Glutamate-Induced Cytoplasmic Ca^{2+} Transients in Neurones Isolated from the Rat Dorsal Cochlear Nucleus

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Abstract. Extracellular application of glutamate elicited cytoplasmic Ca^{2+} transients in freshly dissociated rat neurones of the dorsal cochlear nucleus (DCN) (identified as pyramidal cells) with half-maximal concentration of 513 μmol/l while saturating doses (5 mmol/l) of this neurotransmitter caused transients of 46.1 ± 3.0 nmol/l on an average. The genesis of these glutamate-evoked Ca^{2+} transients required extracellular Ca^{2+}. When $[Mg^{2+}]_o$ was 1 mmol/l, the NMDA receptor antagonist AP5 (100 μmol/l) had no effects while 100 μmol/l CNQX and 10 μmol/l NBQX, inhibitors of the AMPA receptors, greatly decreased the glutamate-induced Ca^{2+} transients (a decrease of 92 and 57 %, respectively). When facilitating the activation of the NMDA receptors (50 μmol/l glycine, 20 μmol/l $[Mg^{2+}]_o$) in the presence of 100 μmol/l CNQX, Ca^{2+} transients of 55.4 ± 13.1 nmol/l could be produced. Block of the voltage-gated Ca^{2+} channels (200 μmol/l Cd^{2+}) decreased the Ca^{2+} transients to approx. 50 %. The data indicate that under our control experimental circumstances the glutamate-induced Ca^{2+} transients of the isolated DCN neurones are produced mainly by Ca^{2+} entry through voltage-gated Ca^{2+} channels and AMPA receptors. However, when the activation of the NMDA receptors may take place, these receptors also contribute significantly to the genesis of the glutamate-evoked cytoplasmic $[Ca^{2+}]$ elevations.

Key words: Glutamate — Cytoplasmic Ca^{2+} transients — Ionotropic glutamate receptors — CNQX — NBQX — AP5

Introduction

The cochlear nucleus of the mammalian brainstem is the site where the axons of the cochlear nerve transmit information to second order sensory neurones. The structurally ordered dorsal cochlear nucleus (DCN) contains several neurone types that form sophisticated networks indicating a function more complex than that of a simple relay station might be (Kane 1974; Zhang and Oertel 1994; Waller et al.

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1996). Several functional studies proved that these DCN networks involve excitatory glutamatergic transmission, and the presence of ionotropic and metabotropic glutamate receptors in the appropriate neurones has been demonstrated, too (Petralia et al. 1996; Waller et al. 1996; Molitor and Manis 1997; Rubio and Wenthold 1997).

It is known that N-methyl-D-aspartate (NMDA)-type ionotropic glutamate receptors are permeable to Ca^{2+} while the α-amino-3-hydroxy-5-methyl-isoxazole proprionic acid (AMPA)-type receptors show considerable diversity in this respect depending on their subunit composition (for a review see Hollmann and Heinemann 1994). It is not surprising, therefore, that neurones possessing these receptors are capable of producing cytoplasmic Ca^{2+} transients on extracellular glutamate applications (see Hattori et al. 1998 and the references cited therein). On the basis of these findings it is plausible to suppose the presence of glutamate-elicited intracellular $[Ca^{2+}]$ elevations in various neurones of the DCN but hitherto no experimental demonstration of these $[Ca^{2+}]$ changes is available in the literature.

We have shown earlier that K^+ depolarizations were able to induce cytoplamic Ca^{2+} transients in neurones which were isolated from the rat DCN and were identified as pyramidal cells (Rusznák et al. 2000). It was also demonstrated that these neurones possessed Ca^{2+} removal mechanisms responsible for the maintenance and recovery of the low resting intracellular $[Ca^{2+}]$ (Harasztosi et al. 2002). Several reports agreed that the pyramidal cells in most species receive glutamatergic excitatory inputs both from the cochlear nerve and from the granule cells (Kane 1974; Zhang and Oertel 1994; Waller et al. 1996). In the present work we provided direct experimental demonstration of cytoplasmic Ca^{2+} transients evoked by extracellular glutamate application in isolated DCN neurones considered to be pyramidal cells. We also proved that Ca^{2+} entry through NMDA and AMPA receptors as well as *via* voltage-gated Ca^{2+} channels were responsible for the genesis of these $[Ca^{2+}]_i$ elevations. Parts of the results have been presented in an abstract (Rusznák et al. 2001b).

Materials and Methods

Isolation of the DCN neurones

The detailed description of the isolation procedure can be found in a recent paper (Rusznák et al. 2001a). The present study was performed by the permission of the Committee of Animal Research of the University of Debrecen (Hungary), the animals were handled according to the appropriate laws.

Briefly, young rats (6–11-day) were decapitated, the removed brains were placed into Na^+-free artificial cerebrospinal fluid (aCSF) of approx. -2 ℃. The adherent tissues (meninges, blood vessels, etc.) were discarded and the brain was cut along its midline. The two cochlear nuclei were excised, their dorsal parts isolated and soaked for 40–55 min in enzyme-containing aCSF (0.03 mg/ml type IA collagenase and 0.12 mg/ml type XIV pronase) at 31 ℃. The incubating solution

was bubbled with 95% O_2/5% CO_2. The enzyme exposure was ceased by transferring the tissue fragments into aCSF containing 1 mg/ml trypsin inhibitor (type I-S). The individual cells were isolated by gentle trituration in HEPES-buffered aCSF.

Chemicals and solutions

The aCSF contained (in mmol/l): NaCl 125; KCl 2.5; glucose 10; NaH_2PO_4 1.25; $NaHCO_3$ 26; $CaCl_2$ 2; $MgCl_2$ 1; myo-inositol 3; ascorbic acid 0.5; Na-pyruvate 2 (all chemicals were purchased from Sigma, St. Louis, USA unless specified otherwise). In the Na^+-free aCSF NaCl was replaced by sucrose (250 mmol/l). The HEPES-buffered aCSF was made of (in mmol/l): NaCl 135; KCl 3; glucose 10; HEPES 10; sucrose 45; $CaCl_2$ 2; $MgCl_2$ 1.

The intracellular $[Ca^{2+}]$ was recorded using membrane-permeable Fura-2-AM (TEFlabs, Austin, USA). In these measurements the HEPES-buffered aCSF was the control external solution, but in these instances its $CaCl_2$ concentration was raised to 5 mmol/l. When K^+ depolarization-induced Ca^{2+} transients were elicited, 35 mmol/l NaCl was replaced by sucrose (control external solution) and the 50 mmol/l K^+-containing solution was prepared by substituting the sucrose with appropriate amount of the 1 mol/l KCl stock.

Glutamate-containing solutions were freshly prepared from a deep-frozen 1 mol/l stock (sodium salt of L-glutamate). AP5 (either DL or D isomers) and glycine stock solutions were prepared using distilled water (50 mmol/l and 100 mmol/l, respectively) while CNQX and NBXQ were dissolved in dimethyl sulfoxide (DMSO) (20 mmol/l and 1 mmol/l, respectively); all these stock solutions were kept at −20 ℃ and the final dilutions were performed prior to the experiments. Nominally Na^+-free external solution was prepared by substituting its NaCl content with sucrose. Because the pH adjustment required NaOH and the glutamate was employed as sodium salt, this solution contained approx. 10 mmol/l Na^+ and was called "low Na^+" solution. In the external solution with reduced Ca^{2+} content ethylene glycol-bis[β-aminoethyl ether] N,N,N′,N′-tetraacetic acid (EGTA) (1 mmol/l) and $CaCl_2$ were added to give a calculated final $[Ca^{2+}]$ of 10 μmol/l. When using Cd^{2+}, the appropriate amounts of $CdCl_2$ were applied from a deep-frozen stock solution (100 mmol/l).

The pH of the solutions was set to 7.2; the osmotic concentration was regularly checked and kept at about 320–330 mosm/l. During the measurements the continuous perfusion of the cells and the quick solution changes were performed using a gravity-driven microperfusion system. Drugs were applied to the vicinity of the neurone chosen for recording.

Intracellular $[Ca^{2+}]$ measurements on dissociated neurones

The suitably long survival of the isolated neurones was achieved by incubating them in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% horse serum. During the incubation period (3–4 h) the cells were kept in a 5% CO_2 atmosphere at 37 ℃ (Rusznák et al. 2001a). Fura-2-AM (3 μmol/l) was added to

the DMEM in the last 30 min. Prior to the experiments the neurones were bathed in HEPES-buffered aCFS at room temperature for at least 30 min.

For recording optical transients the cells were transferred into an appropriate experimental chamber and placed on the stage of an inverted microscope (Diaphot 200, Nikon, Japan). Neurones were selected for recording on the basis of morphological criteria (Harasztosi et al. 1999; Rusznák et al. 2001a). During the selection special emphasis was put on choosing only those cells that had a triangular body of 15–20 μm diameter possessing the remnants of three well-defined processes. These structural marks are in accordance with data reported earlier for DCN pyramidal neurones (Oertel and Wu 1989). Recently very similar images were obtained in rat DCN slices when electrophysiologically identified pyramidal cells were filled with fluorescent markers (data not shown). Based on the above arguments we propose that the isolated neurones used in the present experiments most probably belonged to the pyramidal cell population.

The cytoplasmic $[Ca^{2+}]$ was measured using a Deltascan-1 system (Photon Technology International, New Brunswick, USA) in which a dual wavelength monochromator ensured the illumination with light beams of 340 and 380 nm wavelengths, respectively. The light emission was measured by a photomultiplier at 510 nm and recorded using a digitization rate of 50 Hz. To obtain the Ca^{2+}-related emission, the recorded light intensities were corrected for the background fluorescence and the 340/380 ratios were calculated from point to point (Oscar software, Photon Technology International, New Brunswick, USA). To convert the ratio values to $[Ca^{2+}]$, R_{min}, R_{max} and the apparent K_D values were determined during *in vitro* calibrations. The $[Ca^{2+}]_i$ traces shown in the figures were digitally smoothed.

Statistical analysis

Results are given as mean ± S.E.M. throughout. Statistical analysis was performed applying the appropriate version of Student's *t*-test (Microcal Origin 6.0 software). The level of significance is given on the figures. Fitting of the dose-response data as well as the falling phase of the Ca^{2+} transients was carried out using the Microcal Origin 6.0 software.

Results

Characterization of the glutamate-evoked Ca^{2+} transients

In order to quantify the efficacy of glutamate to evoke cytoplasmic Ca^{2+} transients, this excitatory amino acid was applied extracellularly at different concentrations and a dose-response relationship was established. Three or four glutamate concentrations were applied in a neurone, consequently, data from cells with variable glutamate sensitivities had to be pooled. To decrease the scatter, a standard glutamate concentration (5 mmol/l) was always included, and all transients recorded in a particular cell were normalized to that evoked by this standard concentration.

Fig. 1A presents the dose-response relationship derived from these measurements. Ca^{2+} transients appeared when 50–100 μmol/l glutamate was applied, and

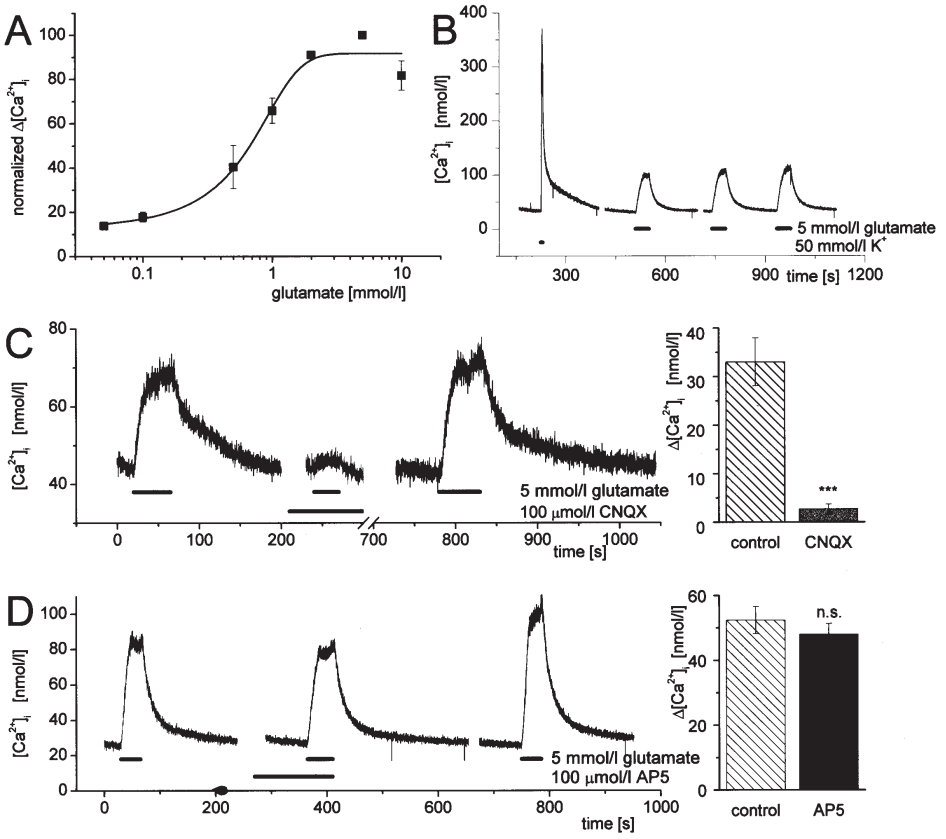

Figure 1. Basic characteristics of the glutamate-induced Ca^{2+} transients. **A.** Extracellular glutamate challenges of various concentrations were applied in 14 neurones. The peak values were determined as deviations from the baseline prior to the glutamate treatment and were normalized to the peak of the Ca^{2+} transient evoked by 5 mmol/l glutamate in the same cell. The calculated relative amplitudes were averaged and plotted as filled squares. The continuous line presents the fit of the dose-response function of the Microcal Origin software to the averages. **B.** Ca^{2+} transients evoked by elevating the extracellular K^+ concentration (to the left) then by the repetitive application of glutamate. Note the eventually unmodified shape of the consecutive glutamate-induced transients. The breaks seen on the records here and in the forthcoming figures indicate intervals during which data acquisition was paused for technical reasons. **C.** 5 mmol/l glutamate-evoked Ca^{2+} transients under control conditions and following 30 s pretreatment with 100 μmol/l CNQX. Wash out of the blocker was demonstrated in the late phase of this experiment. Columns to the right illustrate the statistical evaluation of all analogous measurements ($n = 5$). **D.** 5 mmol/l glutamate-induced Ca^{2+} transients prior to, during and following the application of 100 μmol/l AP5 as well as the synopsis of all similar measurements ($n = 7$; columns to the right). $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; $\Delta[Ca^{2+}]_i$, the changes of the cytoplasmic Ca^{2+} concentration; *** $p < 0.001$; n.s. denotes differences that are not statistically significant.

5 mmol/l glutamate proved to be a saturating concentration. On fitting the averaged data points to a dose-response function, the half-maximal glutamate concentration was 513 μmol/l while the Hill-coefficient was 1.03 indicating a ligand/receptor interaction with 1 : 1 stoichiometry.

As mentioned above, the isolated cells showed variability in their glutamate sensitivity. In order to decrease uncertainties introduced by this phenomenon, the saturating 5 mmol/l glutamate was applied for at least 5 s to reach the maximum effect in each neurone. Fig. 1B indicates that using an incubation pattern of 5 mmol/l/40 s, Ca^{2+} transients with constant amplitude could be observed even if the glutamate application was repeated several times, indicating that neither run-down of the calcium homeostasis nor modification of the glutamate sensitivity had to be encountered.

5 mmol/l glutamate evoked cytoplasmic Ca^{2+} transients in the range of 22.7 and 77.6 nmol/l with an average amplitude of 46.1 ± 3.0 nmol/l ($n = 66$, including controls in all cells tried in the present experiments). In some neurones (see Fig. 1B) both elevated $[K^+]_o$ and glutamate were applied sequentially to allow the comparison of the glutamate-induced Ca^{2+} transients to those evoked by membrane depolarization. In these experiments 50 mmol/l K^+ elicited transients with an average amplitude of 217.7 ± 20.2 nmol/l while the peak of the transients induced by 5 mmol/l glutamate was 60.8 ± 6.8 nmol/l ($n = 9$).

The removal rate of Ca^{2+} following control glutamate-induced transients was approximated by fitting exponential decay functions to their falling phase. This analysis could be carried out in 58 cells. In 37 neurones a single exponential function was more appropriate for fitting, the decay time constant (τ) was 21.5 ± 1.7 s. In the rest of the controls ($n = 21$) application of a function containing two exponential terms gave better fits. In these instances τ_1 was 6.7 ± 0.5 s while τ_2 proved to be 27.2 ± 2.5 s.

Decay rates of K^+ depolarization- and glutamate-induced Ca^{2+} transients could be compared in 8 neurones. Fitting of the depolarization-evoked transients was always better on using the double exponential function. The time constant values obtained ($\tau_1 = 3.0 \pm 0.4$ s, $\tau_2 = 17.8 \pm 2.8$ s) were smaller than those calculated for glutamate-induced transients in 5 cells ($\tau_1 = 8.1 \pm 0.6$ s, $\tau_2 = 26.6 \pm 5.9$ s; fitting of double exponential function to glutamate-evoked Ca^{2+} transients in 3 other cells gave no realistic results). Comparison of time constants obtained from fits of the single exponential function in all 8 neurones also indicated a faster Ca^{2+} removal following K^+ depolarization-induced transients ($\tau = 9.8 \pm 1.2$ s and $\tau = 15.6 \pm 1.8$ s for depolarization- and glutamate-induced transients, respectively).

In order to characterize the role of the various ionotropic glutamate receptors in evoking the Ca^{2+} transients, their antagonists were applied. Fig. 1C illustrates an experiment in which 100 μmol/l CNQX, an inhibitor of AMPA-type glutamate receptors substantially reduced the Ca^{2+} transient evoked by 5 mmol/l glutamate. In 13 similar measurements the peak value of the control transients was 33.0 ± 4.9 nmol/l and this value decreased to 2.7 ± 0.9 nmol/l following CNQX treatment (30 s, $p < 0.001$). Wash out of the CNQX was attempted in 5 cells and in these

instances recovery to $117.7 \pm 19.8\,\%$ of the control transients was observed (the increase relative to the controls is not significant statistically).

The possible contribution of the NMDA receptors was tested by applying 100 μmol/l AP5. As Fig. 1D illustrates, 30–40 s pretreatment with this NMDA receptor antagonist hardly modified the Ca^{2+} transients (control 52.4 ± 4.2 nmol/l while 48.0 ± 3.4 nmol/l in the presence of AP5, $n = 7$, the difference is not statistically significant).

The efficacy of 100 μmol/l CNQX rises the question whether the AMPA receptor-blocking characteristics of this compound are specific. Indeed, recent data indicate that CNQX may block the NMDA receptors, too (Mead and Stephens 1999). To address this question, NBQX, a more specific antagonist of the AMPA receptors was applied at a concentration of 10 μmol/l. The neurones were first treated with AP5 (100 μmol/l) and Cd^{2+} (200 μmol/l) simultaneously. Under these circumstances, 5 mmol/l glutamate evoked Ca^{2+} transients of 25.0 ± 4.8 nmol/l (comments on this value are given later). In the next step NBXQ was added to the incubating medium and the repeated glutamate challenges resulted in Ca^{2+} transients of 12.1 ± 3.0 nmol/l ($n = 6$, $p < 0.01$). Withdrawal of the NBQX from the external solution caused a complete recovery of the Ca^{2+} transients in all neurones tested.

Activation of the NMDA-type ionotropic glutamate receptors

As AP5 did not influence the glutamate-evoked Ca^{2+} transients, one may conclude that there are no functioning NMDA receptors in the isolated neurones. To explore the situation, maneuvers known to facilitate these receptors were performed.

Part A of Fig. 2 presents an experiment in which a control Ca^{2+} transient was followed by a second 5 mmol/l glutamate challenge but in the latter case 50 μmol/l glycine was applied and the Mg^{2+} concentration was decreased to 20 μmol/l. This protocol considerably increased the amplitude of the Ca^{2+} transient. When the control extracellular solution was employed again, the amplitude of the third transient decreased to the control value. Fig. 2B gives a synopsis of these experiments. The control transients had a peak amplitude of 44.5 ± 14.9 nmol/l, while on adding glycine and decreasing $[Mg^{2+}]_o$ the maximum amplitude rose to 91.9 ± 15.3 nmol/l ($n = 5$, $p < 0.05$). On returning to the control external solution the averaged peak of the Ca^{2+} transients was 41.6 ± 19.7 nmol/l (no significant difference when compared to the controls).

In a different set of experiments activation of the NMDA receptors was achieved after blocking the AMPA receptors. In 7 cells, 5 mmol/l glutamate evoked Ca^{2+} transients with peak values of 38.0 ± 7.8 nmol/l on an average (Fig. 2C). Similarly to data shown earlier (Fig. 1C) 100 μmol/l CNQX almost completely blocked the development of the transients (the remaining peak values were 1.8 ± 0.5 nmol/l, $p < 0.001$). In the next step 50 μmol/l glycine was added to the bathing medium in the continuous presence of the CNQX. Under such circumstances, the Ca^{2+} transients increased (16.8 ± 3.8 nmol/l) although their peak values were still significantly smaller than the amplitude of those transients obtained under control cir-

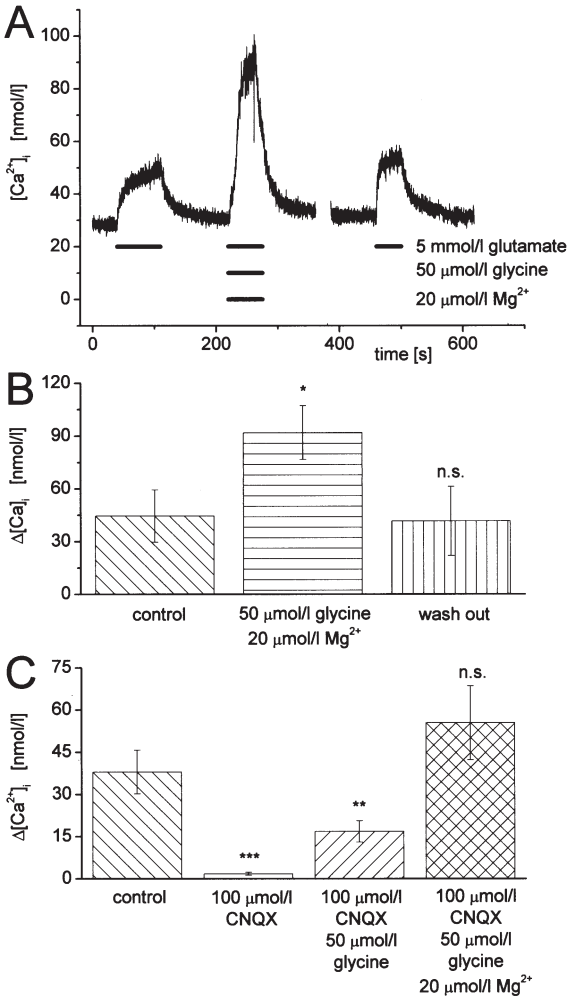

Figure 2. Activation of the NMDA receptors. **A.** Ca^{2+} transients evoked by applying 5 mmol/l glutamate in the external solution. During the second glutamate application 50 μmol/l glycine was present and the Mg^{2+} concentration was reduced to 20 μmol/l. **B.** Synopsis of the experiments carried out as shown in panel A ($n = 5$). **C.** Summary of the experiments ($n = 7$) carried out to demonstrate the activation of NMDA receptors after blocking the AMPA receptors. From the left, the first column represents control transients while the second one the blocking effect of 100 μmol/l CNQX. In the next step 50 μmol/l glycine was added for 2 min in the presence of CNQX and the 5 mmol/l glutamate challenge was repeated (third column). Finally, 50 μmol/l glycine was applied in combination with decreased extracellular Mg^{2+} concentration (20 μmol/l) for 2 min (still in the presence of the CNQX) and the glutamate exposure was repeated again (rightmost column). $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; $\Delta[Ca^{2+}]_i$, the changes of the cytoplasmic Ca^{2+} concentration; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. denotes differences that are not statistically significant.

cumstances ($p < 0.01$). Finally, 50 μmol/l glycine was applied in combination with decreased Mg^{2+} concentration (20 μmol/l) in the continuous presence of CNQX. Under these conditions 5 mmol/l glutamate was able to produce Ca^{2+} transients being somewhat bigger than those found in control external solution (55.4 ± 13.1 nmol/l, no significant difference when compared to the controls).

Mechanism of the AMPA receptor-related cytoplasmic [Ca^{2+}] changes

To identify the possible sources of Ca^{2+} appearing in the cytoplasm during the development of the AMPA receptor-related Ca^{2+} transients, some modifications of the Ca^{2+} transport pathways were performed.

The experiment shown in Fig. 3A indicates that the development of the gluta-

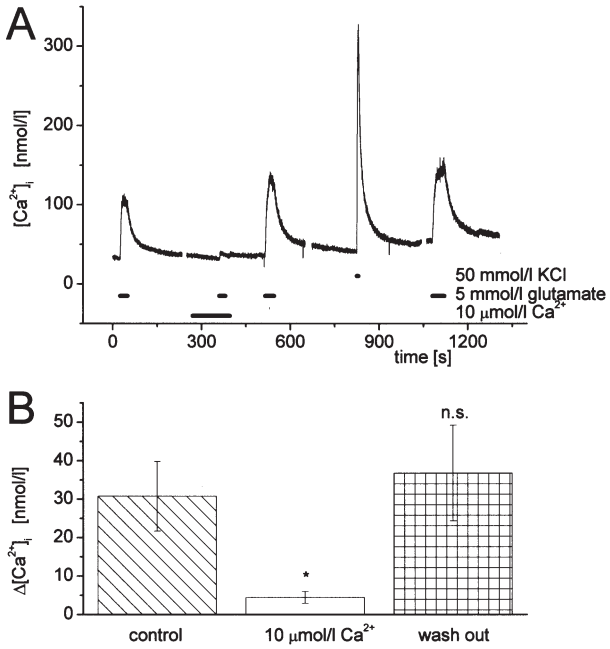

Figure 3. Effects of Ca^{2+} withdrawal on the glutamate-induced Ca^{2+} transients. **A.** Following the relaxation of a control glutamate-induced transient, the external [Ca^{2+}] was decreased to 10 μmol/l for 150 s and during this period the 5 mmol/l glutamate was reintroduced for the same time (20 s) as under control conditions. After restoring $[Ca^{2+}]_o$, 5 mmol/l glutamate, 50 mmol/l K^+ and again 5 mmol/l glutamate were applied consecutively. Note the similar amplitude and time course of the glutamate-induced transients prior to and following the depolarization-induced $[Ca^{2+}]_i$ increase. **B.** Synopsis of the experiments carried out to test the effects of low external [Ca^{2+}] on the glutamate-evoked Ca^{2+} transients ($n = 6$). $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; $\Delta[Ca^{2+}]_i$, the changes of the cytoplasmic Ca^{2+} concentration; * $p < 0.05$; n.s. denotes differences that are not statistically significant.

mate-induced Ca^{2+} transients depends on the presence of Ca^{2+} in the incubating solution. Under control conditions, the average amplitude of the 5 mmol/l glutamate-evoked Ca^{2+} transients was 30.8 ± 9.1 nmol/l (Fig. 3B) while following Ca^{2+} withdrawal of 30–50 s, this value decreased to 4.4 ± 1.5 nmol/l ($n = 6$, $p < 0.05$). Restoration of the external [Ca^{2+}] resulted in normalization of the Ca^{2+} transients (36.8 ± 12.4 nmol/l, no significant difference when compared to the controls).

Part A of Fig. 3 illustrates also an indirect test of the possible contribution of intracellular Ca^{2+} release to the genesis of the Ca^{2+} transients. Following the restoration of the response to 5 mmol/l glutamate, 50 mmol/l K^+ depolarization was evoked in order to facilitate the filling of the Ca^{2+} stores then the glutamate challenge was repeated. It can be seen that the glutamate-related Ca^{2+} transients prior to and following the K^+ depolarization had approximately the same amplitude. In 3 similar experiments the first glutamate-evoked transients reached $35.0 \pm 14.5\,\%$ of the K^+ depolarization-induced transients while the same value for the second glutamate-related transients was $32.1 \pm 5.5\,\%$ (the difference is not statistically significant).

The above data indicate that the primary mechanism responsible for the development of the AMPA receptor-related Ca^{2+} transients might be the entry of Ca^{2+} from the extracellular space. This Ca^{2+} entry, however, might take place *via* different routes. To explore the role of voltage-gated Ca^{2+} channels, the experiments were carried out in which either the extent of depolarization was decreased by reducing $[Na^+]_o$ or the Ca^{2+} channels were inhibited directly.

To prevent any accidental contribution of the NMDA receptors, in these experiments 100 μmol/l AP5 was also present whenever glutamate was applied. Part A of the Fig. 4 illustrates that lowering the external [Na^+] to approx. 10 mmol/l did not influence noticeably the 5 mmol/l glutamate-evoked Ca^{2+} transients. The summary of the data indicates that the first transients had a peak value of 55.6 ± 10.2 nmol/l while those obtained in "low Na^+" solution had maximum value of 68.8 ± 14.7 nmol/l ($n = 5$, the difference is not statistically significant).

Fig. 4B presents data from experiments that tested the role of Ca^{2+} entry *via* voltage-gated Ca^{2+} channels by applying Cd^{2+}, their blocker at a concentration of 200 μmol/l in Na^+-containing solution. Configuration of the 5 mmol/l glutamate-induced transients indicates that Cd^{2+} causes a fully reversible decrease of approx. 50 % in their amplitudes. In 11 similar experiments the control transients had peaks of 52.8 ± 5.8 nmol/l, this value decreased to 27.7 ± 6.7 nmol/l due to the Cd^{2+} block ($p < 0.01$). Wash out of Cd^{2+} was carried out in 6 neurones, in these cases a recovery to $135.3 \pm 20.5\,\%$ of the appropriate controls was observed (the difference is not significant statistically). This figure illustrates that besides decreasing the amplitude of the Ca^{2+} transients, Cd^{2+} also caused a reduction of their rate of rise. This modification was observable in all cases but has not been evaluated quantitatively.

As the experiments shown above tended to exclude the role of the NMDA-type receptors under control conditions and indicated only an approx. 50 % contribution

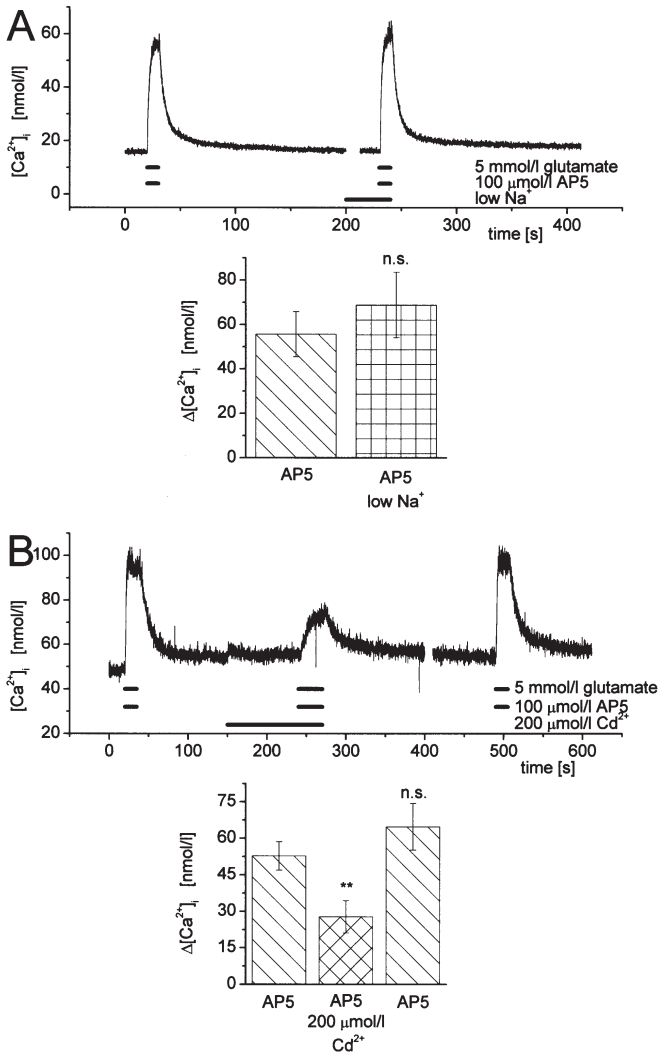

Figure 4. Effects of blocking the voltage-activated Ca^{2+} entry on the glutamate-induced Ca^{2+} transients. **A.** 5 mmol/l glutamate-induced Ca^{2+} transients under control conditions and in external solution with reduced Na^+ content ("low Na^+", see Materials and Methods). During the glutamate treatments, 100 μmol/l AP5 was also present. Synopsis of data from analogous experiments ($n = 5$) is given by the columns to the right. **B.** Ca^{2+} transients evoked by 5 mmol/l glutamate in control external solution (first and third transients) as well as following 200 μmol/l Cd^{2+} pretreatment (90 s; 100 μmol/l AP5 was applied simultaneously with the glutamate). Summary of these experiments ($n = 11$) is illustrated by the columns to the right. Note the considerably slower development of the Ca^{2+} transient in the presence of Cd^{2+}. $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; $\Delta[Ca^{2+}]_i$, the changes of the cytoplasmic Ca^{2+} concentration; ** $p < 0.01$; n.s. denotes differences that are not statistically significant.

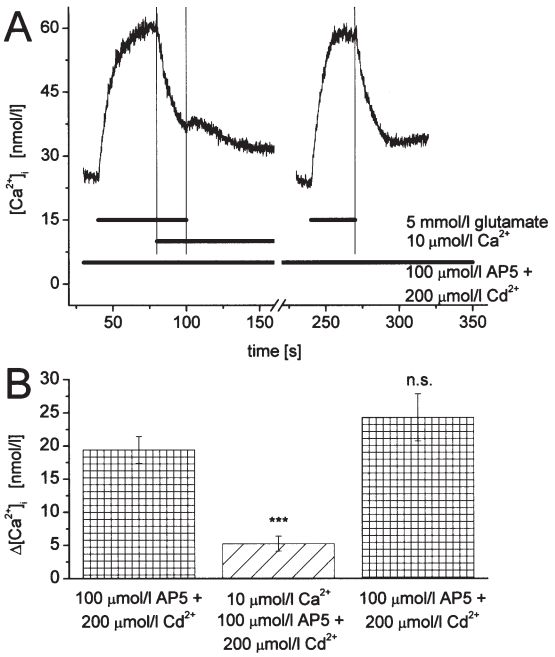

Figure 5. Effects of Ca^{2+} withdrawal on the AMPA receptor-related Ca^{2+} transients. **A.** 5 mmol/l glutamate challenge (60 s) evoked a Ca^{2+} transient in the presence of 100 μmol/l AP5 and 200 μmol/l Cd^{2+}. When the Ca^{2+} transient reached its maximum, the external solution was changed to a new one of identical composition except for a reduced $[Ca^{2+}]$ (10 μmol/l). The low external $[Ca^{2+}]$ was maintained even after terminating the glutamate application. When $[Ca^{2+}]_o$ was restored and 5 mmol/l glutamate applied again, a second Ca^{2+} transient could be evoked. Vertical lines are added to mark the environmental changes on the records. **B.** Synopsis of the experiments performed as shown in panel A ($n = 6$). From the left the columns indicate the peak values of the first Ca^{2+} transients, the intracellular $[Ca^{2+}]$ at the end of a 20–25 s Ca^{2+} withdrawal period and the peak values of the $[Ca^{2+}]$ transients obtained following the restoration of $[Ca^{2+}]_o$. $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; $\Delta[Ca^{2+}]_i$, the changes of the cytoplasmic Ca^{2+} concentration; *** $p < 0.001$; n.s. denotes differences that are not statistically significant.

of the voltage-gated Ca^{2+} channels to the genesis of the Ca^{2+} transients, Ca^{2+} entry through AMPA-type receptors emerged as a similarly decisive mechanism. To prove the role of this route experiments illustrated in Fig. 5 were performed. As part A of the figure shows, these measurements started with evoking 5 mmol/l glutamate transients in the presence of 100 μmol/l AP5 and 200 μmol/l Cd^{2+}. Due to the block of the voltage-gated Ca^{2+} channels, the averaged peak amplitude of the transients was only 19.4 ± 2.0 nmol/l ($n = 6$, Fig. 5B). As the relatively slowly developing Ca^{2+} transient reached its maximum, the $[Ca^{2+}]$ in the external solution was decreased to 10 μmol/l. Fig. 5A shows that the cytoplasmic $[Ca^{2+}]$

started to decrease abruptly even in the continuous presence of the glutamate. The height of the intracellular Ca^{2+} transient decreased in all 6 cells studied (to 5.2 ± 1.1 nmol/l during 20–25 s, $p < 0.001$). After restoring the external $[Ca^{2+}]$, 5 mmol/l glutamate was able again to induce Ca^{2+} transients of the same amplitude as prior to Ca^{2+} withdrawal (24.3 ± 3.5 nmol/l, the increase relative to the controls is not statistically significant). It has to be noted that the secondary rising phases shown on the decays of the Ca^{2+} transients were present in some cells but their mechanism was not analyzed.

Discussion

The present experiments demonstrate glutamate-induced cytoplasmic $[Ca^{2+}]$ increases in freshly isolated DCN neurones that could be identified as pyramidal cells. These neurones are known to possess ionotropic glutamate receptors, the genesis of these transients, consequently, is not surprising. The significant contribution of the AMPA receptors to the Ca^{2+} entry, however, is somewhat unexpected on the basis of their postulated subunit composition (Hunter et al. 1993; Petralia et al. 1996, 2000; Rubio and Wenthold 1997).

Quantitative features of the glutamate-induced cytoplasmic Ca^{2+} transients

The isolated DCN neurones required higher glutamate concentrations to produce cytoplasmic Ca^{2+} transients than reported for several other dissociated or cultured neurone types (Kudo and Ogura 1986; Hattori et al. 1998; Haak 1999; Hay and Lindsley 1999). On the contrary, half-maximal activation of glutamate receptor channels in patches from rat cerebellar Purkinje cells was reached in the presence of 432 μmol/l glutamate (Hausser and Roth 1997), a concentration close to the 50 % efficacy found in our experiments and two thirds of the neurones in chick magnocellular nucleus slices also required glutamate in the mmol/l range as well as the presence of extracellular Ca^{2+} to produce cytoplasmic Ca^{2+} transients (Zirpel et al. 1995). Moreover, the transients in the present experiments were relatively small as, for example, 100 μmol/l glutamate evoked intracellular $[Ca^{2+}]$ increases of 200 nmol/l in rat suprachiasmatic nucleus cells (Haak 1999) and 500 nmol/l in rat hippocampal neurones (Kudo and Ogura 1986).

Freshly isolated DCN neurones can handle effectively depolarization induced Ca^{2+} loads (Rusznák et al. 2000; Harasztosi et al. 2002). It is probable, however, that the isolation process causes a more substantial loss of the glutamate receptors than that of the voltage-gated Ca^{2+} channels (Harasztosi et al. 1999). An indication of this non-proportional loss of the appropriate membrane proteins may be that the amplitude of the 50 mmol/l K^+-evoked Ca^{2+} transients exceeded that of the glutamate-induced ones by 3–4 times in our case, while K^+ depolarizations and saturating glutamate doses were reported to evoke more similar cytoplamic $[Ca^{2+}]$ elevations in several other papers (Tymianski et al. 1993; Zirpel et al. 1995; Metzger et al. 2000). It has to be noted 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 (e.g. Patneau and Mayer 1990).

As for the size of the glutamate-related Ca^{2+} transients, freshly isolated neurones were reported to produce smaller Ca^{2+} transients on NMDA application than those maintained in cell culture (Burgoyne et al. 1988). The low peak amplitudes of our transients were justified by the fact that the calculated Ca^{2+} removal rates were similar to those reported for depolarization-induced transients of the same amplitude (Harasztosi et al. 2002). Nevertheless, the amplitude of the Ca^{2+} transients is influenced by several intrinsic properties and by the experimental conditions. The application of *in vitro* determined parameter values for calculating $[Ca^{2+}]$ also might led to underestimation of the Ca^{2+} transients. These uncertainties, however, do not render impossible the evaluation of pharmacological modifications in self-control experiments.

Contribution of certain Ca^{2+} entry pathways to the genesis of the glutamate-induced cytoplasmic Ca^{2+} transients

The finding that glutamate did not evoke measurable $[Ca^{2+}]_i$ elevations in the absence of extracellular Ca^{2+} indicates the definitive role of Ca^{2+} entry in the development of the transients. Contribution of Ca^{2+} release is rendered unlikely by this observation, by the shape of the dose-response curve and by the finding that the transients were not influenced by the Ca^{2+} content of the intracellular Ca^{2+} stores. It has to be mentioned that preceding K^+ depolarization increased considerably the amplitude of caffeine-induced Ca^{2+} transients in DCN neurones (Rusznák et al. 2000).

CNQX and the more specific AMPA receptor blocker, NBQX, (Mead and Stephens 1999) greatly reduced the glutamate-induced transients although the former compound was apparently more potent. This finding may indicate that besides blocking the AMPA receptors, CNQX acts on the NMDA receptors, too. However, the contribution of the NMDA receptors could be revealed only by facilitating their activation. This fact proves that NMDA receptors are present and functional although they do not seem to contribute significantly to the genesis of the Ca^{2+} transients under our control conditions. The ineffectivity of the AP5 and the resulted contradictions might be explained by the relatively low concentration of this blocker.

When testing the contribution of Ca^{2+} entry through voltage-gated channels, 200 μmol/l Cd^{2+} caused an approx. 50% block indicating a significant role of this pathway while the apparent inefficacy of the Na^+ withdrawal was virtually contradictory. To reconcile the two observations we suppose that the remaining current through the receptor channels was sufficient even under the "low Na^+" circumstances to depolarize the membrane and open the voltage-gated Ca^{2+} channels. Moreover, it can not be completely excluded that Cd^{2+} might influence the AMPA receptors (Joels et al. 1989). Such an effect may be reflected also by the Cd^{2+}-induced slowing of the Ca^{2+} transients.

The above considerations imply that Ca^{2+} permeable AMPA receptors are present in the isolated DCN neurones that we identified as pyramidal cells. The permeability properties of the AMPA receptors depend on their subunit composition, as the presence of GluR2 greatly hinders the Ca^{2+} movement (e.g. Keller et al. 1992; Geiger et al. 1995; Burnashev 1996; Ravindranathan et al. 2000). Experiments using labeling techniques demonstrated that neurones of the DCN generally expressed GluR1-4 subunits although the expression levels show significant variability among the various cell types (Hunter et al. 1993; Petralia et al. 1996, 2000; Rubio and Wenthold 1997) and even in the membrane of the same neurone (e.g. pyramidal cells) (Rubio and Wenthold 1999; Petralia et al. 2000). The postulated subunit composition of the AMPA receptors of the DCN neurones including the pyramidal cells, consequently, does not indicate that these receptors are particularly permeable to Ca^{2+}.

Functional significance of glutamate-related Ca^{2+} signalization in auditory pathway neurones

The functional importance of Ca^{2+}-permeable AMPA receptors of the avian auditory pathway (Otis et al. 1995) was indicated by the finding that these receptors showed higher Ca^{2+} permeability and faster kinetics than those present in some other brainstem nuclei (Ravindranathan et al. 2000). In cochlear nuclear neurones of mice fast, Ca^{2+}-permeable AMPA receptors were characteristic of cells receiving direct auditory nerve input while cartwheel cells of the DCN (that are targeted by parallel fibers) possessed Ca^{2+}-impermeable, slow AMPA receptors (Gardner et al. 1999, 2001). The DCN pyramidal neurones that receive spatially separated and functionally different inputs from both these sources (Kane 1974; Bilak et al. 1996; Waller et al. 1996) expressed Ca^{2+}-impermeable AMPA receptors at both localisations (Gardner et al. 1999, 2001). These findings are in agreement with the data indicating the general presence of GluR2 subunit in the DCN (Hunter et al. 1993; Petralia et al. 1996, 2000; Rubio and Wenthold 1997) and seem to question the results of our experiments independently of whether the identification of the pyramidal cells was unambiguous.

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 $[Ca^{2+}]_i$ 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 (Korada and Schwartz 2000). Third, the auditory pathway neurones in rodents undergo changes along with the maturation of the hearing function (see e.g. Lohmann and Friauf 1996). The younger age of the rats of the present study than that of the mice used by Gardner et al. (1999, 2001) may be accompanied by a lower expression level of the GluR2 subunit. Indeed, a similar transitory contribution of AMPA receptors to cytoplasmic Ca^{2+} transients was noticed in spinal motoneurones of the rat during

the days of their embryonic life when the synaptic refinement of these neurones takes place (Metzger et al. 2000).

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