The average currents resulting from the sum of 50 current traces are shown below the single-channel records. **B** Current-voltage relationship (I–V) of the single channel current amplitude at 22°C. Data points and bars are the mean ±S.D. ($n=10$) of the current at the indicated potentials, from an HP of −40 mV. **C** Arrhenius plot of the single-channel current amplitude vs. absolute temperature ($n=6$–10). **D** Arrhenius plot of the activation time constant τ_{on} vs. absolute temperature. τ_{on} was obtained by fitting the mean current activation by a single exponential function ($n=6$). All data, in this and the following figures (except for the I–V curves), were obtained by depolarising steps to 20 mV from HP = −40 mV; leakage was subtracted from all current traces, and, if not indicated, traces were recorded in the presence of Bay K 8644. Records filtered at 3 kHz

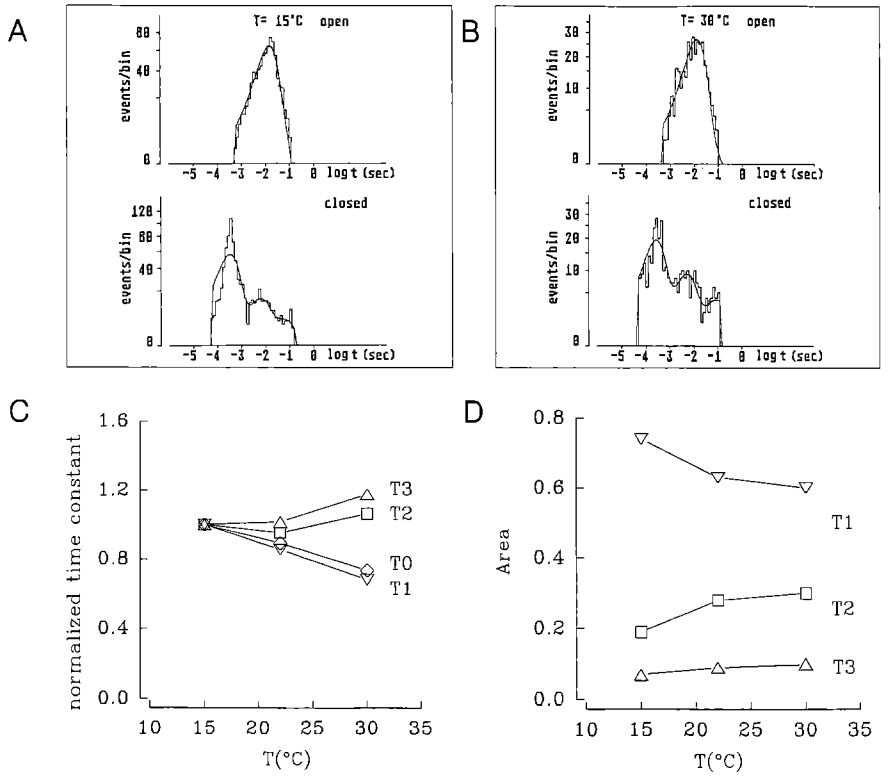

Fig. 2 A–D. Kinetic characteristics of the DHP-sensitive Ca^{2+} channel at different temperatures. Open and closed time distribution at 15 **A** and 30°C **B**. Ordinate: square roots of the numbers of openings and closings. Abscissa: open or closed times on a logarithmic scale. Distribution of open times was fitted by a single exponential, with $\tau_0=15.5$ ms at 15°C and $\tau_0=11.4$ ms at 30°C. The histograms of closed times were fitted by the sum of three exponential functions. Time constants of the exponential components were $\tau_1=0.41$ ms, $\tau_2=6.43$ ms, $\tau_3=53.1$ ms at 15°C, and $\tau_1=0.28$ ms, $\tau_2=6.84$ ms, $\tau_3=62.5$ ms at 30°C. **C** Open and closed time constants versus temperature. Data points are normalised to the values at 15°C. **D** Amplitudes of the exponential components (due to a closed time distribution) vs. temperature

membrane potential. The curve slope fitting the data points is proportional to an apparent activation energy (E_a) equal to 5.5±0.4 kcal/mol. The calculated Q_{10} between 15 and 30°C was 1.4. By taking the mean current activation time constant τ_{on} as a representative parameter of the cumulative distribution of the first-opening latency time, it was found that heating from 15 to 30°C induces a nearly six fold decrease in τ_{on} (see Fig. 1 D). The corresponding E_a and Q_{10} values were 18.7±1.7 kcal/mol and 2.7, respectively. Distributions of open and closed dwell times at two different temperatures are shown in Fig. 2 A, B. The double logarithmic histograms show that the best fit of the open durations is better described by a single-exponential function τ_0, whereas the closed dura-

tions required at least three exponential functions (time constants τ_1, τ_2, τ_3). This and the following analyses of the channel kinetics were made on data obtained by voltage pulses to 20 mV from an HP of −40 mV. From Fig. 2C, it appears evident that an increase in temperature from 15 to 30°C causes a shift in the opening and fast closed time constants (τ_0 and τ_1) towards shorter durations of about 30%, while the intermediate τ_2 and long τ_3 closed duration components are relatively temperature-insensitive. Temperature has no effect on the related number of events, which included the long component, but it increases the number of events belonging to the intermediate component from 20% at 15°C to 30% at 30°C, whereas the numbers of short events decreases from 75% at 15°C to 60% at 30°C (see Fig. 2D). The data obtained are representative of 5 other cells.

Temperature dependence of inactivating DHP-insensitive Ca^{2+} channels

An inactivating DHP-insensitive Ca^{2+} channel, resembling the N-type Ca^{2+} channel, was isolated only in cells not chronically treated with ω-CgTx ($n = 120$). The unitary Ca^{2+} current-voltage relationship (I–V) and traces in control conditions and in the presence of 3 μM Bay K 8644 at 22°C are shown in Fig. 3A. The slope conductance was 13 pS in the indicated voltage range, from a HP of −40 mV. Calcium channels of this type revealed the occurrence of openings in bursts, with longer intervals between bursts and time-dependent activation and inactivation, as can be seen from the single current traces and the averaged records displayed in Fig. 3B for two different temperature values. Single-channel current values from 4–6 cells were plotted versus absolute temperature and then fitted by an Arrhenius equation (not shown). The corresponding E_a value was 4.4 ± 1.6 kcal/mol and the calculated Q_{10} from 15 to 30°C was 1.3. When the temperature was raised, the peak of the averaged single-channel current increased, with a Q_{10} value equal to 1.9 ($n = 4$). The corresponding peak probability of channel opening changed, with a Q_{10} value 1.6. An increase in temperature induces sharp accelerations of channel activation and inactivation. Arrhenius plots of activation and inactivation time constants of the mean unitary channel current versus temperature are shown in Fig. 3C, D. The corresponding E_a and Q_{10} values are 35.0 ± 1.2 kcal/mol and 5.9 for activation, and 15.1 ± 4.1 kcal/mol and 2.0 for inactivation. Temperature affects the time constants and the related areas, obtained by fitting the single channel open and closed times by a single exponential function and three exponential functions, respectively (see Fig. 4). The increase in temperature from 15 to 30°C causes a shift in the open and intermediate closed distributions (τ_0 and τ_2) towards shorter durations (50% of the value normalised to 15°C), whereas the slow duration component τ_3 is relatively temperature-insensitive, and the short one τ_1 increases by about 30% (Fig. 4A). Figure 4B shows that the τ_1 and τ_2 areas are strongly dependent on temperature. The number of events increases from 35% at 15°C to 70% at 30°C for τ_1 and decreases from 50% to 10% for τ_2. The number of events related to the slow component is little affected by temperature. The data shown are representative of 4 cells.

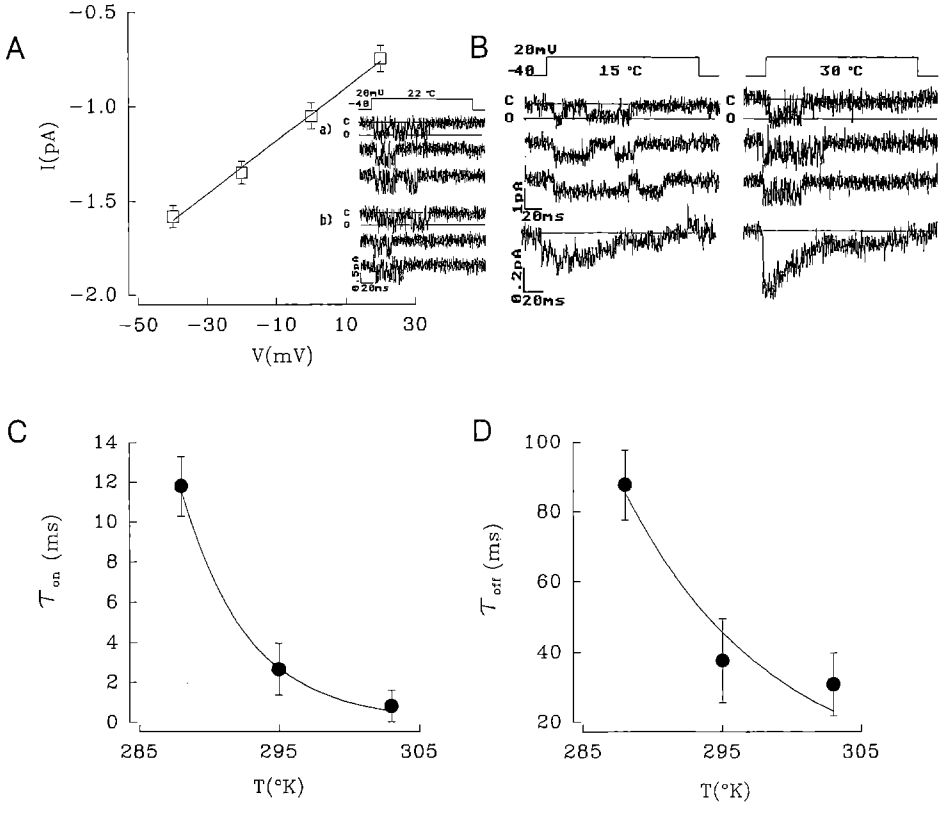

Fig. 3 A, B. Effects of temperature on the inactivating DHP-insensitive Ca^{2+} channel. **A** Representative traces of single current records and I–V relationship of the channel amplitude ($n = 6$) at 22°C. Same experimental conditions as in Fig. 1. The unitary slope conductance was 13 pS. **B** Consecutive sweeps of unitary current records at 15 and 30°C. At the bottom, the related mean current records computed from 30 such traces are shown. The mean single channel current activation and inactivation vs. absolute temperature are shown in panels C and D, respectively (mean ± SD $n = 4$).

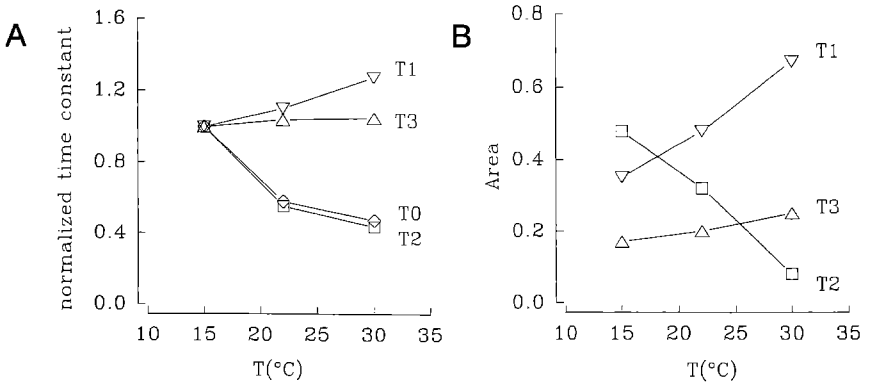

Fig. 4 A, B. Kinetic characteristics of the inactivating DHP-insensitive Ca^{2+} channel at different temperatures. **A** *Open (single exponential)* and *closed (three exponentials)* time-constant values vs. temperature. Data points are normalised to the values at 15°C ($\tau_0 = 3.71$ ms, $\tau_1 = 0.66$ ms, $\tau_2 = 7.93$ ms, $\tau_3 = 103$ ms). **B** Amplitudes of the exponential components (due to a closed time distribution) vs. temperature

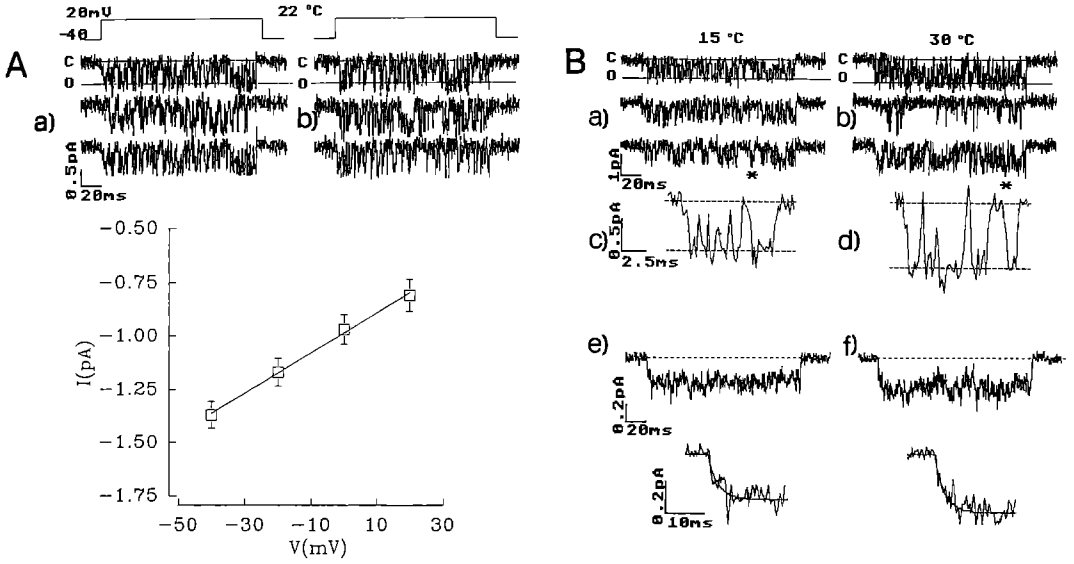

Fig. 5 A, B. Effects of temperature on the non-inactivating DHP- and ω-CgTx-insensitive Ca^{2+} channel. **A** External application of 3 μM Bay K 8644 and 3 μM ω-CgTx does not affect the Ca^{2+} channel characteristics. Control (*a*), presence of Bay K 8644 plus ω-CgTx (*b*). Below, unitary current amplitude plotted vs. membrane potential. Experiments ($n = 4$) were performed at 22°C and from an HP of −40 mV. The slope conductance was 10 pS. **B** Selected traces from one patch, showing the conductance levels and the activity of the channel at two temperature values (*a, b*). Panels (*c, d*) are selected channel openings taken from records in (*a*) and (*b*) as indicated by the asterisks, showing on an expanded time scale to highlight the difference in current amplitude. Panels (*e, f*), mean current records from 30 such traces. Below, best fits of the mean current activations on expanded scales

Temperature dependence of non-inactivating DHP- and ω-CgTx-insensitive Ca^{2+} channels

A non-inactivating DHP-insensitive single channel activity, lasting longer than the pulse duration and with a temperature dependence that was different from those of the above-described types of channels, was observed separately in different patches ($n = 7$). The channel was also active in 3 μM ω-CgTx pre-treated cells ($n = 3$) and was identified in patches where a Bay K 8644 sensitive L-type Ca^{2+} channel was active. The channel displayed a fast opening pattern for several consecutive sweeps before to became temporarily inactive. Figure 5 A illustrates an isolated channel activity in two different cell-attached patches held at 22°C, which indicate Bay K 8644 and ω-CgTx insensitivities, and the I–V relationship of this Ca^{2+} channel type. The unitary current amplitude was resolved and reproducible, and opposite of that expected for imperfect resolution of brief openings due to bandwidth limitation. Indeed, when records are expanded along the time axis (Fig. 5 B panels *c, d*), it is evident that many of the openings are quite well determined. The conductance, as derived from the I–V curve slope, was found to be 10 pS. The temperature dependence of the unitary channel current is not shown, but Arrhenius plots of the values obtained at 20 mV gave an E_a value 4.5 ± 1.2 kcal/mol and a Q_{10} value 1.3. Figure 5 B shows sweeps at two different temperature values with the average of consecutive records indicating that the kinetic pattern of this channel is not dependent on temperature. The activation of the mean currents were fitted by an exponential as shown at the bottom. The time constants were 2.62 and 2.42 ms at 15 and 30°C, respectively. At three temperature values the mean current activation time constant was 3 ± 1 ms ($n = 3$). The probability of the channel being opened did not change significantly in the temperature range examined, and was around 20% in four cells. Taking into consideration the time constants obtained from the open and closed time histograms, one can notice that τ_0 was practically temperature-insensitive, whereas τ_1, τ_2,

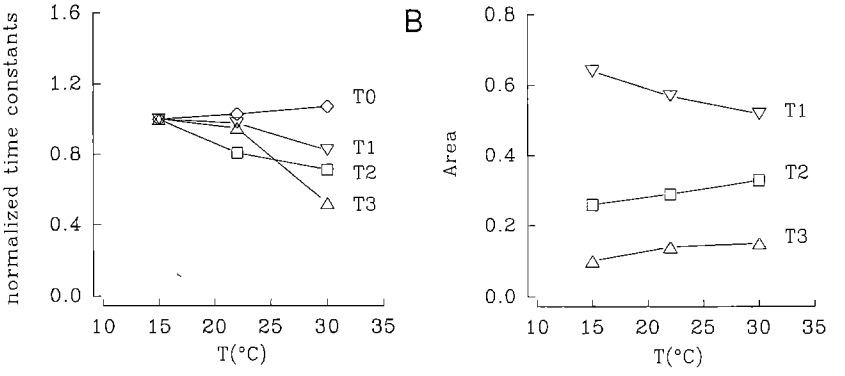

Fig. 6 A, B. Kinetic characteristics of the non-inactivating DHP- and ω-CgTx-insensitive Ca^{2+} channel at different temperatures. **A** *Open* (*single exponential*) and *closed* (*three exponentials*) time constants vs. temperature. The experimental points are normalised to the time constant values obtained at 15°C ($\tau_0 = 0.85$ ms, $\tau_1 = 1.26$ ms, $\tau_2 = 6.46$ ms, $\tau_3 = 72.19$ ms). **B** Amplitudes of the closed-time exponential components in panel **A** vs. temperature

τ_3 were shifted towards shorter durations by about 15%, 30%, 50%, respectively, and only the number of events for τ_1 significantly decreased from 65% to 53% (see Fig. 6A, B). The data shown are representative of 3 neurones.

4. Discussion

Our findings are in qualitative agreement with the results obtained by Fox et al. (1987) and by Kostyuk et al. (1988) concerning the properties of DHP-sensitive Ca^{2+} channels. Some quantitative differences, mainly in terms of unitary current amplitude (Kostyuk et al. 1988), are due to the fact that we used 110 mM Ba^{2+} ions instead of 60 mM Ba^{2+} ions as charge carriers, and to the fact that open and closed time distributions were obtained from current traces recorded at 20 mV and in the presence of Bay K 8644. Moreover, Bay K 8644, which increased the channel mean open time, probably caused an underestimate of temperature effects. By heating from 15 to 30°C, we found an increase in the unitary channel current, with a relatively small Q_{10} value equal to 1.4. The latency time of the first opening was markedly affected by temperature. The rising phase of the mean single-channel current became faster with increasing temperature ($Q_{10} = 2.7$), whereas the decay phase was less dependent on temperature and showed a slow inactivation, probably due to a reduction in the opening probability at temperature values higher than 22°C. The decrease in the opening probability may be the result of shifts in the open and fast intraburst closed time distributions towards shorter durations, in addition to changes in the numbers of events associated with the different closed duration components.

The observed behaviours of the inactivating DHP-insensitive Ca^{2+} channel were instead similar to those found by other researchers (Fox et al. 1987a, 1987b; Kostyuk et al. 1988; Lux and Brown 1984) in chick and mouse sensory neurones and in snail neurones. However, it is interesting to note that the channel activity is already present at a relatively positive holding potential of −40 mV. Regarding the dependence on temperature, no significant effect on the single-channel amplitude was observed. The Q_{10} value 1.3 was similar to that of the DHP-sensitive Ca^{2+} channel. On the contrary, when the temperature was raised, the time constants of the mean single-channel current activation and inactivation showed very fast accelerations. Our results, with Q_{10} values equal to 5.9 and 2.0 for activation and inactivation, respectively, can be compared with earlier results on whole-cell Ca^{2+} currents in mollusc neurones (Brown et al. 1983) and in chick sensory neurones (Nobile et al. 1990). When the temperature was raised up to about 30°C, both groups showed a similar drastic acceleration of the Ca^{2+} current activation but a faster inactivation. This is consistent with the observation that the Ba^{2+} current inactivates more slowly (Lux and Brown 1984). In agreement with the above-mentioned authors, the change in the value of the peak mean channel current during heating seems to be due to an increase in the peak probability of channel opening ($Q_{10} = 1.6$), rather than a change in unit amplitude. The main reasons may be the influence of heating on the latency time of the first opening and the increased flickering during bursts of activity in the first 50 ms of a pulse. Moreover, when temperature is raised, the open and intermediate closed time constants shift towards shorter durations, and, for the intermediate closed duration, the number of events decreases. On the contrary, the short closed duration component becomes slower and the corresponding number of events increases, whereas the long duration component is relatively temperature-insensitive.

Unlike the above-described channel, the non-inactivating DHP- and ω-CgTx-insensitive Ca^{2+} channel is less dependent on temperature. The current amplitude increases with increasing temperature ($Q_{10} = 1.3$), whereas no temperature effect on the mean single channel current is observed. The opening probability and the latency time of the first opening are also temperature-insensitive: during heating, the mean open times remained unchanged and the closed time constants, shifting towards shorter durations, were compensated for by the related numbers of events. We know that recent reports suggest reversible, spontaneous transitions between the inactivation and non-inactivation modes of the HVA DHP-insensitive (N-type) Ca^{2+} channel (Delcour et al. 1993; Plummer and Hess 1991). Such transitions might justify the kinetic characteristics of both the inactivating DHP-insensitive channel and the non-inactivating DHP-insensitive Ca^{2+} channel. Furthermore, the two channel behaviours exhibit similar conductance values. Nevertheless, the activity of the non-inactivating channel observed separately in different patches from ω-CgTx pre-treated cells, and its relative temperature insensitivity, as compared with that of the inactivating channel, allow us to describe that channel

Table 1. Sensitivity of Ca^{2+} channels to temperature

Ca^{2+} channel type	Amplitude		Activation		Inactivation		P_0
	E_a	Q_{10}	E_a	Q_{10}	E_a	Q_{10}	
DHP-sensitive (L)	5.5±0.4	1.4	18.7±1.7	2.7	none	none	↓
Inactivating DHP-insensitive	4.4±1.6	1.3	35.0±1.2	5.9	15.1±4.1	2.0	↑
Non-inactivating DHP-insensitive	4.6±1.2	1.3	none	none	none	none	none

* Activation energy (kcal/mol)

as a third type of channel. The temperature dependence of Ca^{2+} channels parameters is summarised in Table 1.

In conclusion, heating affects transitions among closed states, as well as the open-to-closed state transition, although the DHP-sensitive channel shows a quantitative difference with respect to the inactivating DHP-insensitive channel. Of great interest is the fact that the non-inactivating DHP- and ω-CgTx-insensitive channel is practically temperature-independent, although an increase in temperature affects transitions among closed states and has a very small effect on the open-to-closed transition. In order to characterise the last-mentioned channel by means of other pharmacological tools, further research will be conducted.

Acknowledgements. We wish to thank Prof. E. Carbone for helpful discussions and constructive criticism during the preparation of the manuscript, and Mr. R. Mantovani for valuable technical assistance.

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