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DEACTIVATION KINETICS OF DIFFERENT COMPONENTS OF CALCIUM INWARD CURRENT IN THE MEMBRANE OF MICE SENSORY NEURONES

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SUMMARY

1. Deactivation kinetics of different components of calcium inward current were analysed by measuring 'tail currents' in intracellularly perfused sensory neurones isolated from dorsal root ganglia of newborn mice.

2. Deactivation of a low-threshold inactivating component had a mono-exponential time course with time constants that decreased as the potential was made more negative, and reached a limiting value of 1·0–1·2 ms at extreme hyperpolarizations. Replacement of Ca^{2+} by Ba^{2+} as a charge carrier decreased this value almost twofold.

3. Deactivation of the high-threshold component of calcium current contained two exponential components with time constants of similar potential dependence. The fast time constant became practically constant (0·1–0·2 ms) during repolarizations beyond -50 mV, and the slow time constant became constant (0·80–0·85 ms) at repolarization beyond -70 mV.

4. Replacement of intracellular aspartate with phosphate, or a depolarized holding potential of -40 mV, abolished reversibly the slow component of the tail current; in parallel, the peak current–voltage relation of the current became shifted by 15–20 mV in the depolarizing direction.

5. Extracellular application of the calcium channel agonist Bay K 8644 (2·5–5 μmol/l) did not change deactivation kinetics of the low-threshold current; however, it increased considerably the slow exponential component of high-threshold current deactivation or induced such a component in conditions when the latter was absent before application ($V_h = -40$ mV; cell perfusion with Tris phosphate).

6. The data obtained are analysed on the basis of the presence of three types of calcium channels in the neuronal membrane which possess similar kinetic mechanisms of activation and deactivation but differ in the activation thresholds and the time constants of transitions between open and closed states. The three calcium channels differ also in interaction with different permeant ions and calcium channel agonists.

INTRODUCTION

The relaxation of ionic current after removal of membrane depolarization ('tail current') contains valuable information about the gating mechanisms of voltage-

dependent ion channels. As has been already shown by a number of investigators on intracellularly perfused snail neurones, calcium tail currents recorded near the resting potential include a fast ($\tau \sim 0.5$ ms) and several slow ($\tau = 2$–3 ms) exponential components. Their time constants do not show significant dependence on the potential at which calcium channels were previously opened (Byerly & Hagiwara, 1981, 1982; Tsuda, Wilson & Brown, 1982; Brown, Tsuda & Wilson, 1983; Byerly, Chase & Stimers, 1984).

Recently the studies of calcium channel deactivation were complicated by the detection of several types of calcium channels in the membranes of different electrically excitable cells. Three types of calcium channels were described: low-threshold inactivating (LTI or T-type), high-threshold inactivating (HTI or N-type) and high-threshold non-inactivating (HTN or L-type) (Veselovsky & Fedulova, 1983; Carbone & Lux, 1984; Matteson & Armstrong, 1984, 1986; Deitmer, 1984; Bean, 1985; Fedulova, Kostyuk & Veselovsky, 1985; Nowycky, Fox & Tsien, 1985*a*; Kostyuk, Shuba & Savchenko, 1987). Each type of channel has gating kinetics features of its own. Deactivation of LTI current appears to be monoexponential with a time constant 4–5 times longer than that for high-threshold currents (Armstrong & Matteson, 1985; Veselovsky *et al.* 1985). Hence, the LTI channels were called 'slow deactivating' and the high-threshold ones 'fast deactivating'. The deactivation rate of LTI current was shown by Veselovsky and co-workers to be strongly dependent on repolarization potential. The high-threshold currents deactivation proved to be complicated and included several components. It was also shown that the slow component with a time constant of more than 2 ms could be due in part to deactivation of the non-specific outward current channels (Veselovsky *et al.* 1985).

This paper presents the results of a more extensive study of deactivation kinetics of the three types of calcium channels in the neuronal membrane.

METHODS

Experiments were carried out on isolated nerve cell bodies from dorsal root ganglia of mice (strain CBA/J) aged 1–4 weeks. The enzymatic treatment of ganglia was performed in Eagle's medium containing 2 mg/ml collagenase (type V; Sigma Chemical Co., St Louis, MO, USA) and 0·5 mg/ml pronase E (Serva Feinbiochemica, Heidelberg) and 0·15% (v/v) lactalbumin. The enzyme solution was pre-warmed to 31–32 °C. After 15–25 min (depending on the age of the animal) the enzyme solution was exchanged for Eagle's medium and then ganglia were kept at room temperature. The cells were separated from the ganglia by mechanical agitation immediately before the experiment.

The measurements were made in an extracellular solution containing (in mmol/l): 15 $CaCl_2$, 120 choline chloride, 2 $MgCl_2$, 5 Tris; pH was adjusted to 7·3 with HCl. Ca^{2+} was exchanged for other permeant cations (Ba^{2+} or Sr^{2+}) on an equimolar basis. The intracellular solution contained (in mmol/l): 150 Tris aspartate, 10 EGTA, 10 MOPS (3-(*N*-morpholino)ethanesulphonic acid). In some specially noted cases the intracellular solution contained (in mmol/l) 140 Tris phosphate + 10 EGTA or 150 Tris phosphate only. pH was adjusted to 7·3 with Tris.

The electrical measurements were carried out with intracellular perfusion by means of polyethylene micropipettes, as previously described (Kostyuk, Veselovsky & Fedulova, 1981; Fedulova *et al.* 1985). Neurones had diameters of 40–60 μm. The pipettes had a wall thickness of about 50 μm and conical (40–50 deg) pores with openings of 10–15 μm in diameter.

The input resistance of the resting neurones fixed in the pore was up to 600–700 MΩ with internal Tris phosphate, and 500–600 MΩ with Tris aspartate. The effective value of the series resistance could be decreased by a factor of 2–2·5 when an analog single-exponential compensation circuit was

used. The time constant of the capacity transient was 60–100 μs using maximal possible compensation. Leakage current was subtracted using analog circuitry. Capacitative currents were cancelled out by subtracting summed responses to subthreshold voltage pulses.

Dihydropyridine drugs were dissolved daily in ethanol as a 5 mmol/l stock and then added into the extracellular solution so that the final ethanol concentration was not more than 0·1 %. Application of 0·1 % ethanol alone had no effect on the current studied. Measures were taken to avoid photodestruction of the drugs applied. Temperature was kept at 22 °C during measurements.

RESULTS

Deactivation of low-threshold calcium currents

The low-threshold calcium currents were observed in about 20% of neurones under investigation, as has already been shown (Fedulova *et al.* 1985). To record separately the LTI currents from these neurones, the membrane was held at potentials between −120 and −90 mV, depolarized to −50 or −40 mV, and then repolarized to different potentials.

LTI current deactivation was found to be monoexponential, as has been shown by previous investigations. This feature remained under various holding potentials (−130 to −70 mV), activating depolarizations (up to −40 mV) and different intracellular solutions (Fig. 1*A*). The tail-current amplitude for the LTI currents was proportional to the current value at the end of the activating pulse when the repolarization potential remained the same. The time constant of the tail current showed no significant voltage dependence under repolarizations to very negative potentials (−160 to −140 mV) being 1·1 ± 0·1 ms (mean ± S.E.M., $n = 8$). However, it progressively increased when the amplitude of the repolarization shift diminished (Fig. 1*B*). This general behaviour remained the same when other penetrating cations were used (Fig. 1*C*). Equimolar substitution of external calcium with barium, however, accelerated deactivation more than twofold under strong repolarization. Substitution by strontium had an intermediate effect. Over the potential range showing strong potential dependence, the substitution of penetrating cations produced a small shift of the dependence along the potential axis.

Deactivation of high-threshold calcium currents

To investigate deactivation of high-threshold calcium currents, cells were chosen which, at a holding potential of −120 mV, did not show any hump in the *I–V* curve at testing potentials ranging between −50 and −40 mV. In this case, even if a small LTI current was available, its contribution to kinetic characteristics of the currents activated at a holding potential of −80 to −70 mV could be neglected, since the LTI current undergoes stationary inactivation of 70–80 % at these holding potentials. At these holding potentials the high-threshold current retained its full magnitude, thus allowing an analysis of high-threshold current deactivation in pure form. To fit the time course of the high-threshold tail current, two exponentials were required. The fast time constant of deactivation reached 0·13 ± 0·05 ms ($n = 18$) and the slow one 0·83 ± 0·04 ms ($n = 11$) under repolarization to −80 mV. The slow tail-current component disappeared completely when the holding potential was reduced to −40 mV, but the amplitude of the fast component remained practically unchanged. The two tail-current components depended to a different extent on the magnitude of

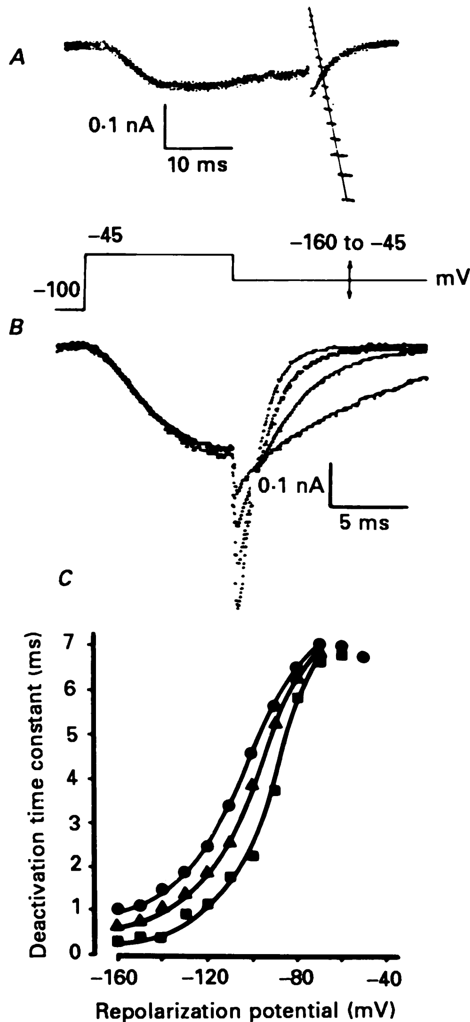


Fig. 1. Potential dependence of deactivation kinetics of low-threshold calcium currents. *A*, example of LTI current recording together with automatic plotting of the tail current on a semilogarithmic scale. *B*, changes in tail-current kinetics at different repolarization potentials. The pulse protocol is presented above the figure. *C*, potential dependence of deactivation time constant measured with different permeant ions: Ca^{2+} (●), Sr^{2+} (▲), Ba^{2+} (■).

activating depolarization shift. An example of high-threshold current recordings is shown in Fig. 2*A*. When plotted on a semilogarithmic scale, the tail currents distinctly revealed the presence of two exponential components (Fig. 2*B*). Their time constants were $0{\cdot}18 \pm 0{\cdot}03$ and $1{\cdot}95 \pm 0{\cdot}04$ ms under repolarization to -55 mV. The amplitude of the slow tail component (determined by regression analysis) was largest at activating potentials in the range of about 0 mV. With stronger depolarizations the amplitude of the fast component became saturated and the amplitude of the slow one decreased. Figure 2*C* presents the dependence of amplitudes of both tail-current components on activating potential shift.

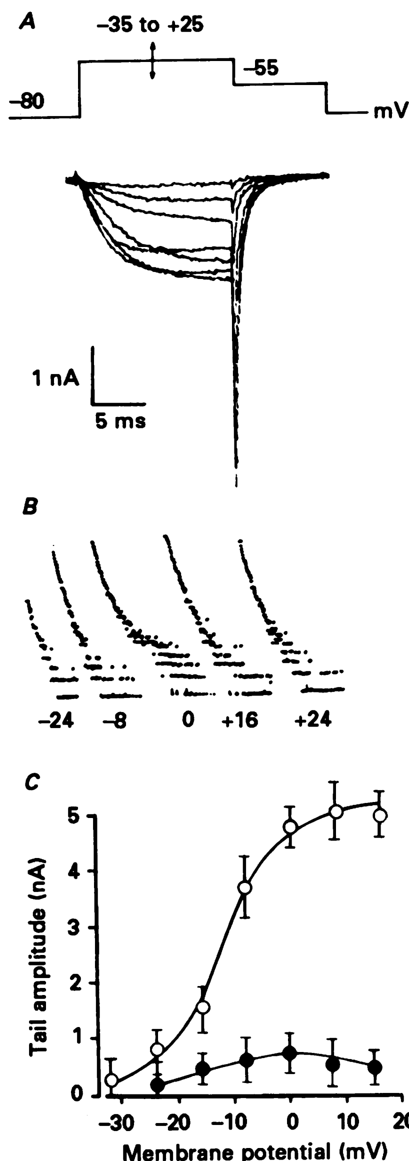


Fig. 2. Separation of the exponential components in the deactivation of high-threshold calcium currents. *A*, examples of high-threshold currents. *A*, examples of high-threshold currents recorded at different activating potential shifts. *B*, tail currents on a semilogarithmic scale. The corresponding depolarization levels are indicated before the records. *C*, amplitudes of the fast (○) and slow (●) exponential components of the tail currents obtained by regression analysis.

The replacement of Tris aspartate as intracellular solution with Tris phosphate reversibly removed the slow component of the high-threshold current deactivation, leaving the time constant of the fast one unchanged. Such replacement also affected the high-threshold calcium current itself; its activation curve became shifted by about 10 mV in the direction of stronger depolarization. The shift did not depend on

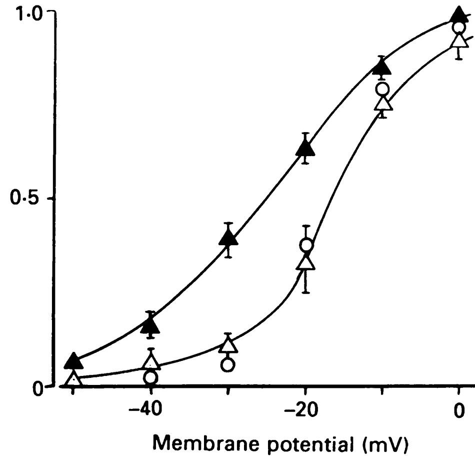


Fig. 3. Effect of different intracellular anions on high-threshold calcium currents. Activation curves obtained from six cells with Tris aspartate (▲) and Tris phosphate (△) solution containing EGTA, as well as with Tris phosphate without EGTA (○) from three cells.

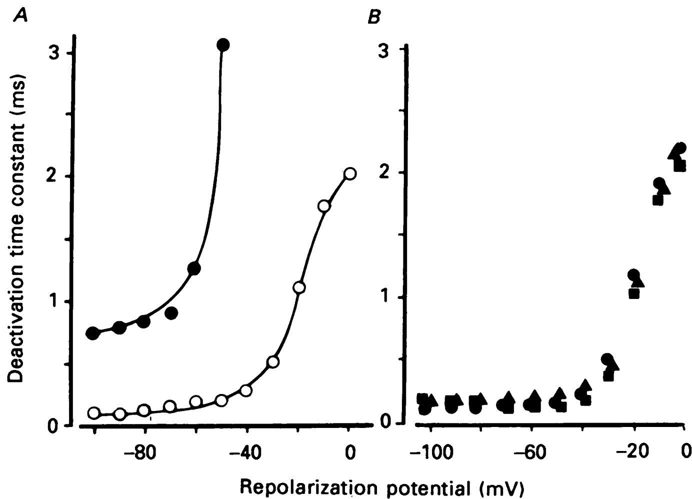


Fig. 4. Time constants of two exponential components in the deactivation kinetics of high-threshold calcium currents. *A*, potential dependence of the time constant of slow (●) and fast (○) components recorded at holding potentials −80 and −40 mV respectively. *B*, potential dependence of the fast time constant with different permeant ions: Ca^{2+} (●), Sr^{2+} (▲), Ba^{2+} (■).

the absence or presence of EGTA in the intracellular solution. The corresponding activation curves are shown in Fig. 3.

The time constants of the two deactivation components are plotted against repolarization membrane potential in Fig. 4*A*. The time constant of the slow component approached a minimum value at potentials more negative than −70 mV, and that of the fast component did so at potentials more negative than −50 mV. It

is necessary to note that the former voltage dependence was determined at a holding potential of −80 mV and the latter at a holding potential of −40 mV. The amplitudes of the high-threshold current and the fast tail-current component significantly increased when external calcium was replaced with barium or strontium. In such conditions it was impossible to distinguish the slow component of the high-threshold current deactivation. No significant differences in potential dependence of the time constant of the fast deactivation component were found for different penetrating cations (Fig. 4*B*).

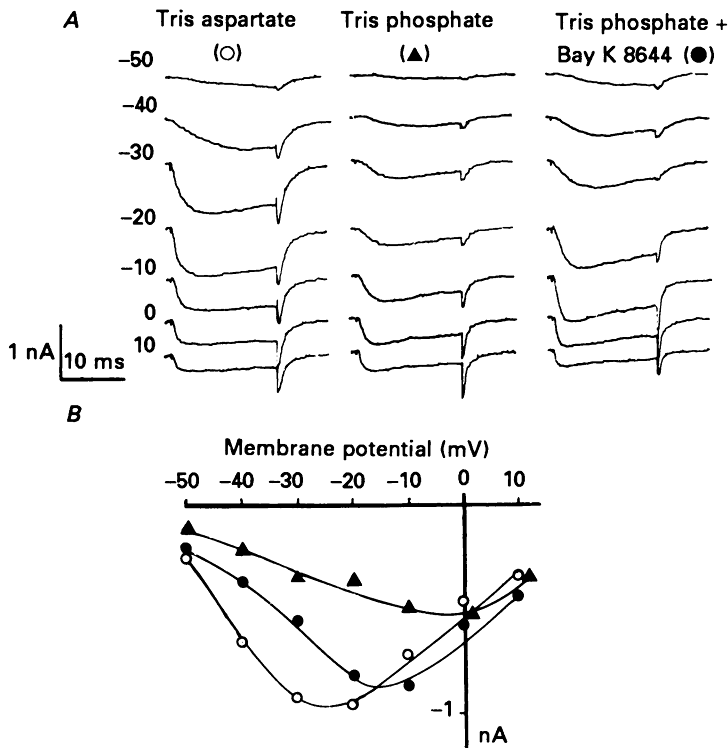


Fig. 5. Effect of Bay K 8644 on high-threshold calcium currents. *A*, examples of currents recorded from the same cell with internal Tris aspartate (left column), Tris phosphate (middle column) and Tris phosphate + extracellular application of 2·5 μmol Bay K 8644 (right column). Depolarization levels are indicated near the records; holding potential, −80 mV. *B*, the peak current–voltage characteristics for the same currents.

Effects of pharmacological substances

The effects of the following calcium channel dihydropyridine agonists and antagonists have been tested by extracellular application: Bay K 8644, nitrendipine, niludipine, nimodipine (all in concentrations of 2·5–5 μmol/l).

No significant changes in low-threshold calcium currents have been detected under the action of all the drugs. However, Bay K 8644 induced a definite change in the kinetics and potential dependence of the high-threshold calcium current, especially when the cell was perfused by Tris phosphate solution. The current–voltage

characteristic of this current, which was shifted in the depolarizing direction during replacement of intracellular Tris aspartate by Tris phosphate, on application of Bay K 8644 was displaced in the opposite direction by 15–20 mV, and a definite increase in the peak amplitude of the calcium current occurred (see an example in Fig. 5 *A* and *B*).

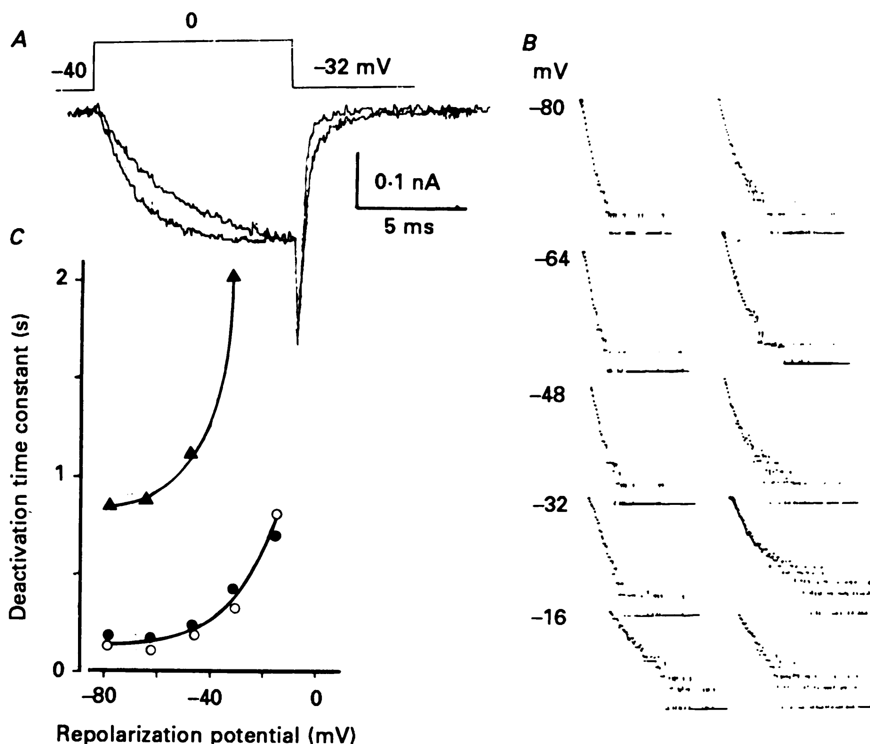


Fig. 6. Effect of Bay K 8644 on deactivation kinetics of high-threshold calcium currents. *A*, currents recorded in a Tris phosphate-perfused cell before (upper record) and after extracellular application of Bay K 8644 (lower record). *B*, automatic semilogarithmic plotting of tail currents recorded at different repolarizing potentials before (left column) and after application of Bay K 8644 (right column). *C*, potential dependence of the time constant of the fast (circles) and slow (triangles) exponential components of the tail current measured before (open symbols) and after (closed symbols) application of the drug.

In parallel, a pronounced increase in the slow exponential component of the high-threshold current deactivation occurred. Moreover, in cases when such component could not be observed ($V_h = -40$ mV), addition of Bay K 8644 induced its appearance. Figure 6*A* presents examples of high-threshold calcium currents elicited in a Tris phosphate-perfused cell at $V_h = -40$ mV without Bay K 8644 (upper record) and after its application (lower record). Figure 6*B* shows automatic plotting on a semilogarithmic scale of tail currents recorded at different repolarizing potentials without Bay K 8644 (left column) and with it (right column). In Fig. 6*C* the potential dependence of the time constants of the two exponential components in the tail current is shown. As can be seen, the kinetics of the fast component before

and after addition of Bay K 8644 remained practically unchanged (open and filled circles, respectively). At the same time, a large slow component appeared after Bay K 8644 application (triangles) with potential dependence similar to that of a slow component recorded in Tris aspartate-perfused cells (compare Fig. 4*A*).

The calcium channel antagonists tested (nitrendipine, niludipine and nimodipine) did not produce detectable inhibition of calcium currents in the potential range tested. On the contrary, some increase of the initial component of the high-threshold current (similar to the effect of Bay K 8644 but less pronounced) could be observed.

DISCUSSION

Adequate speed of potential clamp and reliable separation of different ionic current components are the necessary conditions for reliable analysis of deactivation kinetics of different types of ion channels. The intracellular perfusion technique we used allowed us to recharge the membrane capacity with a τ of about 100 μs. Only the fast component of high-threshold current deactivation had a time constant comparable with clamp rapidity (under repolarization potentials more negative than -50 mV); all the other components had kinetics that were much slower than the clamp.

LTI channels can be activated at a membrane potential of about -60 mV. Choosing the appropriate holding potential and activating depolarizations permits separate activation of this type of calcium channel. It is also possible to inactivate LTI channels completely by decreasing the holding potential to less negative values, thus allowing separate recording of the activity of high-threshold calcium channels.

Separation of two categories of high-threshold calcium channels – inactivating (HTI) and non-inactivating (HTN) – has been achieved on different cell types by recordings of single-channel activity. In our laboratory this has been done on mice sensory neurones (Kostyuk *et al.* 1987). According to these data, only a partial depression of HTI channels can be obtained at a holding potential of about -50 mV. Moreover, under these conditions calcium currents are 'washed out' quite rapidly, probably as a result of a large maintained calcium influx and the limited buffering capacity of the perfused neurones; therefore, the use of long depolarizing pulses for such separation is also problematical.

A new way to separate high-threshold calcium current into individual components seems to be provided by the use of different perfusing solutions. The functioning of a more easily activated component (which may correspond to the HTI or N-current) is likely to be especially quickly depressed during neurone perfusion with Tris phosphate. The effect is probably due to the type of anion introduced into the cell but not to possible differences in the level of intracellular calcium since it did not depend on the presence or absence of EGTA in the perfusing solution.

In principle we cannot reject *ad hoc* a suggestion that the slow component of the high-threshold tail current recorded during cell perfusion with Tris aspartate might be due to a considerable change in the kinetic mechanism of a single type of high-threshold calcium channel under present conditions. But the idea of the presence of two types of high-threshold channel with different kinetics based on single-channel recording data seems to be much more realistic.

If we accept the existence of two types of high-threshold calcium channel in the neurones under study, some suggestions concerning the kinetics of their deactivation can be made.

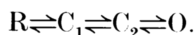
(1) Deactivation of high-threshold channels of a certain type is also predominantly a monoexponential process. In fact, during cell perfusion with Tris phosphate the deactivation of the high-threshold current can be adequately described by a single exponent with a time constant of about 100 μs (at repolarizations to more negative potentials than -50 mV). During perfusion with Tris aspartate, the tail current included distinctly separable exponential components: a fast one similar to that recorded in Tris phosphate, and a new slow one.

(2) Depolarization-induced high-threshold calcium currents underlying the two exponential tail-current components differ in maximum amplitudes. As we found using regression analysis, the initial amplitude of the fast component is more than 5 times larger than that of the slow one. Their amplitudes differ also in the dependence on testing potential level: the slow tail component is related to the current which decreased (inactivated) during strong depolarization while the fast one demonstrated saturation under such conditions.

(3) At very negative repolarization potentials the time constants of exponential tail-current components reach an almost potential-independent minimal value, different for each current. The slow component of the high-threshold current relaxation reaches the value of 0·80–0·85 ms at repolarization potentials between -100 and -80 mV. The deactivation time constant of low-threshold calcium current reaches minimal value (1·0–1·2 ms) only at repolarization potentials more negative than -150 mV.

The suggestion about predominantly monoexponential decay of a current produced by channels of a single type might seem to be in contradiction with some data already published. The complicated kinetics of calcium current deactivation have been described earlier (Tsuda *et al.* 1982; Byerly *et al.* 1984). The authors were inclined to relate the existence of several components of it to the complex gating machinery of this current which includes several intermediate stages. Since the recording system used in our experiments has a limited time resolution, it permitted us to analyse only slow components of the deactivation process reflecting its limiting stages; we do not exclude the possible presence of the other components with very short (below 100 μs) and/or very long (several seconds) time constants.

A successive kinetic model with one open state has been proposed for calcium channels by several investigators (Krishtal, Pidoplichko & Shakhovalov, 1980; Wilson, Brown & Tsuda, 1983; Byerly *et al.* 1984):



$$R \rightleftharpoons C_1 \rightleftharpoons C_2 \rightleftharpoons O.$$

It explains satisfactorily the data obtained in the present investigation as well as data from single-channel recordings. According to this model, channel activation occurs through two or three closed stages (C) with strongly potential-dependent transitions between them, the final transition into the open state (O) being practically potential independent. Analysis of the single calcium channel activity in mice sensory neurones carried out in our laboratory (Shuba & Teslenko, 1987) allowed us to evaluate the transition time constants in the membrane potential range between -15 and $+25$ mV. Extrapolation to more negative potentials indicates

that in this range the rates of potential-dependent transitions to the resting state must be 3–5 times higher than the reversed open time of the channel, and practically no potential-dependent transitions in the direction of the open state occur (which means that closed states are absorbing). This justifies the suggestion that at highly negative repolarization potentials the immediate transition from the open state should limit the deactivation process, whereas at less negative potentials the potential-dependent transitions to the resting state should become important for the tail-current kinetics.

In the present experiments such time constants have been measured precisely in a wide range of repolarization potentials (which is not possible during single-channel recordings), and the results obtained were a good fit with the theoretical predictions.

The plot of the deactivation time constant against potential flattens at very negative repolarization potentials. This confirms the conclusion that both low- and high-threshold inactivating calcium channels close through a practically potential-independent stage; the time constant of deactivation at such repolarization potentials equals the channel mean open time. The mean open times for these types of calcium channel measured at more positive potentials gave values close to our finding (see Fox, Nowycky & Tsien, 1987; Kostyuk *et al.* 1987). The situation with HTN channels is less clear. They may have either no potential-independent transition from the open state (in contrast to the behaviour of other types of calcium channels) or, more likely, their mean open time may be so small (less than 100 μs) that it cannot be measured adequately by our recording system.

The existence of much slower components in calcium tail currents having weak potential-dependent τ was also shown in several investigations of calcium current deactivation in other cell types (Tsuda *et al.* 1982; Byerly *et al.* 1984; Veselovsky *et al.* 1985). In our experiments (Veselovsky *et al.* 1985) it has been shown that such a component may reflect the switching-off of a non-specific outward current probably carried by protons.

The mean open time of LTI channels depends on the type of permeant cation: it is about twice as short when calcium ions are replaced by barium. On the contrary, the substitution of permeant cations does not seem to influence high-threshold channel deactivation kinetics. This difference may also be due to the fact that the mean open time of these channels appears to be extremely short. Even if the type of penetrating cation affects it, this influence cannot be reliably resolved.

Our data confirmed that dihydropyridines have a low efficacy when acting upon calcium channels in the neuronal membrane (Kostyuk, Mironov & Shuba, 1983; Nishi *et al.* 1983). At low membrane potentials some of them act not as antagonists, but as weak channel agonists similar to Bay K 8644. The latter substance, as has already been shown by Tsien and co-workers (Nowycky *et al.* 1985*a*, *b*), has a detectable action only on high-threshold calcium channels. However, a definite answer to the question of which type of high-threshold calcium channels is specifically sensitive to this agonist is not yet possible. On the basis of single-channel recordings Tsien and co-workers proposed a model according to which Bay K 8644 affects high-threshold non-inactivating channels (L-type), increasing the probability of a particular mode in their gating behaviour (long-lasting open states) being linked to drastic changes in current–voltage relations.

On the other hand, a close similarity between the kinetics of the slow tail-current

component induced by Bay K 8644 and the slow tail-current component characteristic of the inactivating high-threshold calcium channels operating in Tris aspartate-perfused cells may indicate that the targets for the effect of Bay K 8644 are the inactivated HTI channels which may be returned to activity by this drug. Obviously, this problem needs further experimental analysis.

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