

**REGULATION OF THE INTRACELLULAR Ca^{2+}
CONCENTRATION IN NEURONES OF THE COCHLEAR
NUCLEUS IN RAT**

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INTRODUCTION

Function and structure of the cochlear nucleus

The primary sensory neurones, the bipolar cells of the spiral ganglion carry auditory information as trains of action potentials from the cochlear hair cells towards upper auditory centers, including cochlear nuclei (CN).

According to research of the latter years it seems to be evident that the CN is not only a relay station in the auditory pathway, but processing of auditory information also begins in this nucleus. Trains of action potentials carried by acoustic nerve here are distributed to different parallel ascending pathways. This distribution of acoustic information is the consequence of the fact that the various secondary sensory neurones project to different brain structures.

CN can be divided into two parts. The dorsal cochlear nucleus (DCN) has important role in spatial localisation of the sound source and in processing temporal characteristics of the acoustical information. The most frequently investigated neurone type in the DCN is the pyramidal cell, while further projection neurones here are the giant and the Pukinje-like cells. The ventral cochlear nucleus (VCN) can be divided into two further parts, namely to an anterior (AVCN) and a posterior (PVCN) part. The AVCN has relay function, the PVCN is responsible for transmitting auditory information and preserving its temporal characteristics in a faithful fashion. While spherical bushy neurones are the most important projection neurones of AVCN, the globular bushy neurones are located in the PVCN together with the octopus cells.

Role and regulation of cytoplasmic Ca^{2+} in neurones

It is evident since long time that increase of intracellular Ca^{2+} is crucial for the activation of numerous cell functions (e.g. muscle contraction, exocrine and endocrine secretion, mitosis). It is also well known that Ca^{2+} channels of the cell membrane can contribute to the electrical properties of excitable cells and Ca^{2+} entering the cells via voltage-activated Ca^{2+} channels can generate action potentials as well.

The increased intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) can modify the gating parameters of certain ionic channels of the surface membrane in neurones (eg. Ca^{2+} -gated K^+ és Cl^- channels), influencing membrane properties, thus, changing excitability of the cell. Increasing intracellular Ca^{2+} concentration has a fundamental role in neurotransmission.

Moreover, the activity of certain intracellular enzymes can depend on the presence of Ca^{2+} (eg. protein kinase C), and the Ca^{2+} -dependent phenomenon termed „Long-Term Potentiation” (LTP) may provide an explanation of the learning process at a cellular level.

Besides the rapid increase of intracellular Ca^{2+} concentration, it is also important that this elevated Ca^{2+} level could return to the resting level in a short time inducing this way the development of Ca^{2+} transients. One source of Ca^{2+} appearing in the cytoplasm is the extracellular space, from where the Ca^{2+} can enter via voltage-gated Ca^{2+} channels activated by depolarization. Moreover, some of the ligand-gated ionotropic receptors located in the postsynaptic membrane of synapses also show Ca^{2+} permeability. Among these channels the glutamate receptors are the most important, but it is worth to mention the nicotinic acetylcholine channels as well. Ca^{2+} release from intracellular stores may also be important although it has variable contribution depending on neurone type.

A common speciality of the regulation processes of the organism is that after the development of the desired effect, the level of the regulating parameter returns to its resting level. This fact has special importance in the case of Ca^{2+} -dependent processes of neurones because these cells can develop cytotoxic degenerative processes due to extremely high or long lasting intracellular Ca^{2+} concentration. It is not surprising therefore, that neurones possess very powerful mechanisms for decreasing intracellular Ca^{2+} concentration.

Ca^{2+} entering the cytoplasm can be temporarily buffered by calcium-binding proteins. The importance of these proteins is confirmed by the fact, that on increasing their concentrations, the cell produce smaller intracellular Ca^{2+} concentration changes. Ca^{2+} uptake to intracellular stores takes place by SERCA (sarco-endoplasmic reticular Ca^{2+}) pump of the endoplasmic reticulum. In the case of bigger Ca^{2+} loads, mitochondria have important roles in Ca^{2+} accumulation. The most evident way for decreasing intracellular Ca^{2+} is, however, its transport to the extracellular space. The Ca^{2+} pump (PMCA) and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger mechanism of neuronal membrane play important roles in this transport.

Aim of the present theses

The main aim of the study reported in the theses was the analysis of the Ca^{2+} homeostasis in neurones of the rat cochlear nucleus, mostly in pyramidal cells. Our questions targeted at three main topics.

1. Characterisation of Ca^{2+} transients developing in the presence of glutamate on pyramidal neurones isolated enzymatically from the dorsal cochlear nucleus. Role of

NMDA- and AMPA-type glutamatergic receptors as well as voltage-gated Ca^{2+} channels in generating Ca^{2+} transients. Comparison of the transients evoked by K^+ and glutamate.

2. Investigation of the presence and distribution of Ca^{2+} -buffering proteins (calbindin [CB], calretinin [CR] és parvalbumin [PA]) in the projection neurones of the CN using immunohistochemical methods on free floating slices. Examination of protein colocalisation with double immunolabeling. Retrograde labeling of neurones for the identification of cell types.

3. Studying the role of cytoplasmic Ca^{2+} removal mechanisms on isolated pyramidal neurones. Verifying the importance of the PMCA, $\text{Na}^+/\text{Ca}^{2+}$ -exchanger and SERCA pump in decreasing cytoplasmic Ca^{2+} concentration using their specific blockers.

MATERIALS AND METHODS

Measurement of the intracellular Ca^{2+} concentration

Pyramidal cells were enzymatically isolated (with collagenase and pronase) from cochlear nuclei of 6-11-days old rats. Cell suspension of dissociated neurones was dropped on a coverslip and incubated for 3-4 hours in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 % horse serum. Cells were filled with 3 $\mu\text{mol/l}$ Fura-2-AM Ca^{2+} -indicator dye.

Recording of $[\text{Ca}^{2+}]_i$ took place in a Deltascan-1 system (Photon Technology International, New Brunswick, USA). The cell filled with Fura-2 dye was alternately exposed to excitation light beams of 340 and 380 nm wavelength, respectively. Intensity of the emitted light was measured at 510 nm, the digitisation rate was 50 Hz. The ratios of light intensities obtained with 340 és 380 nm excitations were calculated by the OSCAR software (Photon Technology International, New Brunswick, USA).

During the analysis of the records only those cells were considered, whose intracellular Ca^{2+} -concentration was smaller than 30 nmol/l. In the course of the experiments the cells were continuously perfused with control solution by using a microperfusion system. The system was utilized also for applying solutions containing blockers, as it provided a fast solution change. The experiments were performed at room temperature.

Immunohistochemistry

30-days-old rats were used for these experiments. After fixation of the brainstem in 4% paraformaldehyde, 60 μm thick sagittal slices were made from the cochlear nuclei. Aspecific binding sites were blocked by 10 % normal horse serum, then the slices were incubated with primary antibodies. Double labeling was performed for the investigation of buffer protein colocalisation. Antibodies conjugated with fluorescent dye were used as secondary antibodies. At the end of the procedure, the slices were stained with DAPI for visualizing nuclei. During our investigations, control experiments were also conducted for the confirmation of the specificity of the primary antibodies (preadsorption, experiments without the primary antibody).

Retrograde labeling

For retrograde labeling of the projection neurones, tetramethylrhodamine-conjugated dextrane was used (molecular weight = 3000). As the axons of the projection neurones are located in the superficial layers of the brainstem, they can be easily cut by making a superficial incision with a thin needle. The dye was injected into these incisions by the same needle.

For permitting retrograde transport through axons of the labeled cells, the brainstem was kept alive at least 8 hours long. Following this period the preparation was fixed in 4% paraformaldehyde, and 50 μm thick sagittal slices were prepared from the cochlear nuclei in the side of retrograde labelling. The slices were stained with DAPI at the end of the procedure

Microscopy

Visualization of the retrograde labeling took place with a fluorescent microscope (Eclipse 600W; Nikon, Tokyo, Japan), while the immunoreactions were visualized with both fluorescent and confocal microscopy (LSM 510; Zeiss, Jena, Germany). Images were taken and analysed with Spot RT v3.5, Zeiss LSM Image Browser and Adobe Photoshop 7.0 softwares. When performing confocal microscopy, several images taken at different horizontal planes from the same cell were merged.

RESULTS

1. Characterisation of the glutamate-induced Ca^{2+} transients in pyramidal cells

1.1. Roles of AMPA- and NMDA-receptors in generating Ca^{2+} transients

During the quantitative analysis of the glutamate-induced Ca^{2+} transients, determination of the dose-response curve of extracellular glutamate was carried out first. In the further experiments, the saturating concentration of glutamate (5 mmol/l) was applied for at least 5 s. This concentration of glutamate generated Ca^{2+} transients with amplitudes between 22.7 és 77.6 nmol/l with an average of 46.1 ± 3.0 nmol/l ($n = 66$). For comparison of different types of stimulation, both high extracellular K^+ concentration and glutamate were used for evoking Ca^{2+} transients in a certain number of neurones. In these cells the average amplitude of transients evoked by 50 mmol/l K^+ was 217.7 ± 20.2 nmol/l, while 5 mmol/l glutamate generated transients with 60.8 ± 6.8 nmol/l peak value ($n = 9$).

The role of different ionotropic glutamate receptors in evoking Ca^{2+} transients was investigated with adding blockers specific for the individual receptor type. CNQX (100 $\mu\text{mol/l}$) specific for the AMPA-receptors decreased the amplitude of the transients evoked by 5 mmol/l glutamate from 33.0 ± 4.9 nmol/l to 2.7 ± 0.9 nmol/l ($n = 13$). The role of NMDA receptors was investigated by the application of 100 $\mu\text{mol/l}$ AP5 that decreased the Ca^{2+} transient from the control amplitude of 52.4 ± 4.2 nmol/l to 48.0 ± 3.4 nmol/l. The difference was not statistically significant ($n = 7$).

As CNQX, used for the inhibition of AMPA-receptors, can also block NMDA-receptors, the effect of a more specific AMPA antagonist, NBQX was also investigated (10 $\mu\text{mol/l}$). After pretreatment with 100 $\mu\text{mol/l}$ AP5 and 200 $\mu\text{mol/l}$ Cd^{2+} , the amplitude of the glutamate-induced transients was 25 ± 4.8 nmol/l and NBQX decreased this value to 12.1 ± 3 nmol/l. The decrease was significant and the effect of the drug was reversible ($n = 6$).

1.2. Activation of the NMDA-type ionotropic glutamate receptors

Because AP5 did not influence the glutamate-induced Ca^{2+} transients, we could not exclude the possibility that isolated neurones lacked functional NMDA-receptors. To investigate the question, the NMDA-receptors were stimulated with various methods and the resulted changes in the Ca^{2+} transients were analysed.

Compared to the amplitude of control Ca^{2+} transients (44.5 ± 14.9 nmol/l), the glutamate transients evoked in the presence of 50 $\mu\text{mol/l}$ glycine and 20 $\mu\text{mol/l}$ Mg^{2+} showed significantly increased peak values (91.9 ± 15.3 nmol/l) but on applying control solution again, this parameter returned to its original level (41.6 ± 19.7 nmol/l).

In some of the experiments, NMDA-receptors were stimulated after the blockade of AMPA-receptors. The amplitude of Ca^{2+} transients evoked with 5 mmol/l glutamate was 38 ± 7.8 nmol/l under control conditions. 100 $\mu\text{mol/l}$ CNQX almost completely eliminated the transients (average amplitude was 1.8 ± 0.5 nmol/l). Following this, we applied 50 $\mu\text{mol/l}$ glycine in the presence of CNQX, and as a consequence, the amplitude of the Ca^{2+} transients increased (16.8 ± 3.8 nmol/l). Finally, we decreased Mg^{2+} concentration of the incubating solution to 20 $\mu\text{mol/l}$ in the presence of the previously applied chemicals and the amplitude of the transients increased further (55.4 ± 13.1 nmol/l).

1.3. Involvement of AMPA-receptors in evoking cytoplasmic Ca^{2+} concentration changes

Our experiments indicated the extracellular origin of Ca^{2+} necessary for the generation of glutamate-induced Ca^{2+} transients. A strong decrease of the amplitude of the Ca^{2+} transients in the presence of lowered extracellular Ca^{2+} concentration provided evidence for this statement.

Some experiments gave information also about the role of Ca^{2+} release from intracellular stores. After the application of glutamate, a Ca^{2+} transient was evoked with 50 mmol/l K^+ -depolarization to load the intracellular Ca^{2+} stores, then the application of the glutamate was repeated. Glutamate-induced Ca^{2+} transients before and after K^+ -depolarization had the same peak values that indicates a minimal role of intracellular Ca^{2+} -stores in generating these transients.

Ca^{2+} entry necessary for evoking AMPA-receptor-dependent Ca^{2+} transients can take place through different pathways. The role of voltage-gated Ca^{2+} channels was investigated with decreasing the extent of depolarization or with blocking the voltage-gated Ca^{2+} channels. In the course of these measurements possible activation of NMDA-receptors was prevented by the application of 100 $\mu\text{mol/l}$ AP5.

On decreasing extracellular Na^+ -level to 10 mmol/l, no significant difference was found in the amplitude of Ca^{2+} transients evoked by 5 mmol/l glutamate: the average value

under control conditions was 55.6 ± 10.2 , while in low Na^+ solution it was 68.8 ± 14.7 nmol/l ($n = 5$).

During the blockade of the voltage-gated Ca^{2+} channels with Cd^{2+} , the amplitude of the transients decreased from 52.8 ± 5.8 nmol/l to 27.7 ± 6.7 nmol/l ($n = 11$). In most of the cases this effect was reversible. Besides decreasing amplitude, the rising rate of the transients was also decreased in cases.

As the possibility of Ca^{2+} entry through NMDA-receptors is low without stimulation of the receptors, and because the voltage-gated Ca^{2+} channels are responsible for 50% of the Ca^{2+} transients, only the AMPA-receptors might serve as the pathway of the further Ca^{2+} entry. To confirm the validity of this hypothesis, Ca^{2+} transients were evoked with 5 mmol/l glutamate in the presence of 100 $\mu\text{mol/l}$ AP5 and 200 $\mu\text{mol/l}$ Cd^{2+} . The amplitude of the transients was only 19.4 ± 2 nmol/l as a consequence of Cd^{2+} inhibition of voltage-gated Ca^{2+} channels. When the transient reached its maximal value, the Ca^{2+} concentration of the extracellular solution was decreased to 10 $\mu\text{mol/l}$ in the presence of glutamate. Despite of the continuous glutamate action, the Ca^{2+} concentration rapidly decreased and after 20-25 s, it reached a value of 5.2 ± 1.1 nmol/l. After restoring the control extracellular Ca^{2+} concentration, glutamate was able again to induce transients with similar amplitude (24.3 ± 3.5 nmol/l) as prior to the Ca^{2+} -withdrawal.

2. Presence and distribution of calcium binding proteins in the CN projection neurones

2.1. Calcium-binding proteins in the DCN

In the case of pyramidal cells, we applied CB/PA and CR/PA double immunolabellings for studying the calcium binding protein expression. The cells exhibited intensive and homogenous PA and CB labelling but the presence of CR could not be shown.

On retrograde labelling of the pyramidal cells, some bigger neurones were also positive in the inner layer of the DCN that were identified as giant cells, according to their projection, size and location. Immunohistochemical studies performed on these neurones showed only CB expression without positive immunoreactions for either PA or CR.

Purkinje-like cells of the DCN project to the cerebellum in numerous species (including rat). This cell type is located near the surface of the DCN and shows strong CB positivity both in its soma and in its abundantly branching dendritic tree. PA labelling of the

soma was intense and homogeneous, while the dendritic tree did not show immunopositivity. According to our results, the Purkinje-like cells do not express CR.

For visualizing immunoreactions of pyramidal and Purkinje-like cells, both fluorescent and confocal microscopy were used.

2.2. Calcium-binding proteins in the VCN

Similarly to the DCN, identification of VCN neurones took place on the basis of their location, size and shape. To investigate the calcium-binding proteins of globular bushy cells, double immunolabellings with PA/CB and PA/CR combinations were applied. Strong PA positivity was shown in the soma of the cells and in the acoustic nerve fibers. The surface of the neurones exhibited intensive patchy immunoreaction which proved to be the consequence of labelling the axon terminals of the acoustic fibers. Neither the somata nor the acoustic nerve fibers showed CB labelling, but the axon terminals gave immunopositivity. Immunoreaction for CR showed similarities to data obtained for PA.

Similarly to globular bushy cells, we applied double immunolabelling with PA/CB and PA/CR combinations on spherical bushy neurones. This cell type exhibited cytoplasmic PA immunopositivity. PA-labelling of fibers around the soma was also evident and in a lot of cases the contours of the soma were marked by the strong positivity of the axon terminals. On the basis of the immunoreactions we concluded that the soma of the spherical bushy cell expressed neither CR nor CB, while fibers and axon terminals near the soma showed immunopositivity both for CR and CB.

In order to confirming the results of experiments conducted on bushy neurones, images were taken also with confocal microscopy. This method provided further evidence for the strong contrast between cell bodies and axon terminals as it indicated CR immunopositivity for the latter ones but found the somata of the spherical bushy cells without positive CR immunoreaction. As the soma of the globular bushy neurones showed intense CR labelling, the presence or lack of CR can be a useful method for distinguishing of these two cell types. Reconstructed images taken with confocal microscopy also confirmed PA positivity of both bushy neurone types, and the acoustic nerve fibers (together with their terminals) became clearly visible as well.

Although CB/CR double labelling provided evidence of CR expression of octopus cell somata, the intensity of this reaction was significantly smaller than that in the neighbouring fibers and axon terminals. It is worth mentioning that these structures showed only weak CB labelling, but intensive CB labelling was present in the octopus cell somata. PA expression

was present only in the axon terminals, this protein was not expressed in the somata of the octopus cells. For comparing the expression levels of the different calcium-binding proteins, a semiquantitative evaluation method was used.

3. Investigation of the Ca²⁺ removal mechanisms

3.1. Kinetics of the decay of depolarization-induced Ca²⁺ transients

Ca²⁺ removal from the cytoplasm of pyramidal cells was studied by fitting the falling phase of Ca²⁺ transients evoked by K⁺-depolarisation and analysing the time constants. To investigate the intracellular Ca²⁺ concentration-dependence of the removal mechanisms, various extracellular K⁺-concentrations were used with different incubation times.

The decay of the Ca²⁺ transients – depending on their peak concentrations – showed three distinct types of kinetics. The falling phase of the transients with small amplitude could be fitted with a single exponential function (type 1), while in the case of Ca²⁺ transients with bigger amplitudes, fitting with a double exponential function was necessary (type 2). The transition from a single to a double exponential decay took place between 45 and 82 nmol/l peak intracellular Ca²⁺ concentration values.

The type 3 behaviour of the declining phase was observed in approximately 10% of the neurones. On applying 50 mmol/l external K⁺ (1-5 s), the initial exponential decay of the Ca²⁺ transients was interrupted by a plateau-like segment then (after decreasing the K⁺ concentration) a second, much slower decline could be observed.

During the examination of Ca²⁺ concentration dependence of the Ca²⁺-removal, the time constant of type 1 transients was 6.43 ± 0.48 s (single τ). The early time constant (τ_1) obtained from fitting type 2 transients to a double exponential function behaved similarly: its value was 3.09 ± 0.26 s in the 0 – 100 nmol/l pool, and decreased at increasingly higher Ca²⁺ transients (to 1.46 ± 0.11 s in the 250 – 300 nmol/l range). The late time constant (τ_2) was 18.15 ± 1.6 s when the peak intracellular Ca²⁺ concentration was between 0 and 100 nmol/l. This parameter did not depend on the intracellular Ca²⁺ concentration as much as τ_1 , its statistically significant reduction could be observed only in the 250 – 300 nmol/l range (to 7.42 ± 0.92 s).

The appropriate fit of the falling phases either to single or to double exponential function required the incorporation of a pedestal. The calculated pedestal became higher as the peaks of the Ca²⁺ transients increased. The intracellular Ca²⁺ concentration measured

prior to the Ca^{2+} transients was sufficiently low and varied only to a small extent (between 15 and 25 nM) during the experiments.

It is known that the presence of high-affinity Ca^{2+} -indicators like Fura-2 influences the time course of intracellular Ca^{2+} concentration changes. To estimate the extent of such a Fura-2-related modification, in some cells Fluo-3, a low-affinity indicator was tried. Transients measured with this method had time constants similar to those obtained by using Fura-2 (in the case of type 2 transients $\tau_1 = 4.16 \pm 0.52$ s, $\tau_2 = 20.78 \pm 4.5$ s, $n = 4$).

3.2. Role of the intracellular Ca^{2+} stores in Ca^{2+} removal

The multiple exponential decline of type 2 and type 3 Ca^{2+} transients indicated that several mechanisms might be involved in removing the Ca^{2+} from the cytoplasm. The significance of the Ca^{2+} uptake by intracellular stores was tested using either thapsigargin (TG) or cyclopiazonic acid (CPA) to block the activity of the sarcoplasmic/endoplasmic reticular Ca^{2+} (SERCA) pump. TG (50 nmol/l) increased the resting intracellular Ca^{2+} concentration while the peak value of the transients showed significant decrease. Although type 2 transients retained their characteristics during the TG treatment, both time constants showed significant increase ($\tau_1 = 2.11 \pm 0.13$ s, $\tau_2 = 14.98 \pm 1.95$ s under control conditions, after TG treatment $\tau_1 = 5.99 \pm 0.96$ s, $\tau_2 = 27.46 \pm 4.21$ s).

The decreased amplitudes of the Ca^{2+} transients raised the question whether this change itself was responsible for the increased time constants. To investigate this question, we estimated the theoretical time constants belonging to the decreased amplitudes of the Ca^{2+} transients. It was found that the measured time constants increased in the presence of TG to a much bigger extent than predicted on the basis of the decreased amplitudes of the Ca^{2+} transients.

To confirm the results obtained with TG, we repeated the experiments using 3 $\mu\text{mol/l}$ CPA and came to the same conclusions. Thus, it seems likely that the decreased amplitude and slower declining phase of the transients are the consequences of the SERCA pump inhibition.

A possible explanation of the decreased peak value of the Ca^{2+} transients might be that both TG and CPA interfered with the Ca^{2+} entry by inhibiting the voltage-gated Ca^{2+} channels. To test this hypothesis, Ca^{2+} currents were measured in isolated pyramidal cells by employing the whole-cell configuration of the patch-clamp technique. The results of these

measurements clearly indicated that neither TG nor CPA exerted any effect on the depolarization-activated Ca^{2+} currents.

The effects of the SERCA blockers on the amplitude of the Ca^{2+} transients can be understood if the peak of these transients is affected by Ca^{2+} release from intracellular stores. Such a contribution of the caffeine-sensitive stores was examined by applying 20 mmol/l caffeine prior to the K^+ -depolarizations. Following the caffeine application, a control K^+ -depolarization was evoked, then the cell was bathed in either TG or CPA. In one neurone population, caffeine evoked a Ca^{2+} transient even in the presence of the SERCA pump blocker and the amplitude of the depolarization-evoked Ca^{2+} transient did not change significantly. In the rest of the cells, caffeine was not able to produce a Ca^{2+} transient in the presence of TG or CPA and the K^+ -depolarization-evoked Ca^{2+} transient showed an approximately 50% reduction.

3.3. Role of the surface membrane in decreasing intracellular Ca^{2+} concentration in pyramidal cells

To inhibit the activity of the $\text{Na}^{2+}/\text{Ca}^{2+}$ exchange mechanism transporting Ca^{2+} to the extracellular space, the extracellular Na^+ concentration was reduced either to 30 mmol/l or to nominally 0 mmol/l. The incubation with low- Na^+ solution started after the K^+ application in order to avoid any interference with the development of the Ca^{2+} transients. As identical results were obtained regardless of both the extent of the extracellular Na^+ concentration reduction and the form of the substitution (Li^+ or sucrose), all data were pooled.

Inhibition of the $\text{Na}^{2+}/\text{Ca}^{2+}$ exchanger mechanism decreased Ca^{2+} removal of the pyramidal cells. The time constants of the declining phase of the Ca^{2+} transients were increased: τ_1 from 2.64 ± 0.32 s to 3.37 ± 0.5 s, and τ_2 from 15.32 ± 1.92 s to 18.14 ± 1.68 s ($n = 10$), but none of these changes proved to be statistically significant. These results show a minimal role of the $\text{Na}^{2+}/\text{Ca}^{2+}$ exchange in cytoplasmic Ca^{2+} removal.

The inhibition of the PMCA pathway was achieved by the extracellular application of 3 mmol/l La^{3+} . To avoid the blockade of the voltage-gated Ca^{2+} channels, La^{3+} was added to the cells when the control extracellular K^+ concentration was restored. La^{3+} dramatically but reversibly changed the falling phase of the Ca^{2+} transients. Following an early decline, the intracellular Ca^{2+} concentration tended to reach a saturation level which was maintained until the termination of La^{3+} application. As the La^{3+} was removed, a second, slower decrease of the intracellular Ca^{2+} concentration could be noticed. The parameters obtained by fitting the control transients were in the usual range ($\tau_1 = 2.57 \pm 0.65$ s, $\tau_2 = 19.85 \pm 3.45$ s, $n = 7$). In

those transients where La^{3+} was applied, both declining segments of the falling phase could be fitted to a single exponential function. The time constant of the first exponential section ($\tau_{\text{La}} = 5.7 \pm 1.07$ s) was somewhat bigger than the control τ_1 , while the time constant after the washout of La^{3+} ($\tau_{\text{wo}} = 12.56 \pm 2.11$ s) was smaller than the control τ_2 (the difference is not statistically significant). Amplitude of the plateau phase was 81.2 ± 18.3 nmol/l, the pedestal seen after the washout (23.2 ± 2.2 nmol/l) was bigger than that determined under control conditions.

DISCUSSION

The main aim of the experiments shown in the theses was to get further information about the regulation of Ca^{2+} homeostasis of neurones in the cochlear nucleus. The work fits well to the efforts of our group to understand the functional features of the parallel ascending pathways formed in the cochlear nucleus and to determine the role of the different neuron types in the organisation of these pathways. To reach this goal it is essential to study the membrane properties and intracellular regulatory mechanisms of the neurones.

A major part of the experiments gave information about the pyramidal cells of the dorsal cochlear nucleus. It can be stated that although these neurones are under significant Ca^{2+} loading as a consequence of their function, they produce relatively small intracellular Ca^{2+} concentration changes. The reason of the low amplitude of the Ca^{2+} transients is partially the buffering ability of the cytoplasmic calcium-binding proteins, and partially the presence of effective Ca^{2+} -removal mechanisms.

1. Glutamate-induced Ca^{2+} transients of isolated pyramidal neurones

The glutamate sensitivity of the pyramidal cells was lower in our experiments than in several other dissociated or cultured neurone types. Moreover, the transients in the present experiments were relatively small compared to the transients described in suprachiasmatic nucleus cells or in hippocampal neurones. The reason of this discrepancy is not clear, it is probable that the enzymatic isolation causes a significant loss of the plasmalemmal glutamate receptors. This hypothesis is rendered probable by the fact that K^{+} -depolarizations were reported to evoke cytoplasmic Ca^{2+} concentration elevations similar to our results in several other papers. It has to be noted also that our data indicate a significant contribution of AMPA-receptors to the genesis of the Ca^{2+} transients and the relatively low glutamate sensitivity of these receptors has already been documented.

The origin of Ca^{2+} resulting the glutamate-induced Ca^{2+} transients is exclusively the extracellular space. The ineffectivity of AP5 in modifying the transients indicate that NMDA receptors do not contribute significantly to the genesis of the Ca^{2+} transients. Contradictory to this finding, the AMPA-receptor blocker CNQX, which acts on the NMDA-receptors as well, decreased the amplitude of the Ca^{2+} transients more effectively than the more specific AMPA-receptor blocker NBQX did. The contradiction might be explained by the relatively low concentration of AP5. Our results about the role of voltage-gated Ca^{2+} channels are slightly contradictory as well: Cd^{2+} caused an approx. 50% block while attenuating the extent of the

depolarization by Na^+ withdrawal was apparently ineffective. In this case we suppose that the remaining inward current through the receptor channels is sufficient even under „low Na^+ ” circumstances to depolarize the membrane. Alternatively, it might be supposed that Cd^{2+} blocked the AMPA-receptors as well.

As a consequence of the aforementioned results, Ca^{2+} entry through AMPA-receptors have to contribute to the genesis of the glutamate-induced Ca^{2+} transients, too. Several experiments demonstrated that neurones of the DCN generally expressed GluR1-4 subunits although the expression levels showed significant variability among the various cell types. The functional importance of Ca^{2+} -permeable AMPA-receptors in the auditory pathway was indicated by the finding that these receptors showed higher Ca^{2+} permeability than those present in some other neighbouring brainstem nuclei. Other observations showed that pyramidal neurones in the DCN contained Ca^{2+} -impermeable AMPA-receptors due to the presence of the GluR2 subunit. Nevertheless, there is a discrepancy between the results of our experiments and the known subunit composition of the AMPA-receptors described in the pyramidal neurones of the DCN.

To solve this apparent contradiction, several possibilities might be encountered. First, a small fraction of the AMPA-receptors may be Ca^{2+} -permeable and their simultaneous activation under the present experimental conditions may result in detectable Ca^{2+} concentration increases. Second, even moderate Ca^{2+} loads may give rise to measurable cytoplasmic Ca^{2+} transients, especially in pyramidal neurones as these cells seem to possess low intracellular Ca^{2+} buffering capacities (this assumption has been questioned as we found high expression levels of calcium binding proteins in the pyramidal cells). Third, the subunit composition of the glutamate receptors in the auditory pathway neurones of rodents undergo changes along the maturation of the hearing function and the younger age of the rats of the present study compared to that of the mice used by the other group may be accompanied by a lower expression level of the GluR2 subunit.

2. Presence and distribution of calcium-binding proteins in the projection neurones of the adult rat cochlear nucleus

Due to their importance, numerous studies are available about the distribution of the calcium-binding proteins in the central nervous system, and in some of them there is information about the cochlear nucleus as well. These data are heterogeneous, because the measurements were carried out on different species or at different ages on a given species. Thus, it seemed desirable to conduct a survey using the same technique in the same species

with fully matured auditory pathway. Besides some traditional approaches, double immunolabelling was utilised to reveal the co-localisation of the calcium binding proteins and confocal microscopy was also introduced.

The results of the present experiments partly support earlier observations that in the cells of the DCN the CB dominates while CR can not be found. It is especially important to estimate the cytoplasmic Ca^{2+} buffer capacity of the pyramidal cells. While earlier observations showed only CB positivity of this cell type in the rat, our data provided evidence about PA positivity as well indicating that the pyramidal cells might possess high Ca^{2+} buffer capacity.

CB and PA positivity of Purkinje-like cells in rat was previously known, while intensive CB positivity and low PA expression level was described in their dendritic tree. According to our data this difference is even more sharp: as almost complete negativity of the dendrites was found both with fluorescent and confocal microscopy. It was also proved that giant cells expressed neither CR nor PA.

The presence of CB is not as dominant in the cells of the VCN as in the DCN neurones while CR can be found in some VCN cells. We found characteristic difference in the buffer protein expression of the two subtypes of bushy neurones. CB-negativity and CR-positivity of the globular cells was previously known while we described the presence of PA in this neurone, so this cell type also possess two different buffer proteins.

The lack of CR and CB of rat spherical bushy neurones was reported previously. Our data indicate the presence of PA in small amounts. This finding has special importance because it shows that this projection neurone contains at least one Ca^{2+} buffer protein also in rat, similarly to other species.

Our data confirmed the presence of CR and CB in octopus cells and proved convincingly the lack of PA, the protein, whose presence was shown in the same cell type in different species.

Despite of the intensive research, there is only little understanding regarding the significance of the calcium-binding proteins. We can state in general that their role is the protection of cells against long-lasting or unusually high cytoplasmic Ca^{2+} concentration increases. This hypothesis is supported by the fact, that these proteins can be found in a great amount in those neurones that are heavily exposed to Ca^{2+} loads due to their strong surface electrical activity or due to the presence of other Ca^{2+} entry pathways. One example for the previous case is the colocalisation of PA with Kv3.1b K^+ channel subunits whose presence is characteristic of those neurons that show high frequency firing. Presence or lack of GluR2

AMPA receptor subunit (and, consequently, that of the Ca^{2+} -permeability of the AMPA-receptors) show coincidence with the expression pattern of calcium-binding proteins as an example for the second case.

3. Removal mechanisms of the cytoplasmic Ca^{2+}

For maintaining physiological functions of neurones, it is crucial to minimize the changes of ionic concentrations due to their activity and to restore the resting values rapidly. In the case of Ca^{2+} , this task is achieved partially by buffer proteins, partially by different transport mechanisms which move Ca^{2+} to the extracellular space or accumulate it temporarily in the ER. For the characterisation of the Ca^{2+} removal, kinetic parameters of the decay phases of depolarization-evoked transients were utilised. Time constants determined in our experiments showed good agreement with the data found in the literature.

Kinetic parameters of the Ca^{2+} transients are strongly effected by the Ca^{2+} buffer capacity of the cell. As these parameters of the Fluo-3 and the Fura-2 transients are similar, buffering effect of the Fura-2 might be minimal. The relatively small Ca^{2+} transients obtained in our experiments indicated high buffer capacity and this finding points toward the significant role of the buffer proteins. Immunohistochemical data proving the co-localisation of CB and PA in pyramidal cells also support this hypothesis. However, the fact that the decay time constants show Ca^{2+} -dependence, may indicate that under these experimental conditions the buffers are close to saturation suggesting that either their capacity is small or their preload is high. Reconciliation of immunohistochemical and kinetic results needs further investigation, mainly more accurate quantitative estimations.

For exploring the roles of the individual transport mechanisms in Ca^{2+} removal, we carried out their selective inhibition and the analysis of the resulting changes. There might be several errors of this approach, e.g. questionable specificity of the blockers. An important problem can be the interaction of the pathways: inhibition of a transport mechanism can decrease the activity of the other pathways, but compensation of the blocked transport via the increasing activity of other pathways may also take place. To explore these possibilities, we tried to inhibit more than one transport mechanism in the same time, but these experiments did not give satisfactory results.

Data about role of the $\text{Na}^+/\text{Ca}^{2+}$ -exchange mechanism show serious discrepancies. Some observations indicate its importance while other data suggest a negligible role of the

exchange mechanism. Our data support the latter view and question the idea about the importance of the $\text{Na}^+/\text{Ca}^{2+}$ -exchange in central structures of the central nervous system.

The role of the PMCA pathway was investigated by applying 3 mmol/l La^{3+} . This ion is thought to inhibit $\text{Na}^+/\text{Ca}^{2+}$ -exchange as well in this concentration, but according to our data about the marginal role of the exchanger, it can be concluded that the dramatic effects of the La^{3+} are the consequences of PMCA inhibition. The fact that the inhibition of this transport mechanism resulted in the generation of a plateau, supports our hypothesis that the kinetic parameters of the decay phase are determined by the balance between the Ca^{2+} -load and the removal capacity.

Storage of Ca^{2+} in the ER can have important role in the termination of the Ca^{2+} transients of the pyramidal cells. The role of this mechanism is indicated by the fact, that inhibition of the SERCA pump clearly decreased the rate of the decay phase. However, we can not completely exclude the possibility that this decrease is only virtual, and the effectivity of the real Ca^{2+} removal is decreased by the opening of some storage-dependent Ca^{2+} channels and by the Ca^{2+} entering through these channels.

An unexpected consequence of SERCA pump inhibition was the decrease of the amplitude of the transients. After excluding some possible explanations, we concluded that the transients might have a component based on Ca^{2+} release. This hypothesis is supported by the finding that TG and CPA treatment increased resting Ca^{2+} level, and that the decrease of the transients by SERCA blocker treatment required the emptying of the Ca^{2+} stores. Moreover, data in the literature indicate that depolarization-induced Ca^{2+} transients might have a Ca^{2+} -induced Ca^{2+} release component.

We can conclude, therefore, that pyramidal neurones possess a well-regulated Ca^{2+} homeostasis and that they are suitable for studying the steps of this regulation. Nevertheless, the repetition of these measurements under more physiological conditions seems to be necessary. It is essential also to collect data about the Ca^{2+} homeostasis of other neurones of the cochlear nucleus that allows the interpretation of links between neuronal functions and Ca^{2+} homeostasis in a more reliable way.

Publications providing the basis of the present theses

Papers

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