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Role played by sodium in activity-dependent secretion of neurotrophins – revisited

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Abstract

In previous experiments, a causal relationship between sodium influx and secretion of nerve growth factor (NGF) was deduced from the observation that the sodium substitute *N*-methyl-D-glucamine (NMDG) abolished any activity-mediated NGF secretion that depends on intact internal calcium stores. However, all available experimental evidence speaks against sodium-mediated calcium mobilization from these stores under physiological conditions. We now report that rapid sodium influx initiated by monensin or ouabain did not induce brain-derived neurotrophic factor (BDNF) secretion from either native hippocampal slices or BDNF-transduced hippocampal neuronal cultures. Additionally, we found marked differences between the replacement of sodium by NMDG and sucrose on the one hand, and choline and lithium on the other. Replacement of 100% (and as little as 10%) sodium by NMDG or sucrose not only blocked the activity-mediated neurotrophin secretion, but itself led to a rapid and substantial increase of neurotrophin secretion. In contrast, the replacement of sodium (10% and 100%) by lithium and choline did not result in a release of neurotrophins, and only 100% replacement blocked the activity-mediated neurotrophin secretion. We conclude that the blocking effects of NMDG and sucrose on neurotrophin secretion do not reflect the sodium replacement, but instead represent an independent blocking effect. These differences were also reflected in part by electrophysiological investigations in individually patched hippocampal neurons. The importance of the present observations lies not only in the reevaluation of the involvement of sodium in activity-dependent neurotrophin secretion, but also in the demonstration that sodium replacement may initiate 'side effects' that are unrelated to sodium replacement.

Introduction

In the context of the modulatory role played by neurotrophins in activity-dependent neuronal plasticity (reviewed by Thoenen, 1995; Berninger & Poo, 1996; Bonhoeffer, 1996; McAllister *et al*., 1999; Schuman, 1999), the mechanism and site of neurotrophin secretion are of paramount importance. It has been demonstrated previously that nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are secreted from hippocampal slices and neurotrophin-transfected primary cultures of hippocampal neurons by depolarization with high potassium, veratridine and glutamate (Blöchl & Thoenen, 1995, 1996; Griesbeck *et al*., 1999). These release mechanisms were shown to be independent of extracellular calcium, but dependent on intact intracellular calcium stores. Supporting these findings, Gärtner *et al*. (2000) reported that the storage compartment of neurotrophins is closely associated with that of intracellular calcium stores. They showed that neurotrophins were predominantly localized in endoplasmic reticulum (ER)-like membrane-confined compartments, but in neither synaptic nor large dense core vesicles. NGF secretion initiated by the sodium-channel activator veratridine and by high-potassium depolarization could be blocked by tetrodotoxin, whereas the glutamate-mediated NGF secretion was not impaired by tetrodotoxin, but by blockers of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (Blöchl & Thoenen, 1995), which represent predominantly ligand-gated sodium channels (Hollmann & Heinemann, 1994). These observations suggested a causal relationship between activity-mediated sodium influx and the subsequent secretion of NGF. This interpretation was strongly supported by the observation that the replacement of sodium by the most frequently used sodium substitute, *N*-methyl-D-glucamine (NMDG, e.g. Simasko, 1994; Chidekel *et al*., 1997; Rose & Ransom, 1997; Sidky & Baimbridge, 1997; Cao & Peng, 1998; Taschenberger & Grantyn, 1998) blocked the high-potassium-, veratridine- and glutamate-mediated NGF secretion from both hippocampal slices and NGF-transfected hippocampal neurons (Blöchl & Thoenen, 1995). However, there is no experimental evidence that sodium influx mobilizes calcium from intracellular stores. Wherever such a relationship has been addressed in neurons, the outcome was always negative (Kennedy & Thomas, 1995; Hoyt *et al*., 1998). In view of this situation, we decided to subject this question to a more thorough analysis. We compared the effect of sodium replacement by NMDG with that of other substitutes, i.e. sucrose, lithium and choline, and evaluated whether other experimental procedures that result in a rapid increase of intracellular sodium (sodium ionophores and blockers of plasma membrane Na^+/K^+-ATPase) initiated the secretion of neurotrophins.

We report here that there is no evidence for a causal relationship between sodium influx and the initiation of the secretion of neurotrophins, as a dramatic sodium influx induced by the sodium ionophore monensin neither initiated a secretion of neurotrophins nor interfered with the high-potassium-mediated neurotrophin secretion. Moreover, the replacement of as little as 10% sodium by NMDG or sucrose, but not by choline or lithium, caused a neurotrophin

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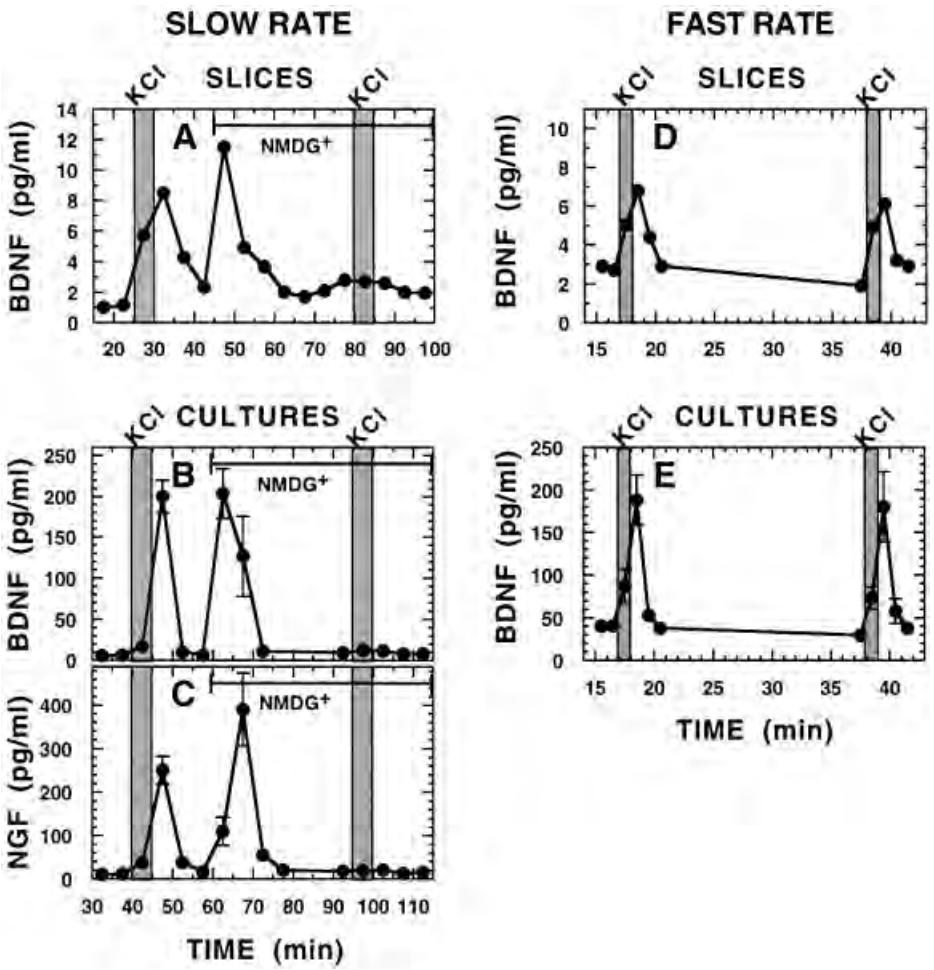

FIG. 1. Effects of the replacement of 100% sodium by NMDG on BDNF release (A and B) and NGF release (C). The effect of a first depolarization by KCl (50 mM) is compared with that of a second depolarization under the influence of the replacement of sodium by NMDG in hippocampal slices (A) and cultures (B and C). The faster perfusion rate (0.5 mL/min) is compared with the slow perfusion rate (0.1 mL/min) in hippocampal slices (D) and cultures (E) on KCl-induced BDNF secretion. The values are the mean ± SD of three to six independent experiments.

secretion of short duration and blocked the subsequent action of high potassium and glutamate.

Materials and methods

Primary cultures of hippocampal neurons

Hippocampal neurons were prepared from E18 Wistar rats according to the procedure by Zafra *et al*. (1990). Briefly, adult pregnant Wistar rats were killed by CO_2 overdose. Hippocampi from E18 embryos were dissected and were incubated for 20 min at 37 °C in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, containing 10 mM glucose, 1 mg/mL bovine serum albumin (BSA), 1 μg/mL DNAse and 12 μg/mL papain, dissociated with a Pasteur pipette (fire-polished tip), and centrifuged (5 min at 1000 r.p.m.). Cells were resuspended in Dulbecco's minimum essential medium (DMEM, Gibco) containing 10% foetal calf serum (FCS, Hyclone, Cramlington, UK). Neurons were plated at a density of 3×10^5 per 0.8 cm^2 on glass coverslips (in a 48-well dish) precoated with poly-D,L-ornithine (0.5 mg/mL). After 2 h, the medium was changed to a serum-free defined medium according to Brewer & Cotman (1989). Because it was observed that non-neuronal cells were more likely to be infected with the adenovirus, cultures were treated with 5 μM cytosine β-D-arabinofuranoside on day 3 for 24 h, which substantially decreased the percentage of non-neuronal cells and hence increased the amount of neuronal cells infected.

In order to obtain reliable and reproducible results, the age of the embryos from which the hippocampal neurons were isolated proved crucial. The same was true for the period for which the neurons were kept in culture. Eighteen-day-old embryos and culture periods of 9–10 days proved optimal for the isolation and culture procedure used in all the present experiments. From older embryos, the yield of neurons was substantially smaller, while the proportion of non-neural cells increased.

In order to compare experiments obtained from cultures infected with different adenoviral stocks, the amount of virus application to cultures was not only determined by plaque-forming units, but also adjusted so that the first 50 mM potassium-mediated BDNF secretion was ~ 250 pg/mL. Infection times were kept between 24 and 30 h.

Hippocampal slices

Slices (350 μm) were prepared from hippocampi of adult Wistar rats (of either sex) in cold, oxygenated modified Hanks buffer (in mM: NaCl, 125; KCl, 5; NaH_2PO_4, 1.2; $CaCl_2$, 1; $MgCl_2$, 1.2; $ZnCl_2$, 1 μM; glucose, 10; *N*-2-[hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES), 25; BSA, 0.25%; pH 7.4) using a MacIlwain tissue chopper. Slices obtained from one hippocampus were placed in a perfusion chamber (Minucell and Minutissue, Bad Abbach, Germany) and constantly perfused with Hanks buffer equilibrated with 95% O_2 and 5% CO_2. After a recovery phase of 15–30 min, the release experiments were started at a flow rate of either 0.1 or 0.5 mL/min.

Adenoviral vectors

Viral vectors were constructed by homologous recombination in 293 cells (McGrory *et al*., 1989; Graham & Prevec, 1991). AdCMV-NGF contained the sequence of mouse preproNGF, and AdCMV-

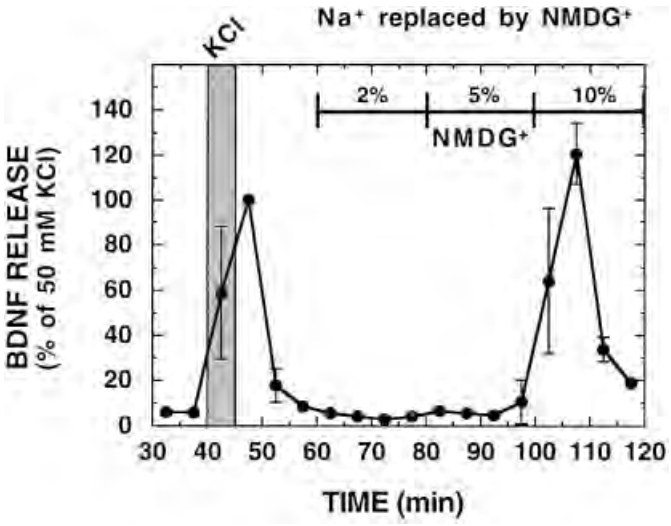

FIG. 2. Dose–response relationship of the short-term effects of NMDG replacement of sodium on BDNF release. This release was compared with that obtained by a first depolarization with KCl. Perfusion rate was 0.1 mL/min. The values are the mean ± SD of three independent experiments.

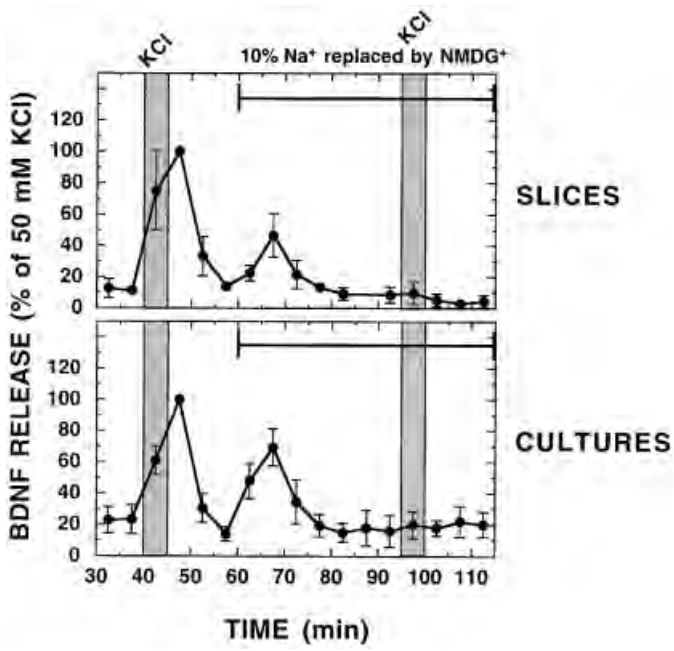

FIG. 3. Long-term effects of the replacement of 10% sodium by NMDG on BDNF release in slices and cultures. The effect of a first depolarization by KCl (50 mM) is compared with that of a second depolarization under the influence of the replacement of 10% sodium by NMDG. Perfusion rates were 0.1 mL/min. The values are the mean ± SD of four independent experiments.

BDNFmyc the sequence of mouse preproBDNF, which was tagged with the 9E10 myc epitope (Evan *et al*., 1985) at the C terminus of the BDNF coding region lacking the last three base pairs. It was shown that the myc epitope interferes with neither the activity (Klose, 1998) nor the sorting of neurotrophins (Di Polo *et al*., 1998; Möller *et al*., 1998). The neurotrophin cDNAs between the cytomegalovirus (CMV) promoter and the bovine growth hormone terminator were cloned into the shuttle plasmid pXCJL1/2. Shuttle plasmids and the plasmid pJM17, which contains the entire circularized genome of human adenovirus type 5, were cotransfected into 293 cells. After homologous recombination, recombinant clones were isolated by plaque purification, amplified and purified in CsCl density gradient. Stocks were prepared with titres ranging from 3×10^{10} to 5×10^{11} plaque-forming units/mL.

Release experiments

After 9 days in culture, neurons plated on 0.8 cm^2 coverslips were infected with ~ 10^8 plaque-forming units of either AdCMV-NGF or AdCMV-BDNFmyc in a volume of 300 µL serum-free defined medium. On the following day, neurons were used for the release experiments. Cultured hippocampal neurons or hippocampal slices were perfused with modified Hanks buffer at a perfusion rate of 0.1 mL/min or 0.5 mL/min in a perfusion system, as described by Blöchl & Thoenen (1995). The viability of neurons before and after the release experiments was compared. Living and dead cells were visualized using fluorescein-diacetate and propidium iodide, respectively, according to the procedure of Jones & Senft (1985). Samples derived from perfused hippocampal cultures were collected at 5-min intervals at a perfusion rate of 0.1 mL/min, or at 1-min intervals at a rate of 0.5 mL/min. BDNF or NGF concentration was determined by ELISA. Depolarization was achieved by replacing 50 mM NaCl in the perfusion medium by 50 mM KCl, or by addition of 50 µM glutamate. Investigations addressing extracellular sodium were conducted by the iso-osmotic replacement of sodium by NMDG, sucrose, choline or lithium.

For the evaluation of the role of calcium in the release, $CaCl_2$ was omitted from the perfusion buffer. The specific high-affinity calcium chelator bis-(*o*-aminophenoxy)-ethan-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) or its membrane-permeable ester BAPTA-AM (Tsien, 1981) was added at a concentration of 10 µM.

Calculations were carried out as follows: a first depolarization by KCl was used as a reference (100%) and, after the collection of three samples, perfusion was performed in either Hanks buffer or one of the modifications described in the Results section. After an equilibration and recovery period of 30 min (0.1 mL/min perfusion rate) or 15 min (0.5 mL/min), samples were again collected and a second depolarization was initiated either under control conditions or in one of the modifications. In order to compare the higher perfusion rate with the lower, the neurotrophin secretion was expressed as a percentage relative to the first depolarization (100%). In experiments run with ~ 10^8 plaque-forming units of AdCMV-BDNFmyc, the 100% value ranged between 100 and 250 pg/mL BDNF.

Enzyme immunoassays (ELISAs)

NGF and BDNF were quantified by a two-site immunoassay according to Blöchl & Thoenen (1995) and Canossa *et al*. (1997), for NGF and BDNF, respectively. For details of the characterization of the monoclonal mouse anti-BDNF antibodies, see Kolbeck *et al*. (1999).

For BDNF, microtitre plates (Nunc, Germany) were coated overnight at 4 °C with 200 µL of 0.05 M carbonate buffer, pH 9.7, containing mAb against BDNF #1 (1 µg/mL). Unbound antibodies were removed and plates blocked with 300 µL modified Hanks buffer containing 2% BSA and 0.1% Triton X-100 for 2 h at room temperature. Samples (170 µL) and standards (2–1000 pg/mL) were each incubated overnight at 4 °C together with the peroxidase-conjugated mAb against BDNF #9 (30 µL, diluted in Hanks buffer containing 6.67% BSA and 0.033% Triton X-100). Plates were washed three times with 300 µL PBS containing 0.1% Tween-20, and developed using 200 µL BM Blue peroxidase substrate (Boehringer Mannheim). The reaction was stopped after 15 min by addition of

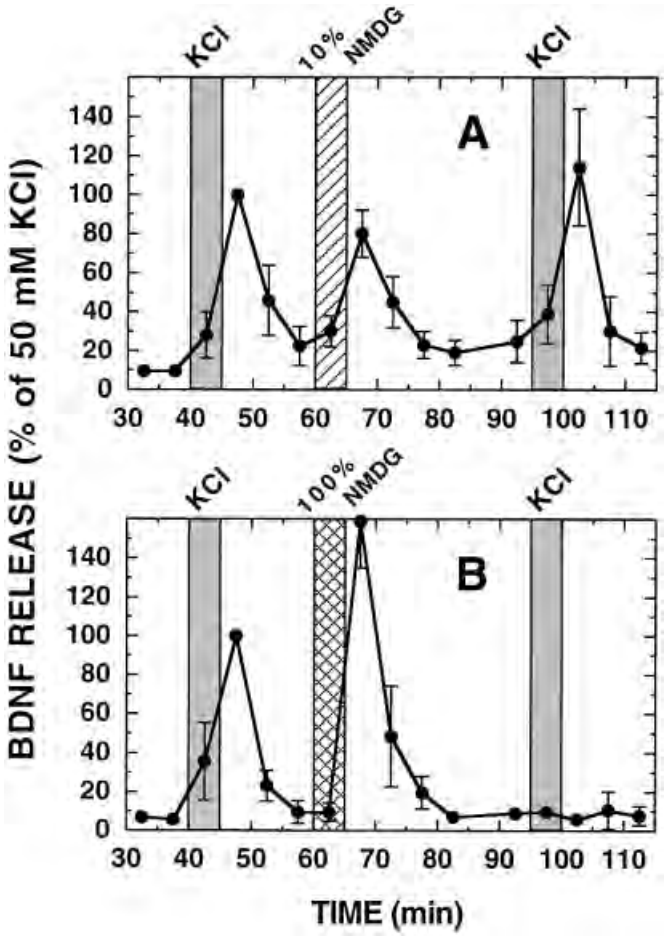

FIG. 4. Effects of a short, 1-min replacement of 10% (A, single-hatched bar) and 100% sodium (B, cross-hatched bar) by NMDG on the depolarization-(KCl-)induced BDNF secretion. The values are the mean ± SD of three independent experiments.

50 µL 1 M H_2SO_4. Absorbance, in optical density units (OD), was read at 492 nm.

For NGF, microtitre plates were coated with 200 µL of a mAb against NGF (27/21, 1 µg/mL) in 0.05 M carbonate buffer, pH 9.7, and incubated overnight at 4 °C. After washing with 250 µL 0.05 M Tris–HCl buffer, pH 7.0, containing 0.2 M NaCl and 0.1% Triton X-100, plates were blocked with coating buffer containing 2% BSA for 2 h at room temperature. Blocking buffer was removed and 150 µL standards (2–1000 pg/mL) and samples were incubated in modified Hanks buffer overnight at 4 °C. Wells were washed twice in 250 µL washing buffer and incubated overnight at 4 °C with 150 µL of a peroxidase-conjugated mAb against NGF (27/21). Plates were then washed three times with 250 µL washing buffer and developed using 200 µL BM Blue peroxidase substrate (Boehringer Mannheim). The reaction was stopped after 30 min by addition of 50 µL 1 M H_2SO_4. Absorbance, in optical density units (OD), was read at 492 nm.

Sodium and calcium imaging

For intracellular loading and fluorescence ratio imaging, the fluorescence dyes SBFI-AM (acetoxymethylester of sodium-binding benzofuran isophthalate; Molecular Probes, Leiden, The Netherlands) for sodium and FURA-2-AM for calcium imaging, dissolved in dimethyl sulphoxide/10% pluronic acid P-127 (Molecular Probes) was used (Minta & Tsien, 1989). Hippocampal cells (plated on 1.5 cm^2 coverslips, 10 days in culture) were loaded with 20 µM SBFI-AM for 90 min or with 2 µM FURA-2-AM for 40 min in Hanks buffer at room temperature. At the end of the loading period, the cells were transferred to an RC-20 experimental chamber (volume, 36 µL; Warner Instrument, Hamden, CT, USA) and perfused with Hanks buffer solution (standard flow rate, 1 mL/min). The chamber and the microscope table were warmed to 37 °C. Cells were equilibrated with Hanks buffer for 5–10 min before starting the experiments.

The experimental chamber was mounted on a P1 platform (Warner Instrument) fixed to a Zeiss Axiovert stage adapter. Cells were visualized with a Zeiss Fluar 40 ×/1.30 oil objective, using an inverted microscope (Axiovert 100, Zeiss). Fluorescence was determined at the excitation wavelengths of 340 and 380 nm with an intensified charge coupled device camera (C24000-87, Hamamatsu, Middlesex, UK), and images were processed with the Argus fluorescence ratios. Images of cell bodies were taken at a sampling rate of 0.75/s, with eight frames being averaged for each image. Unless stated otherwise, experiments were repeated on at least four different coverslips, each allowing analysis of three to seven individual cells.

Electrophysiology

For electrophysiological recordings, cultured hippocampal neurons (10 days old) were used, plated at the same density as for the release experiments (3×10^5 per glass coverslip of 10 mm^2). The recordings were performed with the whole-cell perforated-patch technique in current-clamp mode. Amphotericin B (Sigma) was used for perforation. The electrodes were tip-filled with internal solution, and then back-filled with internal solution containing amphotericin B (200 µg/mL). The internal solution contained (in mM): KCl, 110; $MgCl_2$, 5; HEPES, 40; ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.6 (pH 7.2). External bath solutions were the same as for the release experiments. The recording chamber was constantly perfused with fresh medium at a flow rate of 0.5 mL/min. Cells were patched in voltage-clamp mode (Axoclamp 2B amplifier, Axon Instruments). After a stable patch was obtained, the recording was performed in current-clamp mode to monitor the membrane potential constantly. Data were collected at a sample rate of 5 kHz and stored on a computer for further analysis. Before and after washout of the different sodium replacement media with normal media, a 50-ms depolarizing pulse of 0.05–0.1 nA was given. An agar bridge was used as an indifferent electrode. In a typical experiment, similar to neurotrophin-secretion experiments under fast-flow conditions (0.5 mL/min), cells were equilibrated for at least 10 min with normal Hanks buffer, depolarized for 1 min with the addition of 50 mM KCl, washed for 10 min, and different sodium substitutes applied for 15 min.

Results

Characterization of the experimental procedures

The present investigations are based on an ELISA for BDNF that has recently become available (Canossa *et al*., 1997; Kolbeck *et al*., 1999). Monoclonal anti-BDNF antibodies made it possible to determine BDNF reliably and reproducibly in perfusion fluids and tissue samples. In addition, transduction of hippocampal neurons with adenoviral vectors (Griesbeck *et al*., 1997) permitted a standardized, reproducible transduction of BDNF and NGF expression. The fact that BDNF levels in the hippocampus are ~ 35 times higher than those of NGF (Korsching *et al*., 1985; Nawa *et al*., 1995; Hoener & Varon, 1997; Kolbeck *et al*., 1999) permitted a more reliable determination of BDNF secretion from native hippocampal slices (Fig. 1A)

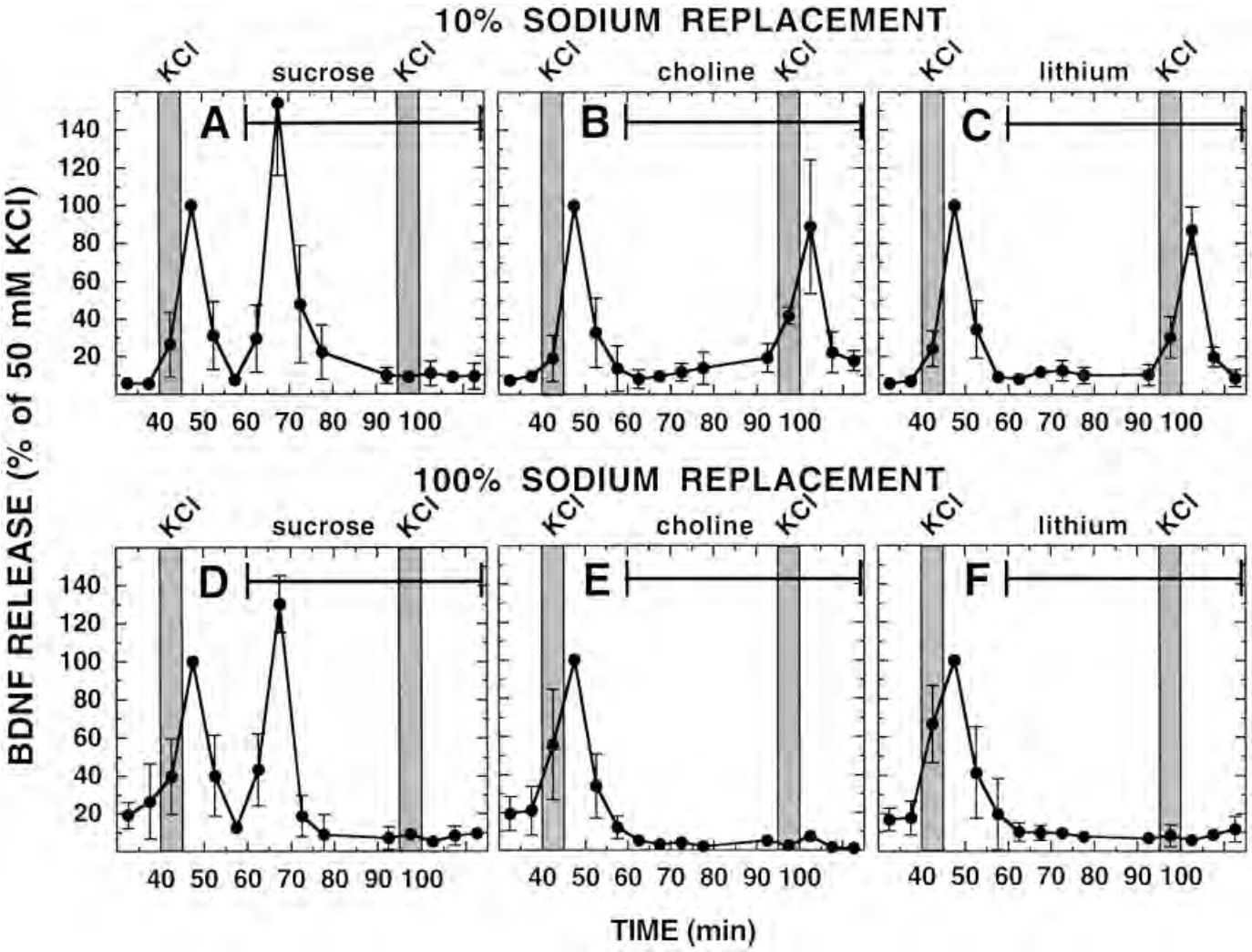

FIG. 5. Comparison of different sodium substitutes with respect to their effects on BDNF release. The effects of a first depolarization by KCl (50 mM) are compared with those of a second depolarization under the influence of the replacement of 10% sodium (A–C) and 100% sodium (D–F) by sucrose (A and D), choline (B and E) and lithium (C and F). The values are the mean ± SD of three independent experiments.

compared with NGF in previous experiments. Those levels were at the limit of detectability. It further demonstrated that transduction with adenoviral vectors did not change the release characteristics in comparison with those of BDNF from native, non-transduced hippocampal slices. Moreover, release characteristics of adenoviral-transduced hippocampal neurons were the same for BDNF and NGF (Fig. 1B and C). Thus, the release characteristics effected by high potassium were the same for non-transduced hippocampal slices, and both NGF- and BDNF-transduced hippocampal cultures (see also Griesbeck *et al*., 1999).

In most experiments dealing with activity-dependent neurotransmitter release from hippocampal slices, relatively high perfusion rates (0.5–5 mL/min) have been used (Elverfors *et al*., 1997; Malcangio *et al*., 1997; Martire *et al*., 1997). We therefore evaluated whether increasing the perfusion rate from 0.1 mL/min (slow rate) to 0.5 mL/min (fast rate) changes the principal characteristics. As shown in Fig. 1D and E, the secretion of BDNF at the fast rate occurred within one to two fractions (1–2 min) in both hippocampal slices and BDNF-transduced cultures, and was similar to the results obtained using a slower rate (one to two fractions within 5–10 min). The higher perfusion rate permitted a more precise resolution of the time course of secretion. Using the same experimental procedure and a duration of depolarization (50 mM KCl) of 1 or 5 min, Griesbeck *et al*. (1999) demonstrated that the release is independent of the length of depolarization.

Figure 1 also shows that the maximal levels of BDNF and NGF secretion per fraction in adenoviral-transduced hippocampal cultures were 200–250 pg/mL per fraction, with basal release levels of 10–20 pg/mL. BDNF secretion in slices was 3–10 pg/mL with basal release levels of 1 pg/mL.

Although similar results were obtained in different sets of experiments under the given experimental conditions, we nevertheless expressed the results in terms of the percentage of maximal BDNF release after a first depolarization induced by 50 mM KCl. In this way, it was possible to compare slices and transfected cultures on the one hand, and different perfusion rates on the other.

Replacement of sodium by NMDG

In agreement with previous experiments for NGF (Blöchl & Thoenen, 1995), complete replacement of sodium by NMDG inhibited depolarization-induced BDNF secretion from hippocampal slices, and BDNF and NGF secretion from transduced hippocampal cultures

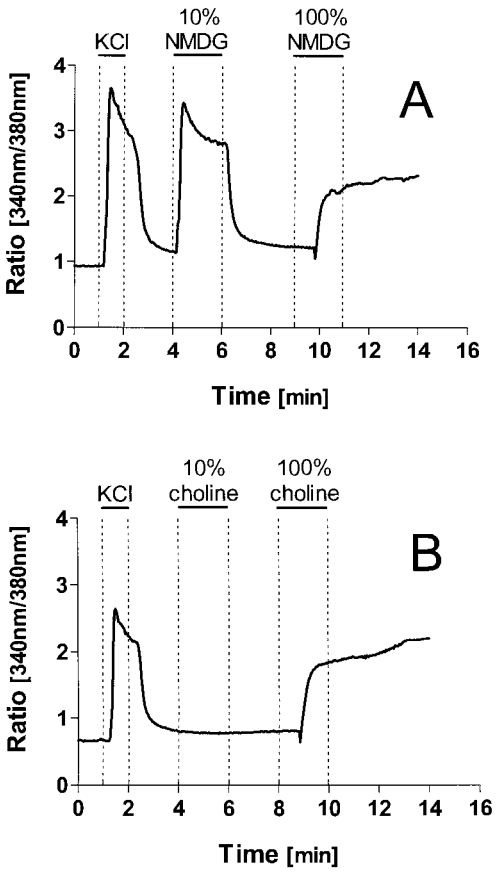

FIG. 6. Influence of the replacement of 10% and 100% sodium by *N*-methyl-D-glucamine (A) or choline (B) on $[Ca^{2+}]_i$ was compared with that of an initial depolarization with 50 mM KCl. Hippocampal neurons were loaded for 40 min with 2 μM Fura2-AM, and $[Ca^{2+}]_i$ was estimated from the respective ratio of the fluorescence at 340 and 380 nm excitation wavelengths. Images were taken at a sampling rate of 0.75/s; seven cells were averaged for each image. A representative experiment (out of three experiments) is shown.

(Fig. 1A–C). In addition to this inhibition, the replacement of sodium by NMDG itself resulted in neurotrophin secretion. For BDNF, this was shown in slices as well as in cultures (Fig. 1A and B).

In view of the dramatic secretion of neurotrophins by replacing 100% of the sodium (125 mM) by NMDG, we decided to evaluate the dose dependency of this effect by using 2, 5 and 10% of NMDG. Replacement of 2 and 5% did not result in a detectable release; however, the replacement of 10% of sodium by NMDG initiated a BDNF secretion that was similar to that produced by 50 mM KCl (Fig. 2). When sodium was replaced shortly (15 min) after the initial KCl-induced BDNF secretion, the amount of BDNF released was significantly smaller (Fig. 3) compared with that after the usual washing period of 55 min (Fig. 2). Similar results were obtained by Griesbeck *et al.* (1999).

The 10% replacement elicited similar effects in both slices and cultures. In both systems, depolarization-mediated BDNF secretion by 50 mM KCl was totally inhibited by continuous replacement of 10% of sodium by NMDG (Fig. 3), but not by 5% (two experiments, data not shown).

KCl-mediated BDNF secretion was unimpaired after replacement of 10% sodium by NMDG for only a brief period (i.e. 5 min), whereas 100% replacement of sodium for 5 min irreversibly blocked KCl-initiated BDNF secretion within the time limit of the duration of the experiment (i.e. 40 min, Fig. 4).

Continuous replacement of 10% sodium by NMDG prevented not only the KCl-induced BDNF secretion, but also the secretion initiated by 50 μM glutamate (in two experiments, data not shown).

Comparison between sodium replacement by NMDG, sucrose, choline and lithium

In view of the very surprising observations resulting from the replacement of sodium with NMDG, we evaluated the effects of other sodium substitutes that are commonly used in electrophysiological experiments (Woodward *et al.*, 1988; Concalves & Carvalho, 1995; De la Fuente *et al.*, 1996; Shillingford *et al.*, 1996; Martire *et al.*, 1997; Sidky & Baimbridge, 1997). These substances were compared with respect to their ability to secrete neurotrophins on their own and to prevent KCl-induced neurotrophin secretion. The replacement of sodium by sucrose resulted in effects very similar to those seen using NMDG. The continuous replacement of both 10% and 100% of sodium by sucrose caused BDNF secretion and prevented the 50 mM KCl-induced secretion (Fig. 5A and D). Replacement of sodium by choline or lithium presented a completely different picture. These sodium substitutes did not cause any BDNF secretion on their own, nor did the continuous 10% replacement by the substitutes influence KCl-mediated BDNF secretion (Fig. 5B, C, E and F).

Only the continuous 100% sodium replacement by choline and lithium inhibited depolarization-induced secretion, as observed after the replacement by NMDG and sucrose (Fig. 5E and F).

The effects of the different substitutes themselves were further analysed by calcium imaging. An initial depolarization-induced $[Ca]_i$-increase by 50 mM KCl was compared with 10% replacement of sodium by the different substitutes. In three out of five experiments the 10% replacement of sodium by NMDG caused a similar intracellular calcium response to that of 50 mM KCl (Fig. 6A), which was also observed after the replacement of 10% sodium by sucrose in two out of two experiments (data not shown). No change in $[Ca]_i$ was observed with the replacement of 10% sodium by choline (Fig. 6B) or lithium (data not shown) in three experiments each. These results correlate well with the secretion of BDNF observed in Fig. 5, suggesting that 12.5 mM NMDG and sucrose cause an increase in intracellular calcium, and therefore the release of neurotrophins.

A different result was obtained by replacing 100% sodium by NMDG, sucrose, choline and lithium. The replacements all caused an increase in $[Ca]_i$ delayed by more than 1 min compared with the immediate response seen with KCl and the 10% replacement of sodium by NMDG, in which the peak was rapidly attained. Even extensive washing with Hanks buffer containing sodium did not decrease the $[Ca]_i$ concentration, suggesting a persisting effect.

Electrophysiological consequences of the replacement of sodium by NMDG, sucrose, choline and lithium

The differences seen in the perfusion experiments were investigated at the electrophysiological level (Fig. 7). Intracellular recordings were made using the whole-cell perforated-patch technique in current-clamp mode. During baseline conditions, the neuron had a stable resting membrane potential of –62.8 mV (SEM ± 1.0; $n = 12$), but typically fired with high spontaneous activity. We recorded from different cells in terms of spiking behaviour. Most of the cells ($n = 8$) were bursting with an interval of 10 bursts/min (SEM ± 0.3). The average number of action potentials within the burst was 42 (SEM ± 1.9) with an average frequency of 19 Hz (SEM ± 0.8). Four cells showed no bursting. The firing frequency of these cells was 0.22 Hz (SEM ± 0.06). Wash-in of KCl (50 mM) depolarized the neuron strongly, and spike activity was completely abolished due to a

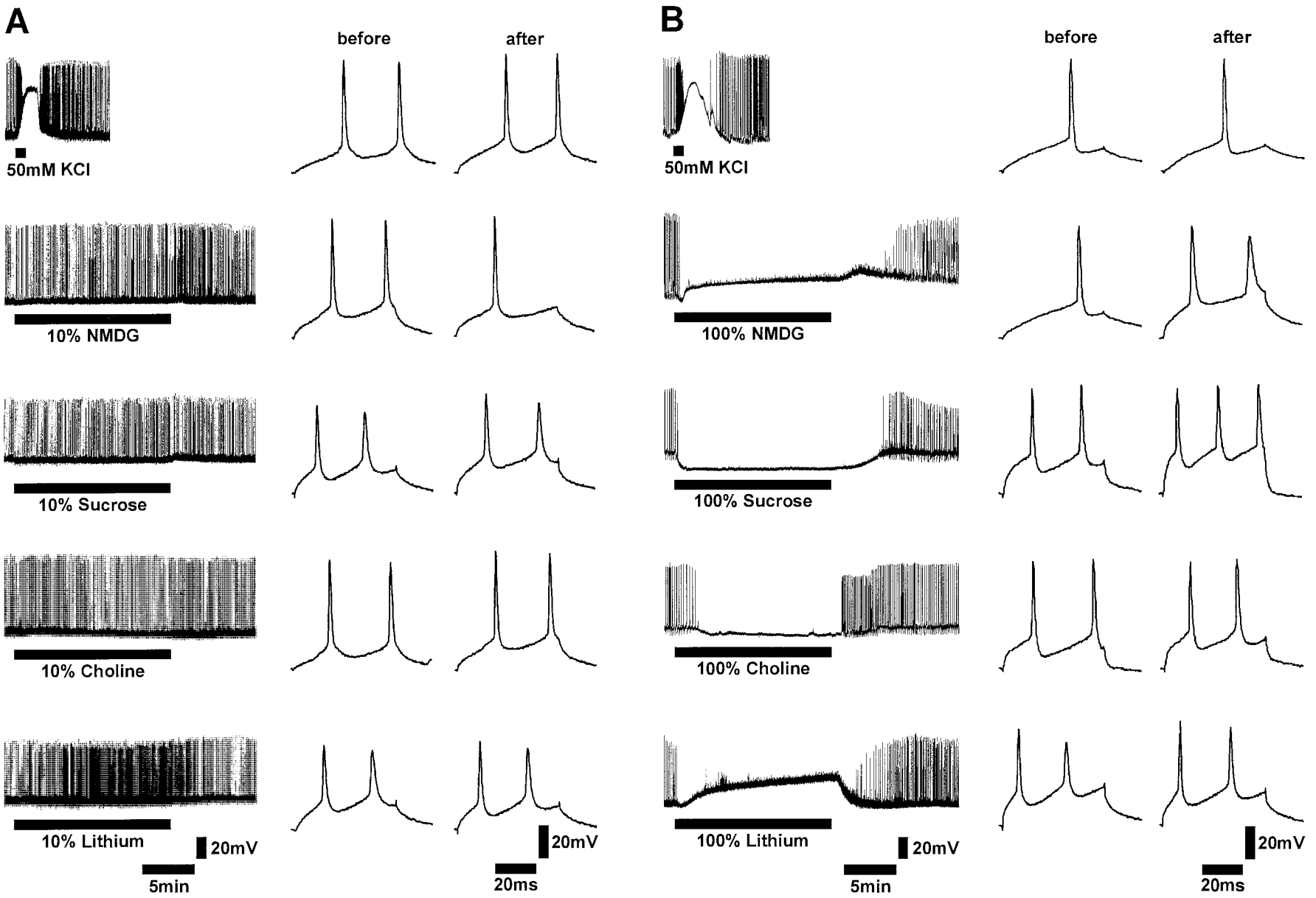

FIG. 7. Electrophysiological monitoring of a single cultured hippocampal neuron during different sodium replacements in the perfusion medium. Different sodium substitutes were applied for 15 min after a 10-min equilibration with normal Hanks buffer, 1-min depolarization with 50 mM KCl and a 10-min washing period. Shown here are the recording traces (perforated-patch recording, current-clamp, membrane potential –60 mV) of different neurons during application of the different solutions. (A) The 10% sodium substitution, (B) 100% substitution of sodium by the different substitutes NMDG, sucrose, choline and lithium. On the right side of each panel, responses of the neurons in normal medium to an intracellular depolarization pulse (50 ms, 0.1 nA) after each medium exchange are shown, demonstrating that the membrane properties of the cell remain stable over the course of the experiment. One representative experiment (out of at least three) is shown for each substitution of sodium. Only one representative KCl-mediated depolarization is shown in each panel.

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depolarization block. Average depolarization by 50 mM KCl was 46.8 mV (SEM ± 2.0; $n = 12$).

Replacement of 10% sodium by the different substitutes had no significant effects on the cells, whereas 100% abolished all spontaneous action potentials. Replacement of 100% sodium by NMDG, sucrose and lithium immediately affected the cells, whereas almost no effect was seen during the first 2 min of sodium substituted by 100% choline. Accordingly, the neurons also recovered almost instantly after choline or lithium were washed out. In contrast, it took more than 7 min for the action potentials to recover after replacement of 100% sodium by NMDG or sucrose. Depolarizations of 0–20 mV and up to 30 mV were seen using NMDG and lithium, respectively, whereas the replacement of sodium by sucrose caused hyperpolarizations of up to –15 mV. It should also be mentioned that, in some cells, there was a dramatic reduction of spontaneous action potentials by replacing 30% sodium by NMDG and sucrose, but not by choline or lithium.

Wash-in of 50 mM KCl during the period of 100% sodium replacement caused an immediate depolarization of the neurons in a similar fashion as in the presence of sodium (data not shown).

It has to be emphasized that these observations in individually patched cells were made not in isolated individual neurons, but in neurons that were relatively densely plated in a similar manner as used for the release experiments. Similar results were obtained with cultures of 10 times lower density than normal (3×10^4 cells per 0.8 cm^2).

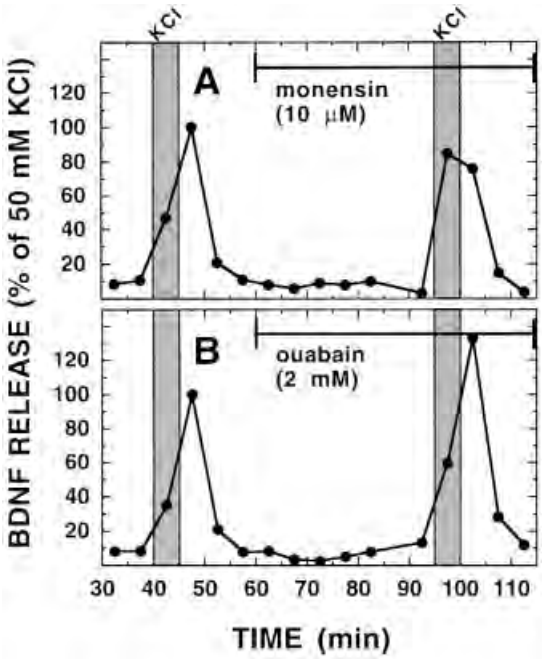

FIG. 8. Effect of sodium influx on activity-dependent BDNF secretion. The effect of a first depolarization by KCl (50 mM) on BDNF release is compared with that of a second treatment under the influence of the addition of 10 μM monensin (A) and 2 mM ouabain (B). One representative experiment (out of three independent experiments) is shown.

Effect of monensin and ouabain on high potassium- and glutamate-mediated neurotrophin secretion

In order to address the question whether sodium influx per se causes the secretion of BDNF, the effects of monensin (a sodium ionophore) and ouabain (a blocker of plasma membrane Na^+/K^+-ATPase) were analysed.

Incubation with 10 μM monensin did not result in a secretion of BDNF, even when incubated for longer periods (30 min), and did not interfere with KCl-induced BDNF secretion (Fig. 8). The same was true for 2 mM ouabain administered over a period of 30 min. In order to determine whether monensin and ouabain did in fact cause an intracellular sodium increase under our perfusion conditions, we determined the time course of sodium influx using sodium imaging.

The results presented in Fig. 9 demonstrate that monensin, ouabain and glutamate are responsible for a substantial increase in

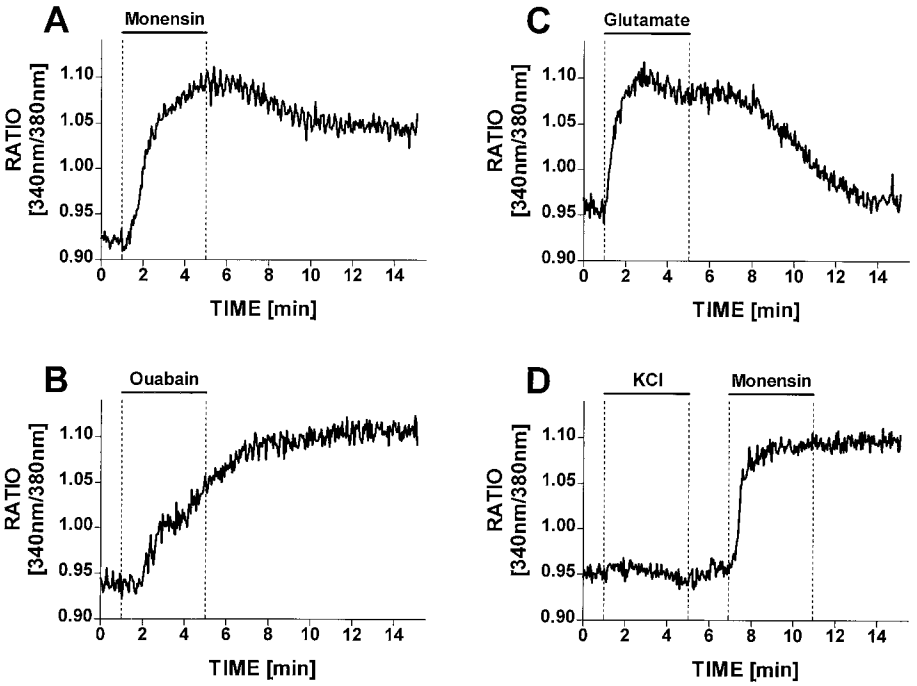

FIG. 9. Sodium imaging: effects of monensin, ouabain, glutamate and KCl on SBFI fluorescence in hippocampal neurons. Hippocampal neurons were loaded for 90 min at 25 °C with SBFI-AM in Hanks buffer, and intracellular sodium concentration was estimated from the respective fluorescence ratios at 340 and 380 nm excitation wavelengths. Monensin (A, 10 μM), ouabain (B, 2 mM) and glutamate (C, 50 μM) caused a marked and rapid increase in intracellular sodium, whereas KCl (C, 50 mM) had no detectable effect. Images were taken at a sampling rate of 0.75/s. Averages of four cells in a representative experiment (out of three experiments) are shown.

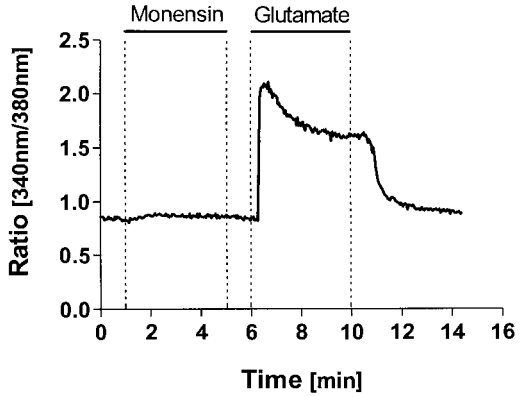

FIG. 10. Influence of monensin (10 μM) on $[Ca^{2+}]_i$ was compared with that of glutamate (50 μM). Hippocampal neurons were loaded for 40 min with 2 μM Fura2-AM, and $[Ca^{2+}]_i$ was estimated from the respective ratio of the fluorescence at 340 and 380 nm excitation wavelengths. Images were taken at a sampling rate of 0.75/s; six cells were averaged for each image. A representative experiment (out of four experiments) is shown.

intracellular sodium. Maximal sodium concentrations at similar levels were reached within 3 (monensin), 7 (ouabain) and 2 min (glutamate), respectively, using a perfusion rate of 0.5 mL/min. In contrast, 50 mM KCl did not cause any detectable increase in intracellular sodium, whereas – after a short washing period – the cells still reacted to monensin, resulting in a rapid sodium influx. Upon depolarization with KCl, a small increase in intracellular sodium has been observed in other laboratories (Rose & Ransom, 1997; Takahasi *et al*., 1997). In these, an increase of ~10–20% was determined compared with maximal levels reached by using veratridine, a level that might be below our detection limit.

Together, these experiments demonstrate that BDNF secretion is not influenced by sodium influx per se.

Discussion

The goal of the present investigations was to resolve the discrepancy between the observation that the replacement of sodium by NMDG interfered with the activity-mediated secretion of neurotrophins (Blöchl & Thoenen, 1995) and the negative experimental evidence that sodium influx directly mobilizes calcium from intracellular stores under physiological conditions. We therefore compared the replacement of sodium by NMDG with that by other sodium substitutes. Moreover, we analysed the consequences of the very rapid sodium influx initiated by the sodium ionophore monensin, and blockade of the plasma membrane K^+/Na^+-ATPase by ouabain, which also results in a substantial and rapid increase in intracellular sodium.

Our experiments demonstrated that the different sodium substitutes had very distinct effects on neurotrophin secretion. Much to our surprise, the replacement of as little as 10% of sodium by NMDG or sucrose (but not 5%) was sufficient to block the neurotrophin secretion initiated by high potassium or glutamate. In contrast, the replacement of sodium by choline or lithium did not impair the activity-dependent neurotrophin secretion. Therefore, the blocking effect of NMDG, which initially was thought to prove the sodium dependence of activity-mediated NGF secretion (Blöchl & Thoenen, 1995), does not reflect the sodium replacement. Instead, it represents an effect that is independent of sodium replacement by NMDG. This was further supported by the observation that, at normal sodium concentrations, the addition of 10% NMDG or sucrose equivalents had the same effect on the release as the corresponding replacement of sodium by these substitutes (seen in three and one experiments, respectively; data not shown). In addition to the blocking effect, 10% of NMDG and sucrose substitution initiated a short-lasting neurotrophin secretion of their own similar to that produced by 50 mM potassium (Figs 3 and 5). This was also observed after the addition of 10% NMDG or sucrose to the normal sodium concentration.

In contrast, both lithium and choline substituting 10% of sodium neither interfered with the activity-dependent neurotrophin secretion, nor initiated a neurotrophin release on their own.

However, it is essential to realize that for a sodium replacement-independent action of NMDG and sucrose, the replacement of 10% sodium corresponds to a rather high concentration of 12.5 mM. Although the detailed elucidation of the underlying molecular mechanisms was beyond the scope of the present investigations, preliminary experiments provided evidence that the neurotrophin secretion resulting from 10% NMDG or sucrose substitution of sodium was mediated by a release of calcium from intracellular stores, as this effect was independent of extracellular calcium but was blocked by the administration of 10 μM BAPTA/AM.

In calcium imaging studies with some cell populations an increase in intracellular calcium was seen upon replacing 10% sodium by NMDG or sucrose, but not by choline or lithium (Fig. 6).

One hundred per cent replacement of sodium resulted not just in a somewhat larger increase of neurotrophin secretion on its own. In addition, 100% replacement for not more than 5 min resulted in an irreversible blockade of the activity-dependent secretion of BDNF within the time-frame of the duration of the experiment (Fig. 4B). This irreversible blocking effect did not result from a depletion of the BDNF stores, as the 100% replacement of sodium by choline or lithium did not cause any secretion of BDNF, but still inhibited the activity-dependent secretion of BDNF.

The various sodium substitutes showed distinctly differing electrophysiological manifestations with 100% sodium replacement (Fig. 7). A 10% replacement did not result in a detectable influence on either the frequency or the shape of the spontaneous action potentials, whereas a 100% replacement abolished them very rapidly. Therefore, the 10% replacement of sodium, which leads to a blockade of the activity-mediated neurotrophin secretion, is not related to the observed electrophysiological manifestations, which only became apparent at 100% sodium substitution. In contrast to lithium and particularly choline the recovery after changing to a normal perfusion medium was very much delayed but reappeared after ~7–10 min in the presence of normal perfusion medium. This demonstrates that the irreversible blockade of high potassium-mediated secretion of neurotrophins within the time frame of the duration of the release experiments (Fig. 4B) does not result from an irreversible damage of the hippocampal neurons by a 100% replacement of NMDG or sucrose, which is further supported by the absence of an increase of propidium iodine-positive neurons after 100% sodium substitution by NMDG or sucrose. However, it indicates that both NMDG and sucrose have side effects on physiological parameters, for which the recovery period is longer.

The absence of a causal relationship between sodium influx and the mobilization of calcium from intracellular stores was further strongly supported by sodium imaging experiments. Changes in the activity-dependent sodium influx were not detectable after 10% replacement of sodium, whereas the sodium ionophore monensin, which resulted in a dramatic influx of sodium, neither induced a secretion of neurotrophins of its own nor blocked the activity-mediated secretion of neurotrophins. The same was true for ouabain, which, by blocking the sodium/potassium pump of the plasma membrane, produced a rapid increase of intracellular sodium concentrations, whereas

glutamate caused a rapid and marked increase in intracellular sodium concentrations similar to those produced by monensin. In contrast, 50 mM potassium did not result in a detectable increase in intracellular sodium. On the one hand, we have no explanation why a 50 mM KCl depolarization did not result in a measurable increase in intracellular sodium under our experimental conditions, whereas other laboratories, using very similar experimental conditions, found a consistent though very small increase in the intracellular sodium concentration (Rose & Ransom, 1997; Takahashi *et al*., 1997). On the other hand, this result additionally supports the secretion of neurotrophins to be independent of sodium influx (KCl causes secretion of neurotrophins, but no sodium influx). As monensin has been previously described to increase not only intracellular sodium but also intracellular calcium (Mulkey & Zucker, 1992; using crayfish neuromuscular junctions), we measured the change in intracellular calcium upon application of 10 μM monensin. In four attempts we have not seen any elevation in intracellular calcium (Fig. 10). The subsequent application of 50 μM glutamate, which causes a sodium influx similar to that of monensin, caused an immediate elevation of intracellular calcium.

If the results of our present experiments are brought in line with previous investigations performed by other laboratories (Kennedy & Thomas, 1995; Hoyt *et al*., 1998), they demonstrated unambiguously that there is no evidence for a relationship between sodium influx and the mobilization of calcium from intracellular stores, the prerequisite for the activity-dependent secretion of neurotrophins. Of particular importance are the experiments of Kennedy & Thomas (1995) and Hoyt *et al*. (1998), who analysed these aspects in cultured hippocampal and forebrain neurons under experimental conditions that were similar to ours.

In two other studies, using neurohypophysial nerve endings, the release of neuropeptides was found to be dependent on high intracellular sodium concentration in docked vesicles, but independent of calcium (Stuenkel & Nordmann, 1993; Thiron *et al*., 1999).

In addition to their significance for the reinterpretation of previous experiments, which seemed to indicate that there is a causal relationship between sodium influx and activity-dependent neurotrophin secretion, the present investigations are also of more general importance. For the selection of sodium substitutes in electrophysiological experiments, in which the sodium substitutions are selected in an arbitrary manner, the possible 'side effects', unrelated to the sodium substitution, have to be borne in mind. This is true not only for the quite dramatic actions of NMDG and sucrose at relatively low (10% sodium replacement) concentrations, but also for the use of the other substitutes in high concentrations. In particular, complete replacement of sodium by choline may have muscarinic effects (Alkondon *et al*., 1997). Lithium also has a broad spectrum of biochemical actions predominantly resulting from the blockade of inositol monophosphatase and downstream effects, e.g. depletion of intracellular inositol and the inhibition of cyclic AMP formation (Wood & Goodwin, 1987; Berridge & Irvine, 1989; Berridge *et al*., 1989; Atack, 1996).

In conclusion, the present experiments have demonstrated that there is no direct relationship between sodium influx and the mobilization of calcium from intracellular stores, the mechanism that is responsible for the activity-mediated secretion of neurotrophins. However, the frequently used sodium substitutes NMDG and sucrose (but not choline and lithium) have pharmacological effects of their own (presumably releasing calcium from internal stores) that are independent of the sodium replacement and that, at a concentration of 12.5 mM (corresponding to 10% sodium replacement), blocked the depolarization-mediated secretion of neurotrophins.

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; BAPTA, bis-(*o*-aminophenoxy)-ethan-*N*,*N*,*N*′,*N*′-tetraacetic acid; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CMV, cytomegalovirus; DMEM, Dulbecco's minimum essential medium; EGTA, ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; FCS, foetal calf serum; HEPES, *N*-2-[hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]; NGF, nerve growth factor; NMDG, *N*-methyl-D-glucamine; PBS, phosphate-buffered saline.

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