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CALCIUM CURRENTS IN AGED RAT DORSAL ROOT GANGLION NEURONES

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SUMMARY

1. The whole-cell voltage clamp technique was used to record calcium currents in the somatic membrane of rat cultured dorsal root ganglion neurones.

2. Neurones were enzymatically isolated from animals of three age groups (neonatal, 2–7 days; adult, 7 months; and old, 30 months) and maintained in primary culture 3–14 days.

3. The neurones isolated from neonatal and old rats showed two distinct types of Ca^{2+} currents, a low-threshold transient current and a high-threshold sustained current, whereas neurones from old rats showed only a high-threshold calcium current.

4. The density of the high-threshold calcium current was $28{\cdot}4 \pm 6{\cdot}3$ pA/pF (mean $\pm$ S.E.M., $n = 54$) in neonatal, $39{\cdot}1 \pm 7{\cdot}2$ pA/pF ($n = 62$) in adult and $11{\cdot}0 \pm 4{\cdot}6$ pA/pF ($n = 64$) in old dorsal root ganglion neurones.

5. We found no difference in elementary high-threshold Ca^{2+} current characteristics in neurones from different age groups. The single-channel conductance was (with 60 mM Ca^{2+} in the recording pipette) $16{\cdot}0 \pm 2{\cdot}7$ pS (mean $\pm$ S.E.M., $n = 9$) in neonatal, $16{\cdot}2 \pm 1{\cdot}7$ pS ($n = 11$) in adult and $16{\cdot}4 \pm 1{\cdot}2$ pS ($n = 12$) in old neurones.

6. Current–voltage relations and kinetics of high-threshold calcium currents showed no detectable age-dependent difference.

7. The run-down of high-threshold calcium currents in dorsal root ganglion neurones from old rats was practically insensitive to intracellular administration of cyclic AMP and ATP. The same intervention caused a significant deceleration of Ca^{2+} current run-down in the majority of neonatal and in some adult cells.

8. We suggest that the disappearance of the low-threshold calcium current and reduction of high-threshold calcium current with ageing is due to a depression of calcium channel expression during late ontogenesis. The decrease of sensitivity of high-threshold calcium channels to phosphorylation by cyclic AMP-dependent protein kinase in aged neurones could also be a reason for altered turnover between silent and functional pools of calcium channels, which may underlie the age-dependent decline in the density of high-threshold calcium channels.

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INTRODUCTION

The process of ageing is associated with significant changes in the excitable properties of neuronal cells (Frolkis, Stupina, Martynenko, Toth & Timchenko, 1984; Morales, Boxer, Fung & Chase, 1987; Scott, Leu & Cinader, 1988). These changes should be aligned to alteration of expression of ion channel molecules, changes in channel functioning, or a combination of both. Alterations in calcium channel function may be of special importance in this respect. Calcium channels are philogenetically one of the oldest ion channels, and they have been described in most excitable and non-excitable tissues (see for review Tsien, 1983; Kostyuk, 1986; Miller, 1987; Morad, Nayler, Kazda & Schramm, 1988). Transmembrane calcium flux is essential for maintaining the cytoplasmic free Ca^{2+} concentration. Disturbances in calcium homeostasis may produce abnormalities in numerous calcium-dependent processes, such as various enzymatic reactions, release of neurotransmitters, and neuronal excitability.

There are some data indicating the alteration of calcium homeostasis controlling systems including transmembrane calcium fluxes with ageing (Fifkova & Cullen-Dockstader, 1986; Gibson & Peterson, 1987; Martynez, Vitorica, Bogonez & Satrustegui, 1987). However, in most experiments on the movement of calcium ions across the neuronal membrane $^{45}Ca^{2+}$ uptake by synaptosomes was measured (Martinez, Vitorica & Satrustegui, 1988; Peterson, Ratan, Shelanski & Goldman, 1989). Direct measurements of calcium currents in aged neurones are quite controversial; some authors described a decrease of calcium currents in senile hippocampal neurones (Reynolds & Carlen, 1989) while others observed an increase of this current in hippocampal (Landfield, Campbell, Hao & Kerr, 1989; Pitler & Landfield, 1990) and molluscan neurones (Frolkis, Martynenko & Timchenko, 1991).

In this study we attempted to characterize in more detail calcium currents in cultured sensory neurones isolated from dorsal root ganglion (DRG) of rats of different age groups.

METHODS

Culture of DRG neurones

Unidentified neurones from the lumbar dorsal root ganglia of rats of three age groups (neonatal, 2–7 days; adult, 7 months and old, 30 months) were used for preparing a primary culture.

Rats were killed by decapitation and dorsal root ganglia were quickly removed and placed in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MA, USA). After wash-out of blood they were transferred into a DMEM-based isolation medium supplemented by enzymes: 0·1% protease, Type XIV, (Sigma, USA) for neonatal rats, 0·25% protease and 0·2% collagenase (Fluka, Switzerland) for adult, and 0·3% protease and 0·4% collagenase for old animals. Ganglia were incubated in isolation medium at 35 °C for 15–20 min for the neonatal, and 50–55 min for adult and old age groups. After the end of enzymatic treatment they were washed and pipetted into DMEM. The cell suspension was plated on flame- and ultraviolet-presterilized glass coverslips or on sterile plastic Petri dishes (Nunk, Denmark). After 1·5–2 h incubation of cell suspensions in a 5% CO_2 + 95% air environment at 35 °C, dishes were filled with 2 ml of DMEM medium enriched with 10% embryonic calf serum (Vector, Ukraine). Then cultures were kept at 35 °C in a humidified atmosphere of 5% CO_2 + 95% air. From the second day in culture, enough single cells adhered to the coverslips so that recordings could be performed.

Electrophysiological techniques and data analysis

Membrane currents in DRG neurones were studied using the patch clamp technique in the whole-cell or cell-attached configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). A plastic

dish containing cultured cells in 1 ml of extracellular solution was mounted on the stage of an inverted phase contrast microscope. Recordings were performed at room temperature. For patch clamp measurements we used patch pipettes pulled from borosilicate glass (outer diameter 1·7 mm, wall thickness 0·3 mm, Hilgenberg, Germany) using the conventional two-step procedure. Pipettes were coated with Sylgard to reduce additional capacitance and pipette tips were heat polished. These pipettes had a tip diameter of 1·5–2 μm and a resistance between 4 and 6 MΩ when filled with intracellular solution. For cell-attached recordings, pipettes were filled with the high-calcium balanced salt solution (see below). Current and voltage signals were amplified with conventional electronics (EPC-7 amplifier, List Electronics, Darmstadt, Germany), filtered at 1–3 kHz (8-pole Bessel, −3 dB) and sampled at 2–10 kHz by a 12 bit, 125 kHz DMA Labmaster interface (Axon Instruments, CA, USA) connected to an AT-compatible computer system. The acquisition and data analysis were controlled by a pClamp software, version 5.5.1 (Axon Instruments, CA, USA). The computer system also served as a stimulus generator. All experiments were done with capacitance and series resistance compensation to improve the voltage clamp conditions. Extracellular solutions were changed by pressure ejection from an adjacent extracellular micropipette.

As a rule, at the beginning of the experiment, immediately after establishing a whole-cell recording configuration and before the cell was dialysed, the resting membrane potential (RP) of the neurone was determined in a current clamp mode. If the resting potential was more positive than −30 mV the cell was discarded.

Measurement of calcium currents (I_{Ca})

Exitability of rat dorsal root ganglion neurones is determined by the generation of several inward and outward currents (Kostyuk, Veselovsky & Fedulova, 1981*a*; Kostyuk, Veselovsky, Fedulova & Tsyndrenko, 1981*b*; Kostyuk, Veselovsky & Tsyndrenko, 1981*c*). The compositions of the intracellular solutions (see below) were designed to exclude outward potassium currents. After forming a whole-cell recording configuration and wash-out of potassium ions (monitored by disappearance of outward potassium current which was usually completed within 1–2 min) a complex inward current was observed when the cell was bathed in a normal Tyrode solution. This current consisted of an initial fast peak corresponding to the fast sodium current followed by a prominent slower component. Addition of 0·1 μM tetrodotoxin (Calbiochem, Switzerland) to the external solution produced almost a complete block of the fast sodium current, while only the slower component remained. The latter was a result of superposition of slow sodium and calcium currents. To remove the slow sodium current, external Tyrode solution was replaced by a sodium-free solution; after this procedure the calcium inward current could be measured in a pure form.

Solutions

All solutions were freshly prepared from refrigerated stock solutions, and filtered through 0·22 μm filters (Schleicher & Schuell, Germany) before use. Basic Tyrode solution contained (mM): NaCl, 140; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 1·1; Hepes–NaOH, 10; pH, 7·4. The sodium-free extracellular solution contained (mM): $CaCl_2$, 1·8; $MgCl_2$, 2; *N*-methyl-D-glucamine chloride or choline chloride, 130; TEA-Cl, 10; Hepes–TrisOH, 10; pH, 7·4. The pipette solution dialysing the cell interior contained (mM): caesium aspartate, 60; CsCl, 60; $MgCl_2$, 4; Hepes–CsOH, 10; EGTA, 10; pH, 7·2. For some recordings we used a pipette solution which was optimized to prevent the run-down of Ca^{2+} currents; it contained in addition cyclic AMP (10^{-5} M), Na_2ATP (3 mM), and $MgCl_2$ was increased to 5 mM. For monitoring of resting and action potentials in cultured DRG neurones patch pipettes were filled with potassium intracellular solution (mM): KCl, 140; $MgCl_2$, 4; EGTA, 10; Hepes–KOH, 10; pH, 7·2.

For experiments in cell-attached patch clamp configurations the pipette solution was as follows (mM): $CaCl_2$, 60; $MgCl_2$, 1·1; TEA-Cl, 50; Hepes–CsOH, 10, pH, 7·2. During cell-attached experiments cells were bathed in hyperpotassium solution for zeroing the transmembrane potential (mM): KCl_2, 140; $CaCl_2$, 1·8; $MgCl_2$, 1·1; Hepes–KOH, 10, pH, 7·2. All reagents were from Sigma Chemical Co. (MO, USA).

RESULTS

Size and membrane capacity of investigated cells

Immediately after isolation all neurones were almost spherical in shape. During the first two days in culture they adhered to the coverslips and started to develop

neuritis, but the shape of the soma remained spherical. The mean diameter of neurones was $24{\cdot}5 \pm 12{\cdot}7$ μm (mean $\pm$ S.D., $n = 56$), $53{\cdot}8 \pm 16{\cdot}1$ μm ($n = 69$) and $58{\cdot}4 \pm 17{\cdot}9$ μm ($n = 66$) for the first, second and third age groups respectively. The distribution of the somatic diameters for these groups is shown in Fig. 1.

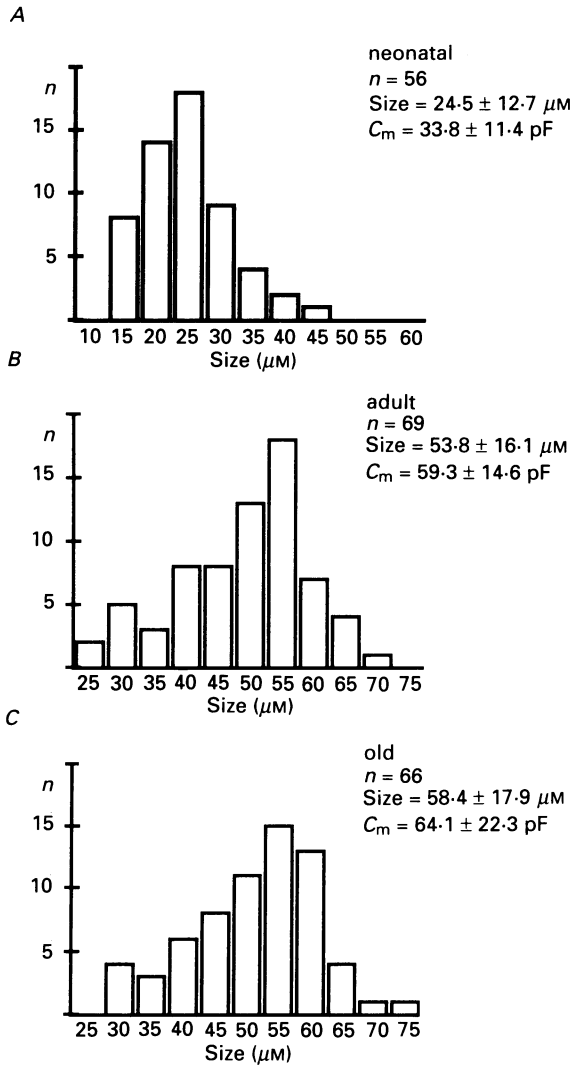

Fig. 1. Distribution of the diameters of cultured dorsal root ganglion neurones obtained from neonatal (*A*), adult (*B*) and old (*C*) rats. The mean values for cell diameter and corresponding membrane capacitance are shown in the graphs.

The membrane capacitance (C_m) of investigated neurones was calculated from the integral (corresponding to the moved charge) of the transient capacitative currents. The capacitative currents were measured by depolarizing the cell membrane from -100 to -90 mV without using the feedback circuit for series resistance

compensation and electronic capacitance compensation. The mean capacitance was 33·8±11·4 pF for neonatal neurones, 59·3±14·6 pF for adult, and 64·1±22·3 pF for aged cells. The distribution of cell capacitance was very similar to the distribution of the diameters of the neuronal somata.

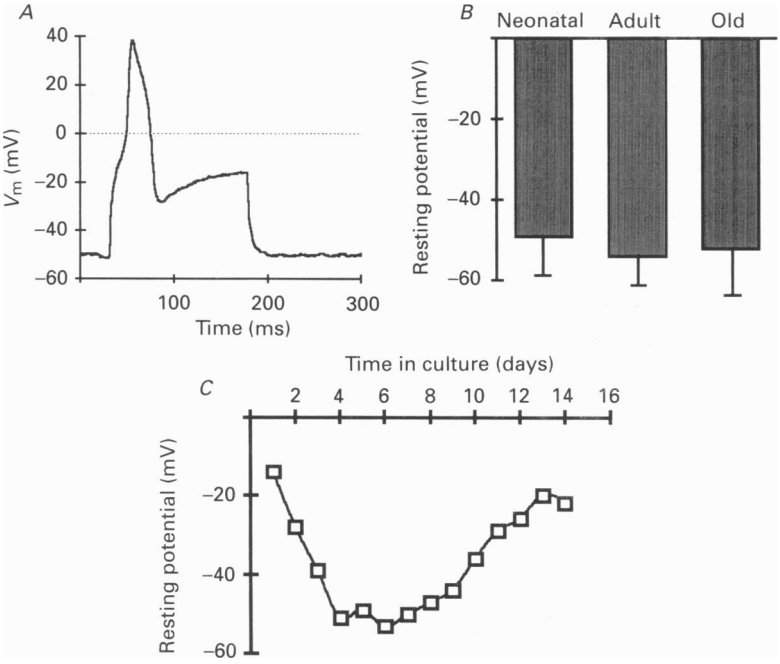

Fig. 2. General electrophysiological properties of cultured DRG neurones. *A*, action potential recorded in response to current injection from DRG neurone isolated from 30-month-old rat. *B*, average values of resting potential of DRG neurones from different age groups. Average data were collected from measurements performed between days 4 and 9 of cell culture. *C*, variations in resting potential values during culture of DRG neurones. Values represent the means of seven experiments. Measurements were performed in neurones isolated from 30-month-old rats.

Resting and action potential of DRG neurones

Resting membrane potential (RP) and action potential (AP) of DRG neurones were measured using current clamp mode in whole-cell patch clamp configuration. We found no significant changes in the resting potential value measured between days 4 and 9 in culture in different age groups (Fig. 2*B*). Resting potential was −47·4±9·3 mV (mean±S.E.M., $n = 43$), −52·1±8·7 mV ($n = 49$) and −50·7±11·2 mV ($n = 51$) for the first, second and third age groups, respectively. It has to be noted that the RP level changed during the culturing procedure (Fig. 2*C*): the resting potential became significantly more negative during the first three days after cell isolation, probably reflecting cell adaptation to the culture conditions. It then become stable, and after 7–9 days in culture started to become more positive again.

No substantial differences in the parameters of APs were observed in the three age

groups: in all groups the AP demonstrated a high upstroke velocity and a prominent overshoot (30–40 mV; Fig. 2*A*). Such AP parameters were seen consistently in cells maintained in culture for 4–9 days. Based on these data, we concluded that this was the best time for performing voltage clamp experiments in culture in all age groups, and all experiments described below were performed on such neurones.

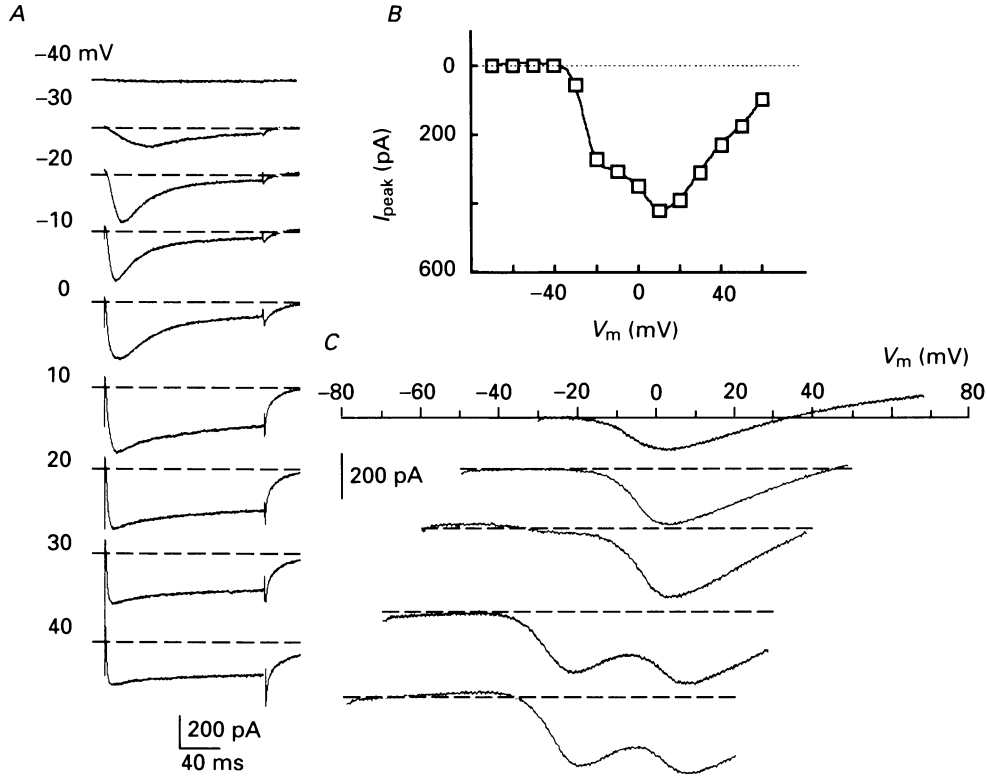

Fig. 3. Low- and high-threshold calcium currents in dorsal root ganglion neurone isolated from neonatal rat. *A*, representative family of calcium current records. Holding potential −80 mV. Voltage clamp pulses of 200 ms duration to the potentials indicated near current traces. Note clearly distinguished transient and sustained current components. *B*, peak current (I_{peak}) to voltage (V_m) relationship for the cell illustrated in *A*. *C*, family of current records in response to the ramp stimulation (amplitude 100 mV, duration 100 ms) from holding potentials between −80 and −30 mV. Note the disappearance of the first hump in the ramp I–V curves at more positive holding potentials.

Lack of the low-threshold calcium current in old neurones

The co-existence of multiple types of Ca^{2+}-selective channels has been shown in DRG neurones (Bean, 1989; Carbone & Swandulla, 1989; Kostyuk, 1989; Hess, 1990). We examined the types of calcium currents present in cultured dorsal root ganglion neurones from different age groups using the conventional voltage clamp protocol for the separation of high-threshold (I_{HT}) and low-threshold (I_{LT}) calcium current components.

The main finding was an age-dependent disappearance of the low-threshold calcium current: neurones from neonatal and adult animals possessed both I_{LT} and I_{HT}, while neurones from old rats lacked the I_{LT}. This is illustrated in Figs 3–5. Figure 3 shows calcium currents measured from neonatal DRG neurones in response to

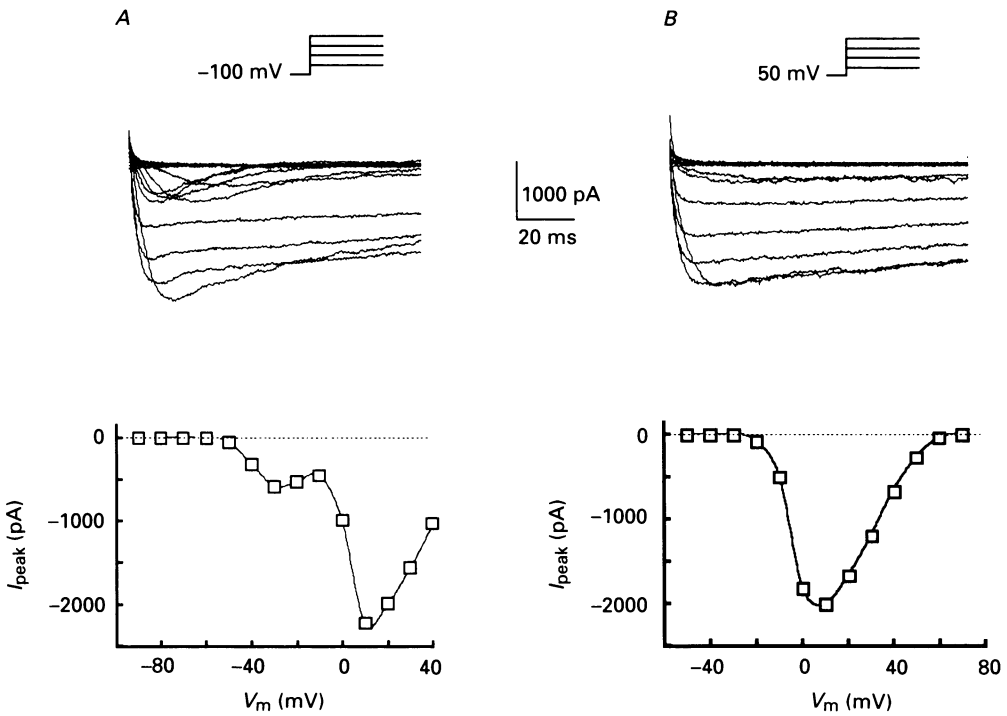

Fig. 4. Separation of low- and high-threshold calcium currents in the dorsal root ganglion neurone obtained from adult rat. *A*, family of calcium currents recorded at the holding potential −100 mV (top) and corresponding peak current (I_{peak}) *versus* voltage (V_m) curve. *B*, calcium currents (top) and corresponding peak current (I_{peak}) *versus* voltage (V_m) curve recorded from the same cell at holding potential −50 mV.

increasing step depolarizations from a holding potential of −80 mV together with the corresponding current–voltage curves. They evidently show the presence of two calcium current components with different kinetics and voltage dependence. The low-threshold component activated at membrane potentials more positive than −40 mV and reached a maximum at −20 mV. This current had relatively fast inactivation kinetics and was strongly dependent on the holding potential (V_h); shifting V_h to values positive to −50 mV led to its complete disappearance. Such an effect is clearly shown in Fig. 3*C* which represents a family of whole-cell currents in response to ramp depolarization from progressively decreasing holding potentials. The disappearance of the first hump on the ramp response indicates the depression of I_{LT} due to steady-state inactivation. The high-threshold component (I_{HT}) of the calcium current, activated with depolarizations to potentials positive to −20 mV,

reached maximal values between 0 and +10 mV. It displayed a much slower inactivation and remained when holding the cell at −50 or −40 mV. About 65% of all neurones examined from neonatal rats possessed both I_{LT} and I_{HT}; the amplitude of I_{LT} reached on average 40% of the amplitude of the high-threshold calcium current. Some neurones expressed only the I_{LT}.

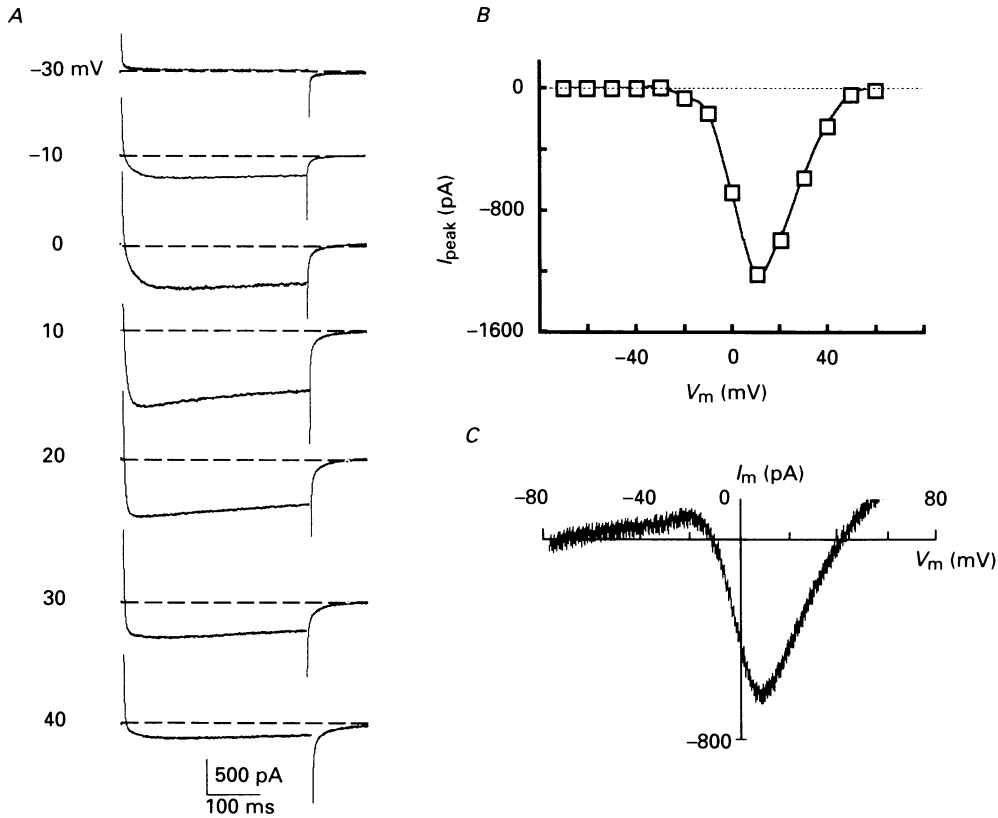

Fig. 5. One component of calcium current in the membrane of a dorsal root ganglion neurone, isolated from a 30-month-old rat. *A*, representative family of calcium current records. Holding potential −80 mV, with 400 ms voltage clamp pulses to the potentials indicated near current traces. Note the existence of only one current component. *B*, peak current (I_{peak}) to voltage (V_m) relationship for the cell illustrated in *A*. *C*, ramp current to voltage curve from the holding potential −80 mV.

In the second age group (7-month-old rats) the expression of I_{LT} channels was lower: only 30% of neurones examined displayed this calcium current component (Fig. 4). However, in this age group it was still possible to find a number of neurones which expressed only the low-threshold calcium current.

Finally, in the group of aged neurones we did not find a single cell (from sixty-six tested) with measurable low-threshold calcium current. Peak current–voltage relations as well as ramp *I–V* curves (Fig. 5) indicated the presence of only a high-threshold calcium current.

From these observations we conclude that a progressive decrease of low-threshold calcium channel expression occurs during ageing which, in the long run, causes a complete disappearance of I_{LT} in aged sensory neurones.

Age-dependent decrease of high-threshold calcium current

To compare kinetic and voltage-dependent characteristics of the high-threshold calcium currents, we chose neurones which expressed only the I_{HT}. The I_{HT} of dorsal

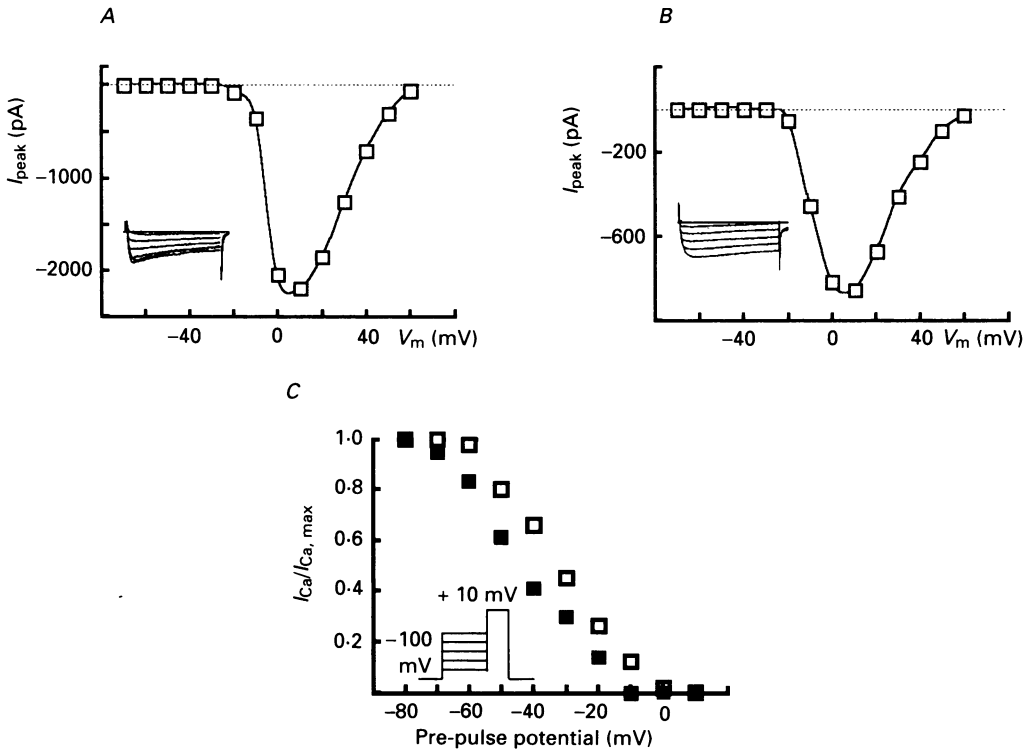

Fig. 6. Voltage-dependent properties of high-threshold calcium current in dorsal root ganglion neurones isolated from adult and old rats. *A*, peak current (I_{peak}) *versus* voltage (V_m) relationship recorded from a DRG neurone obtained from a 7-month-old rat. This neurone displayed only the high-threshold calcium current. Holding potential −80 mV. Inset; corresponding family of calcium current records. *B*, as in *A*, except from a DRG neurone obtained from a 30 month-old rat. *C*, the steady-state inactivation of the high-threshold calcium current, recorded in DRG neurones from adult and old rats. The steady-state inactivation curve was determined using conventional voltage clamp protocol (inset). The mean normalized current ($I_{Ca}/I_{Ca,max}$) was plotted as a function of the holding potential for cells isolated from 7-month-old ($n = 7$, □) and 30-month-old ($n = 9$, ■) rats.

root ganglion neurones was recorded at holding potentials between −80 and −60 mV. The activation threshold for I_{HT} in neurones from all age groups was around −20 mV, and *I–V* curves peaked at about +10 mV. The only difference in voltage-dependent parameters was a 10–15 mV shift of the steady-state inactivation

curve in aged neurones in the hyperpolarizing direction (Fig. 6). No age-dependent differences in kinetics of high-threshold calcium currents were observed (Fig. 7).

However, the density of I_{HT} (determined as a ratio between peak I_{HT} amplitude and C_m in all investigated cells, measured at a holding potential of −50 mV) in aged

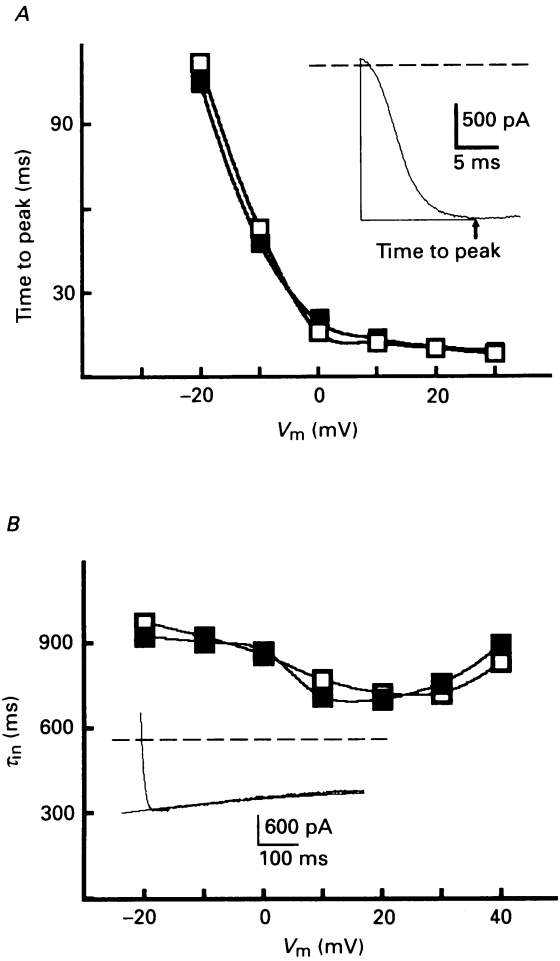

Fig. 7. Kinetic parameters of high-threshold calcium current in dorsal root ganglion neurones isolated from adult and old rats. *A*, time to peak (T_p) determined as shown in inset and plotted against membrane potential (V_m) for neurones obtained from 7-month-old (□) and 30-month-old (■) rats. Values represents the means of eleven experiments. *B*, inactivation time constants (τ_{in}) for high-threshold calcium current measured in neurones of different ages. Time constants were obtained by fitting traces with a mono-exponential function as illustrated for the sample current trace shown in the inset. The inactivation time constant for adult ($n = 6$, □) and old ($n = 9$, ■) rats were plotted as a function of membrane potential (V_m).

DRG neurones was significantly lower: the high-threshold Ca^{2+} current density was 28·4±6·3 pA/pF (mean±S.E.M., $n = 54$) in neonatal, 39·1±7·2 pA/pF ($n = 62$) in adult and only 11·0±4·6 pA/pF ($n = 64$) in aged DRG neurones.

Elementary calcium currents

To check for a possible alteration of calcium channel proteins with ageing we also analysed single events in neurones from different age groups. These experiments showed practically no changes in single high-threshold Ca^{2+} channel characteristics

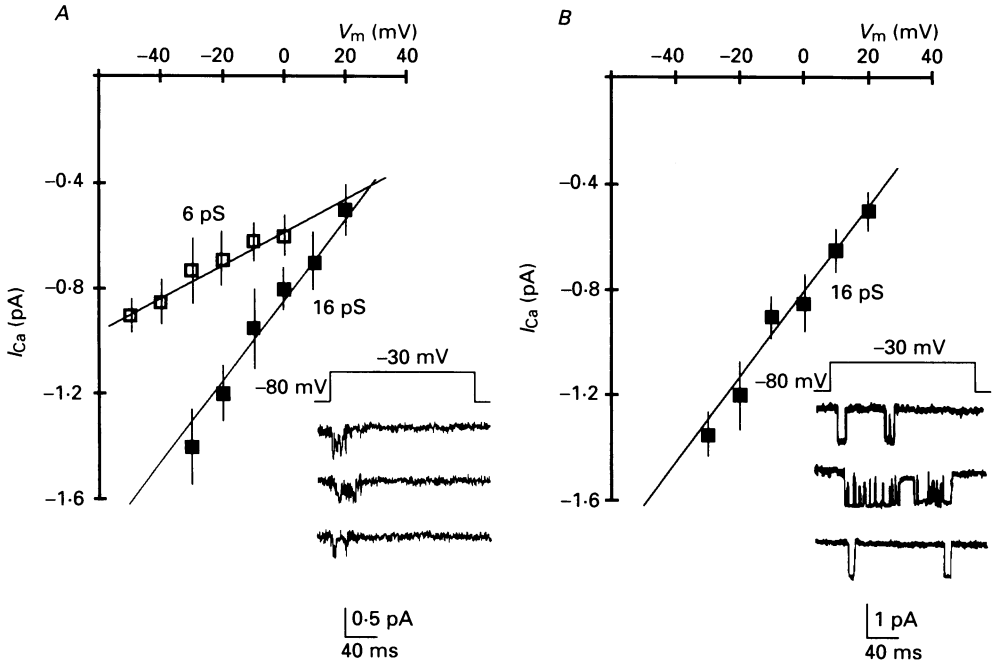

Fig. 8. Conductance of elementary calcium currents in DRG neurones isolated from adult and old rats. Single calcium currents were measured in a cell-attached patch clamp configuration at 60 mM of Ca^{2+} in the recording pipette. The resting potential of neurones was zeroed by cell incubation in hyperpotassium external solution. Unitary current amplitudes (I_{Ca}) were plotted against membrane potential (V_m). *A*, *I–V* curves for low-threshold and high-threshold single-channel currents measured in adult DRG neurones. Continuous lines indicate the slope conductances, which were $6{\cdot}17 \pm 0{\cdot}63$ pA ($n = 9$) for I_{LT} channels and $16{\cdot}2 \pm 1{\cdot}7$ pS ($n = 11$) for I_{HT} channels. Inset; example of activity of the low-threshold single calcium channels, measured in response to step depolarization as indicated. Current records were filtered at 1 kHz. *B*, *I–V* curves for high-threshold single-channel currents measured in old DRG neurones; slope conductance ($16{\cdot}4 \pm 1{\cdot}2$ pS, $n = 12$) is indicated by continuous line. Inset; example of the activity of the high-threshold single calcium channels, measured in response to step depolarization as indicated. Current records were filtered at 1 kHz.

with age (Fig. 8). The I_{HT} single-channel conductance was (with 60 mM Ca^{2+} in the recording pipette) $16{\cdot}0 \pm 2{\cdot}7$ pS ($n = 9$) in neonatal, $16{\cdot}2 \pm 1{\cdot}7$ pS ($n = 11$) in adult and $16{\cdot}4 \pm 1{\cdot}2$ pS ($n = 12$) in old neurones. No significant difference was found in the kinetic properties of unitary high-threshold calcium currents in the different age groups.

Consistent with the data from the whole-cell observations, single-channel

experiments failed to reveal the low-threshold-activated elementary calcium currents in neurones isolated from old animals (in twenty-one patches tested), while it was possible to record regularly the activity of single low-threshold calcium channels in the membrane of dorsal root ganglion neurones obtained from neonatal and adult animals (Fig. 8*A*).

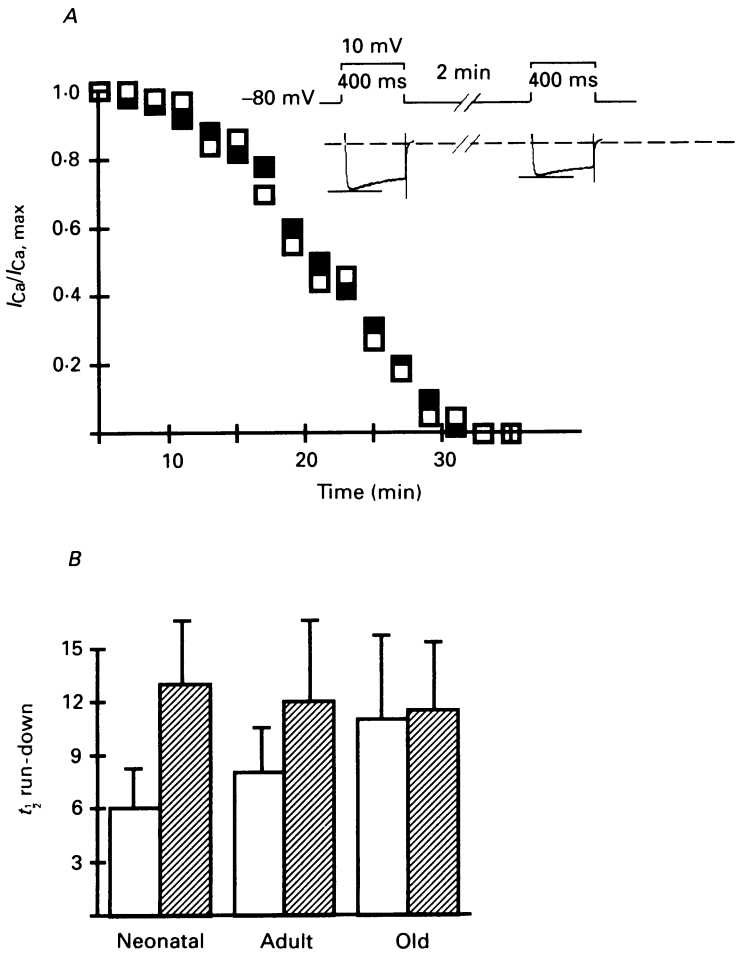

Fig. 9. Run-down of high-threshold calcium current and the effect of cyclic AMP. *A*, run-down of the high-threshold calcium current in DRG neurones, obtained from 30-month-old rats in the absence (□) and presence (■) of cyclic AMP+ATP in intracellular solution. Currents were recorded at 2 min intervals after the establishment of the whole-cell recording configuration. Currents were activated by voltage jumps to +10 mV (corresponding to the maximum of I_{HT} *I–V* curve) from a holding potential of −80 mV (stimulation protocol is shown in the inset). Peak currents were normalized to the current measured after establishment of the whole-cell recording configuration and complete wash-out of potassium currents and plotted as a function of recording time. *B*, dependence of the run-down half-time (time by which a 50% decrease of I_{HT} had occurred) in the presence of cyclic AMP+ATP (▨) in the intracellular solution in DRG neurones isolated from rats of different age groups. Control, □.

I_{Ca} run-down

The functional activity of calcium channels is controlled by a number of metabolic processes in the cytoplasm. The exchange of soluble components between the cytoplasm and the recording pipette during intracellular dialysis leads to the disappearance of this metabolic support of calcium channels and a progressive decrease (run-down) of I_{HT} amplitude (Fedulova, Kostyuk & Veselovsky, 1981; Belles, Malecot, Hescheler & Trautwein, 1988). In various cell types this decrease of calcium current amplitude during intracellular perfusion could be slowed down or even reversed by addition of substances involved in channel-protein phosphorylation to the intracellular solution. We found that metabolic dependence of high-threshold calcium current which is very pronounced in dorsal root ganglion neurones isolated from neonatal rats became insignificant in neurones obtained from old animals. This is shown in Fig. 9, where the run-down of calcium currents measured in the presence or absence of cyclic AMP and ATP together in the intracellular solution are compared. Mean run-down half-time was practically unaffected by cyclic AMP and ATP in aged DRG neurones, while it was clearly slower in neonatal neurones. These findings are in line with the data of Fedulova, Kostyuk & Veselovsky (1991) who also described a loss of the ability of cyclic AMP to restore calcium currents in a significant number of adult neurones as compared with neonatal ones. Obviously, with ageing this effect becomes even more prominent.

DISCUSSION

Down regulation of calcium channel expression during late ontogenesis

The present study shows two important changes in calcium permeability during ageing. The low-threshold calcium current in rat dorsal root ganglion neurones decreases progressively during late ontogenesis, and becomes undetectable in neurones isolated from very old animals. The density of the high-threshold calcium current also decreases with ageing; these two age-linked effects are summarized in Fig. 10.

The first conclusion is based on the following data: (1) the density of I_{LT} was largest in neonatal DRG neurones; in neurones isolated from adult animals this density was reduced almost three times, and in the cells prepared from old rats the low-threshold calcium current was undetectable; (2) similarly, the amount of neurones which possess I_{LT} becomes reduced during ageing from 67% in neonatal rats to 30% in 7-month-old animals and to zero in 30-month-old rats.

Several reasons could account for the disappearance of low-threshold calcium current with ageing: (1) decrease of the number of corresponding channels in cellular membrane due to a slow-down of channel-protein synthesis, less effective incorporation of channel proteins into the neuronal membrane, or acceleration of degradation and removal of the channels from the membrane; (2) inhibition of I_{LT} channel functioning by some substances appearing with ageing; (3) alteration of the low-threshold calcium current due to the cultivation and isolation procedure.

The last possibility seems to be unlikely for the following reasons: (i) the resting potential, which represents the functional activity of various membrane ion-

transporting systems, did not differ significantly in all age groups; (ii) old neurones as well as young and adult showed the ability to generate normal action potentials, which means that the main systems determining the excitability of a neurone are still functioning properly. Hence we can conclude that the functional state of dorsal root

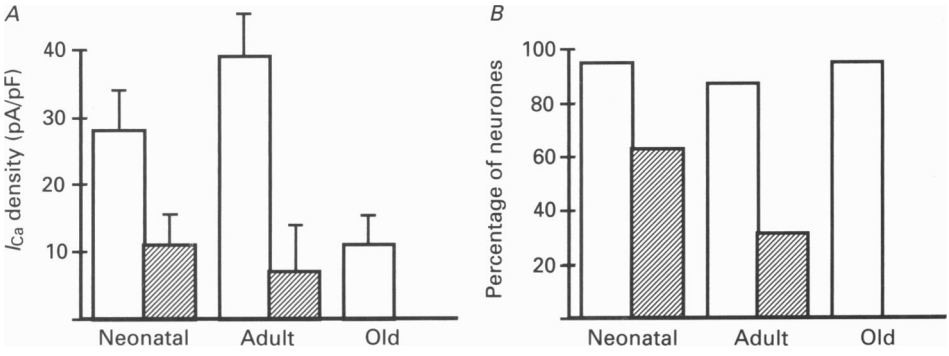

Fig. 10. Density of low- (▨) and high-threshold (□) calcium currents in rat DRG neurones isolated from different age groups (*A*), and percentage of neurones which expressed these components of calcium current (*B*).

ganglion neurones in culture is similar to that found in the native tissue, and that their maintenance in culture does not alter the mechanisms of transmembrane ionic permeations.

The first two possibilities could not be checked directly. We have found no data in the literature about the ion channel turnover in old tissue. Definitely, there are substantial changes in phospholipid constitution of cellular membrane with ageing (Zs.-Nagy, 1982; Malone & Szoke, 1982). Such changes in the composition of the bilayer might significantly alter the functional activity of ionic channels (Burnashev, Undrovinas, Fleidervish, Makielski & Rosenshtraukh, 1991). However, if this is the case we should expect some changes in basic characteristics of other components of the ionic permeability of aged dorsal root ganglion neurones. The absence of any signs of alteration of gating properties in high-threshold calcium channels as well as sodium channels contradicts this suggestion.

Simultaneously with the disappearance of I_{LT} in the membrane of old DRG neurones we found a remarkable decrease in the amplitude and density of high-threshold calcium current. The lowering of I_{HT} density with ageing could be a result of: (1) a decrease of single-channel conductance or alteration of channel voltage dependence and/or kinetics; (2) reduction of the number of functionally active I_{HT} channels due to the down-regulation of channel synthesis or an increase in the pool of 'hibernating' calcium channels.

Single-channel experiments argue against the possibility of an alteration of single calcium channel properties with ageing: channel conductance, as well as voltage dependence and kinetics remained unchanged in all three age groups of neurones.

Therefore, we conclude that a reduction in the number of functionally active calcium channels may underlie the observed decrease of I_{HT} with ageing. All the above listed suggestions explaining the fading of low-threshold channels can also be

applied for the explanation of a decrease of high-threshold channels. However, the situation in the case of high-threshold calcium channels seems to be more complicated in comparison with low-threshold channels. The diminution of the whole-cell calcium current may be due to the elevation of resting intracellular free Ca^{2+} concentration in aged neurones (Landfield, 1989; Kiristchuk, Pronchuk & Verkhratsky, 1992). Elevated cytoplasmic calcium content could induce the development of Ca_i^{2+}-dependent inactivation of calcium channels (Kostyuk & Krishtal, 1977; Eckert & Chad, 1984). Such a mechanism responsible for the decrease of calcium current amplitude was demonstrated on hippocampal neurones of old rats (Reynolds & Carlen, 1989). However, in this case the experiments were done by means of a single electrode voltage clamp without intracellular perfusion. In our case we established an effective intracellular perfusion by solutions containing relatively high concentrations of EGTA. Introducing this buffer into the intracellular medium excludes the influence of Ca_i^{2+}-dependent inactivation. On the other hand the persistently elevated intracellular calcium concentration in aged neurones could serve as a signal which induced the down-regulation of Ca^{2+} channel expression (DeLorme, Rabe & McGee, 1988).

We also found that in old neurones calcium channels lose their sensitivity to cytoplasmic phosphorylation. In neonatal and partly in adult DRG neurones the cyclic AMP-dependent phosphorylation seems to prevent the transition of calcium channels between active and 'silent' forms of calcium channels, while in aged cells this mechanism does not work. However, we could not also exclude age-dependent changes in the activity of cytoplasmic phosphorylation-dephosphorylation processes which may underlie observed phenomena.

At the same time, it is difficult to make any suggestion about the physiological significance of the observed changes in the calcium permeability with ageing. We found no age-dependent changes in the parameters of the action potentials of DRG neurones; this means that age-dependent down-regulation of calcium channels probably did not affect the cellular electrogenesis, but we could suggest that their role is in the realization of the intracellular messenger function of Ca^{2+} ions. Naturally, this problem needs further investigations.

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