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Simultaneous detection of L-glutamate and nitric oxide from adherently growing cells at known distance using disk shaped dual electrodes

Sonnur Isik [a], Jaime Castillo [b], Andrea Blöchl [c], Elisabeth Csöregi [b], Wolfgang Schuhmann [a,*]

[a] *Anal. Chem.-Elektroanalytik and Sensorik, Universitätsstr. 150, D-44780 Bochum, Germany*
[b] *Department of Analytical Chemistry, Lund University, SE-22100 Lund, Sweden*
[c] *Lehrstuhl für Molekulare Neurobiochemie Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany*

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Abstract

An ex vivo system for simultaneous detection of nitric oxide (NO) and L-glutamate using integrated dual 250 μm platinum disk electrodes modified individually with suitable sensing chemistries has been developed. One of the sensors was coated with an electrocatalytic layer of Ni tetrasulfonate phthalocyanine tetrasodium salt (Ni-TSPc) covered by second layer of Nafion, which stabilises on the one hand the primary oxidation product NO^+ and prevents interferences from negatively charged compounds such as NO_2^-. For glutamate determination, the second electrode was modified with a crosslinked redox hydrogel consisting of Os complex modified poly(vinylimidazol), glutamate oxidase and peroxidase. A manual *x–y–z* micromanipulator on top of an inverted optical microscope was used to position the dual electrode sensor at a defined distance of 5 μm from a cell population under visual control. C6 glioma cells were stimulated simultaneously with bradykinin or VEGF to release NO while KCl was used to invoke glutamate release. For evaluation of the glutamate sensors, in some experiments HN10 cells were used. To investigate the sensitivity and reliability of the system, several drugs were applied to the cells, e.g. Ca^{2+}-channel inhibitors for testing Ca^{2+}-dependence of the release of NO and glutamate, rotenone for inducing oxidative stress and glutamate antagonists for analysing glutamate release. With these drugs the NO and glutamate release was modulated in a similar way then expected from previously described systems or even in-vivo measurements. We therefore conclude that our system is suitable to analyse stress-induced mechanisms in cell lines.

Keywords: Dual electrode; Simultaneous detection; Nitric oxide; Glutamate; Drugs; Antagonists

1. Introduction

Glutamate is an important neurotransmitter involved among others in learning and memory processes, while NO acts as a retrograde messenger, which is released upon stimulation of the postsynaptic cell and used to modulate synaptic transmission [1,2]. In addition, NO is known to interfere with the regulation of blood pressure and may activate immune response. Both substances are thought to contribute to the pathogenesis of several diseases for example glutamate in stroke, epilepsy, schizophrenia and Parkinson disease [1,3,4], and NO in hypertension, hyperglycaemia, arteriosclerosis, arthritis, reperfusion injury and cancer [5–7]. Both substances are also known to be released in response to oxidative stress [8,9]. Suitable, sensitive electrochemical methods are necessary to analyse the biochemical background of these diseases and might also serve as efficient systems for testing the response to oxidative stress in different cell systems. For this purpose, it is important to develop methodologies to determine both compounds with low detection limit, high selectivity in complex samples, fast response time, the possibility for miniaturization with potential in-vivo capability, and easy handling. To obtain real quantitative and reproducible information about the concentrations of glutamate or NO after cell stimulation, besides the amount of secreted substances and possible reactions with compounds present in the immediate proximity of the release site, the distance between cells and active surface of the sensor is a very important parameter determining the sensor signal. To cope with this fact, SECM [10–12] and manual or computer controlled *x–y–z* manipulator with positioning resolution below 1 μm [13,14] were used for positioning sensors in very close and known distance to the cell

* Corresponding author. Tel.: +49 234 322 6200; fax: +49 234 321 4683.
E-mail address: wolfgang.schuhmann@rub.de (W. Schuhmann).

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or cell population. This assures the successful detection of NO released from cells avoiding significant losses of NO by diffusion or reaction with other substances such as O_2.

The detection of simultaneous release of NO and glutamate from an adherently growing cell population was shown in a previous study using a planar microelectrode array [15]. A nanopore membrane, which was used as biocompatible surface for cell cultivation concomitantly preventing the direct contact of the cells with the sensor surfaces, decreases transmitter diffusion causing a slowed-down response time and a decreased collection efficiency for NO and glutamate.

In this communication, an integrated needle-type dual electrode assembly was applied which can be accurately positioned at known distances from adherently growing cells using a manual micromanipulator mounted on an inverted optical microscope. The dual electrode setup allowed for the simultaneous monitoring of the release of glutamate and NO upon stimulation of the cells with relevant drugs (e.g. KCl or veratridin, a Na^+-channels agonist [16], for stimulating release of glutamate, and bradykinin or VEGF for invoking the release of NO). Additionally, the dependence of NO release from intra- and extracellular Ca^{2+} was investigated by incubating the cells with Ca^{2+}-channel blockers prior to release experiments. It is known that oxidative stress enhances the response of glutamate at glutamatergic *N*-methyl-D-aspartate NMDA receptors [17]. To analyse possible effects of oxidative stress on glutamate and NO release, we used rotenone, which blocks the oxidative phosphorylation within the cells and thereby produces H_2O_2 [18–20]. In addition glutamate antagonists were applied, which are known to interfere in vivo with neurotoxicity of glutamate [21–23].

2. Experimental

2.1. Chemicals

NaCl, KCl, NaH_2PO_4, $MgCl_2$, $CaCl_2$, $NaHCO_3$, glucose, HEPES (Riedel-de-Haen, Seelze, Germany) were used to prepare Hank's buffer. Stock solutions of drugs and glutamate antagonists were dissolved in dimethylsulfoxid (DMSO) (Riedel de-Haen, Seelze, Germany). Glutamate oxidase (EC 1.4.3.11, Cat. No. 7804) was from Yamasa Corporation (Tokyo, Japan) and horseradish peroxidase (EC 1.11.1.7, Cat. No. P-6782, Lot. No. 111K74351) was obtained from Sigma (Deisenhofen, Germany). PVI-dme-Os was synthesized according to an earlier published procedure [24]. To crosslink glutamate oxidase, horseradish peroxidase and the Os-modified redox polymer a bifunctional crosslinker (poly(ethylene glycol)diglycidyl ether; PEGDGE; Polyscience, Warrington, PA, USA) was used. Ni tetrasulfonate phthalocyanine tetrasodium salt (Ni-TSPc), KOH, bradykinin and vascular endothelial growth factor-E (VEGF-E) were purchased from Aldrich (Steinheim, Germany). Arginine, L-glutamic acid, rotenone, nifedipine, veratridine, and flunarizine were purchased from Sigma (Steinheim, Germany). Non-competitive AMPA antagonists [25] such as 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI-52466), 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine (GYKI-53655) and talampanel (GYKI-53773; (R)-7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H[1,3]dioxolo[4,5][2,3]benzodiazepine) were provided from IVAX Drug Research Institute, Budapest, Hungary.

2.2. Sensor preparation

Preparation of the disk shaped Pt dual electrode was previously described in detail [11]. After pulling of a θ-type borosilicate glass capillary with a home made capillary puller, two 250-μm diameter Pt wires were inserted into the capillary from the back and the tip was sealed by heating under vacuum. The electrical contact to the Pt wire was achieved by means of a thin Cu wire using tin powder. The electrode was carefully polished using emery paper and an alumina suspension (0.3 μm particle size; Leco, Kirchheim, Germany) ensuring that the surface of the tip was kept perpendicular to the polishing wheel. The polished electrodes were rinsed with H_2SO_4, KOH and H_2O. The sensing chemistry for NO and glutamate detection was deposited sequentially. First, for establishing the NO-sensor the dual electrode was inserted into a solution of 2 mM NiTSPc dissolved in 0.1 M KOH. Polymerization of NiTSPc on one of the 250 μm Pt electrodes was achieved using multi-sweep cyclic voltammetry in a potential range between 0 and 1.2 V (30 cycles). Polymeric Ni-TSPc is a well-known electrocatalyst for the oxidation of NO [10,26,27]. As a second layer, 5% Nafion in ethanol was applied in the format of a dip droplet and allowed to dry. Following the NO-sensor preparation, the second 250-μm electrode was modified with a glutamate-sensing bi-enzyme sensor architecture developed before [15,28,29]. The sensing layer consists of a mixture of horseradish peroxidase (5 mg ml^{-1}), glutamate oxidase (10 mg ml^{-1}), PVI-dme-Os (2.5 mg ml^{-1}) and PEGDGE (2.5 mg ml^{-1}, in water used within 15 min). About 1 μl of this mixture was placed under microscopic control on the Pt surface using a microsyringe forming a small droplet covering the entire Pt surface. After drying the sensor was stored for 20 h at room temperature to induce crosslinking. Afterwards, the sensor was stored at 4 °C before use.

2.3. Cell preparation

C6-glioma cells were cultivated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal calf serum, 2 mM glutamine and 100 U penicillin/streptomycin in an atmosphere of 10% CO_2. HN10 neuroblastoma cells were cultivated in RPMI 1640 medium with the same supplements used for C6-glioma but in an atmosphere of 5% CO_2. Passages of the cells were performed all 3 days using 2 mM EDTA in PBS for detaching the cells. 24 h prior to electrochemical experiment the cells were seeded in a density of 105 cells per cover slip (2*2 cm^2).

2.4. Apparatus and electrochemical measurements

A CHI 1030 eight-fold potentiostat (CH Instruments, Austin, USA) was used to polymerize Ni-TSPc on top of the Pt surface using multi-sweep cyclic voltammetry (CV). The simultaneous amperometric measurement of NO and glutamate was carried out with a Biometra EP-30 bipotentiostat (Biometra, Göttingen,

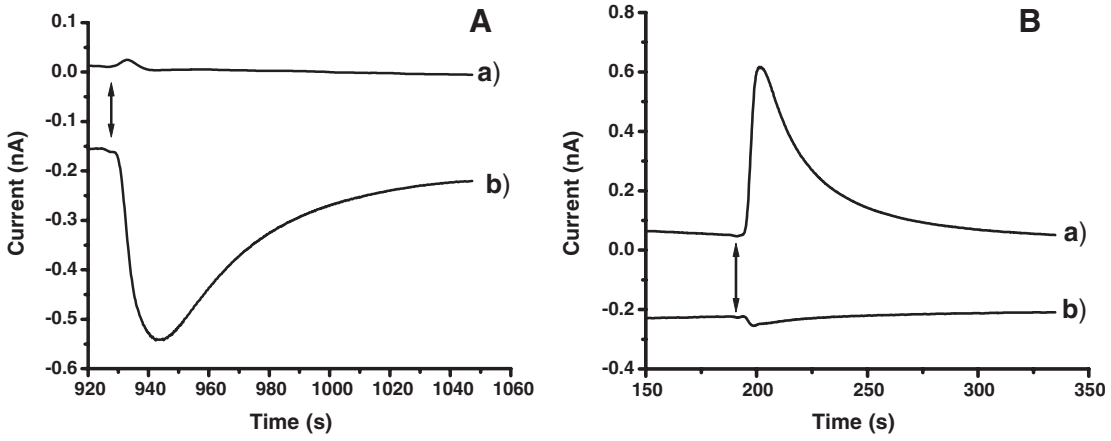

Fig. 1. Cross-talk evaluation between the glutamate and NO sensor in the absence of cells; (A) represents addition of a concentration of 3 μM glutamate and (B) represents addition of a concentration of 10 μM NO: (a) NO sensor, (b) glutamate sensor.

Germany). A potential of −50 mV was applied to the glutamate sensor and a potential of +750 mV was applied to the NO sensor. These potential values are sufficiently low (for glutamate detection) and high (for NO oxidation) that small potential changes at the pseudo-Ag/AgCl reference electrode upon addition of Cl^- do not lead to changes in the diffusion limited current. A manual *x–y–z* micromanipulator (Märzhäuser, Wetzlar, Germany and Physik Instrumente, Waldbronn, Germany) was used for positioning the dual electrode in known distance from the cell layer. The micromanipulor was fixed to the base plate of an inverted microscope (Axiovert 25C, Carl Zeiss Jena, Germany) which was used for controlling the electrode positioning. Usually, a slow approach of the sensor tip was made until contact to the cells was achieved. The electrode was then removed by 5 μm and moved in *x–y* direction to detect glutamate and NO release from cells which did not have any contact to the electrode before.

3. Results and discussion

3.1. Electrode characterization

A Pt dual barrel electrode with active electrode surfaces of 250 μm each was modified with appropriate sensing chemistries

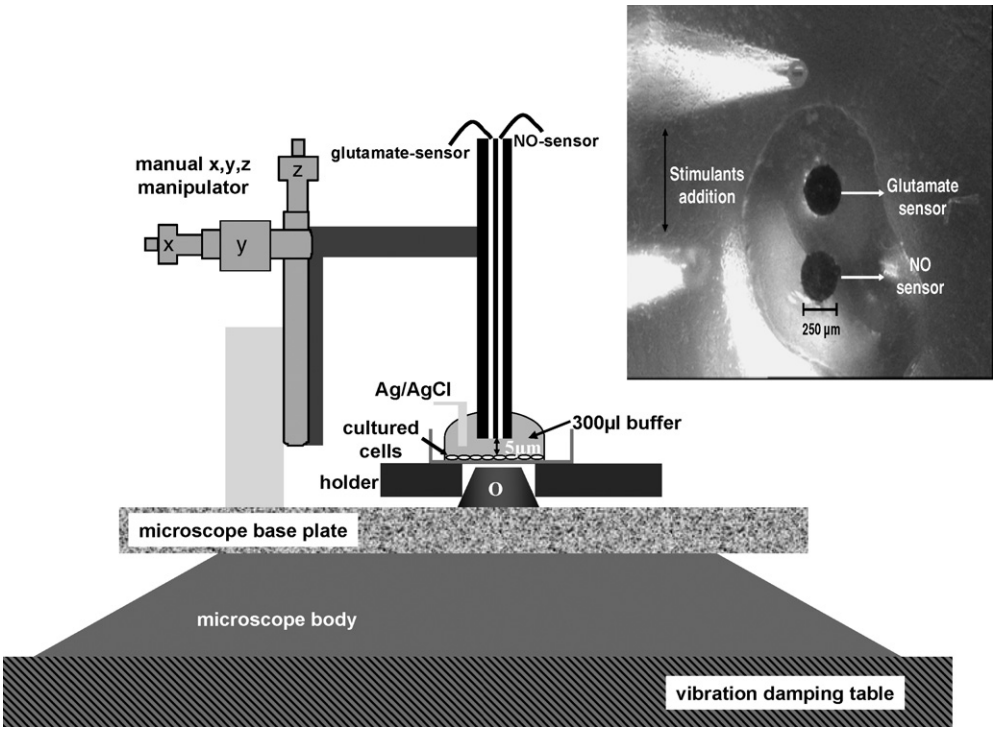

Fig. 2. Schematic representation of the experimental set-up showing the inverted microscope for visual approach and distance control, the micromanipulator with attached dual-barrel electrode and the electrochemical cell with a 300 μl droplet as bulk volume. The inset is showing the electrode surfaces as seen at the distance of 5 μm from the adherently growing C6-glioma cell layer.

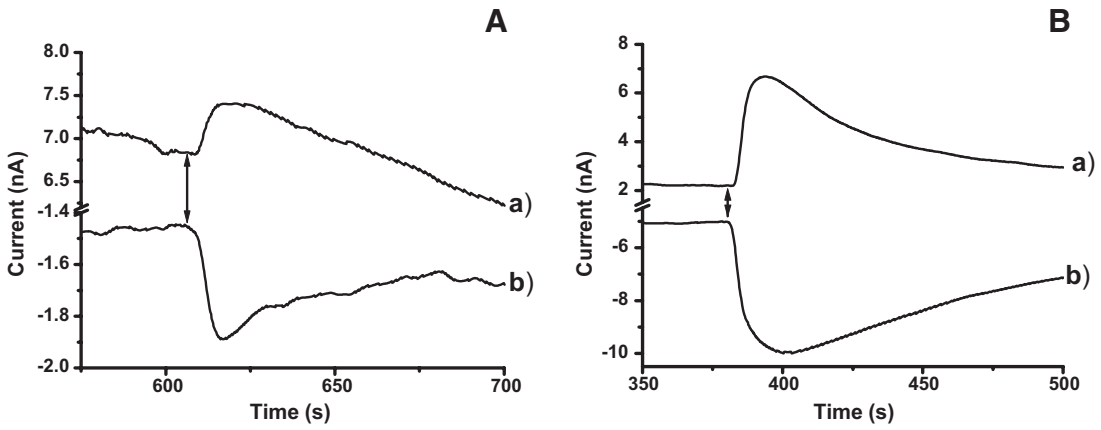

Fig. 3. (A) Simultaneous detection of (a) NO at +750 mV vs. pseudo Ag/AgCl and (b) glutamate at −50 mV vs. Ag/AgCl pseudo reference after stimulation of C6 glioma cells with a mixture of bradykinin and KCl. (B) detection of (a) NO and (b) glutamate release after stimulation of C6 glioma stells with a mixture of VEGF-E and KCl.

for the simultaneous detection of NO and glutamate. The working potentials of −50 mV for the reduction of the $Os^{2+/3+}$ couple at the glutamate sensor and of +750 mV for electrocatalytic oxidation of NO at the phthalocyanine-modified NO sensor were applied vs. a pseudo Ag/AgCl reference/counter electrode using a bipotentiostat.

The electrode was approached to a bare glass slide in absence of cells to a working distance similar to the later experiments with cells (see below). To rule out possible cross-talk between the two sensing chemistries, glutamate or NO stock solution, respectively, were added by means of a micropipette. Addition of glutamate (3 μM final concentration) induced an immediate response of the glutamate sensor but no response at the NO sensor (Fig. 1A). Vice versa, NO addition let to an exclusive response of the NO-sensor but not of the glutamate sensor (Fig. 1b).

3.2. Simultaneous detection of glutamate and NO release from C6-glioma cells

We already have shown earlier that in the case of C6-glioma cells K^+-stimulated glutamate release did not affect NO-release, while NO bradykinin stimulated release did not induce glutamate release [15].

A cover slip with adherently growing C6-glioma cells was located on the platform of an inverted microscope and covered with a droplet of 300 μl buffer. Under visual control, the tip of the dual electrode was moved towards the cells until a contact between the microelectrode and the cell layer was seen. This distance is referred to as relative distance zero. Using the z-micromanipulator the dual-barrel electrode was retracted to a position of 5 μm apart from the cells. Fig. 2 shows a schematic representation of the used set-up and an image of the dual electrode positioned at a distance of 5 μm to the cells.

After positioning of the tip of the dual electrode at the predefined distance from the cell population the working potentials were applied to both sensors. After a stable background current was attained, 35 μl of a mixture of the stimulants, namely bradykinin and K^+, were carefully injected in close proximity to the electrodes into the 300 μl buffer solution on top of the cell layer. The final concentration of KCl and bradykinin were 100 mM and 20 μM, respectively. As expected, the stimulation of the cells by K^+-ions and bradykinin induced simultaneously the release of glutamate and NO causing a current change at the related sensor (Fig. 3a). These results unequivocally demonstrate the capacity of the dual sensor configuration to simultaneously detect glutamate and NO released from the cells. The compounds released from the adherently growing cell population upon stimulation are diffusing away from the cells into the bulk solution under formation of a complex diffusion zone. Moreover, re-uptake of glutamate by the cells and follow-up reactions of NO (e.g. with molecular oxygen) modulate this diffusion profile. Hence, all experiments are giving current values instead of glutamate or NO concentrations. As a matter of fact, the accurately positioned electrodes allow for a comparison of the experiments due to a comparable position of the active sensor surface with respect to the releasing cells.

VEGF-E (vascular endothelial growth factor E) triggers the synthesis and release of NO by activation of a constitute NO synthase [30]. Therefore, similar to the activation using

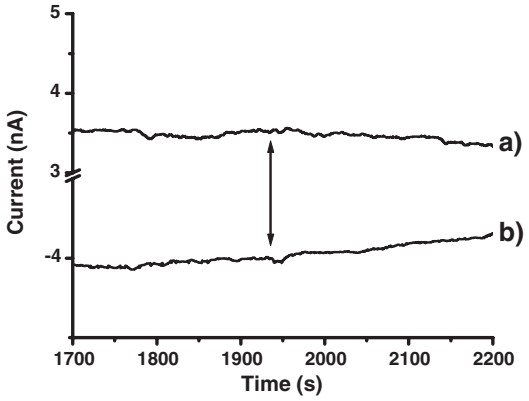

Fig. 4. Control experiment for evaluation of the impact of the stimulating compounds VEGF-E and KCl on the current response at (a) the NO sensor at +750 mV vs. pseudo Ag/AgCl and (b) the glutamate sensor at −50 mV vs. Ag/AgCl pseudo reference in the absence of cells.

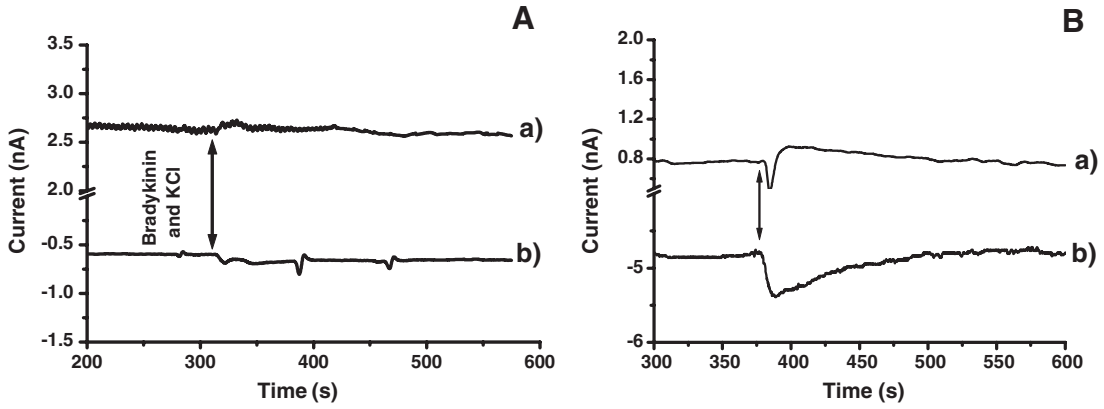

Fig. 5. (A) Effect of the incubation of C6 glioma cells with 50 μM flunarizine for 30 min. Simultaneous detection of (a) NO at +750 mV and (b) glutamate at −50 mV after stimulation with a mixture of bradykinin and KCl. (B) Similar experiment performed after incubation of C6 glioma cells with 50 μM nifedipine for 30 min.

bradykinin, VEGF-E was tested as an alternative stimulator for provoking NO release. The addition of 35 μl of a mixture of VEGF-E and KCl leading to final concentrations of 100 mM K^+ and 15 ng/ml VEGF-E, respectively, demonstrated the successful stimulation of simultaneous NO and glutamate release from the cell layer invoked by membrane depolarization and activation of the VEGF receptor 2 (Fig. 3b).

In order to prove that the addition of the stimulants does not lead to any changes in the current at both sensors a similar experiment was performed by adding a mixture of VEGF-E and KCl in the absence of cells (Fig. 4).

3.3. Effect of different inhibitors on the release of glutamate and NO from C6-glioma cells

Massive releases of neurotransmitters into the extracellular space are associated with acute membrane depolarization caused by postsynaptic Na^+ and Ca^{2+} influx via voltage-operated as well as ligand-gated channels. This initiates a cascade of events that generate tissue damage, cell swelling, generation of free radicals, increased NO production, impaired mitochondrial activity and possibly apoptosis [31,32]. In addition, Na^+ and Ca^{2+} channels are important components in neurotransmission regulating the propagation of the action potential along the axon. When this depolarization reaches the nerve terminal, Ca^{2+} channels open and trigger exocytosis of synaptic vesicles which release neurotransmitters. Hence, modulation of these channels can alter neurotransmitter release [16]. In order to investigate if the modulation of this Na^+ and Ca^{2+} channels will alter the release of NO and/or glutamate in the in-vitro cell culture system, C6 glioma cells were incubated with three different compounds namely rotenone, flunarizine and nifedipine prior to the detection of stimulated NO and glutamate release by means of the positioned dual barrel electrode.

To evaluate the Ca^{2+} dependence of stimulated NO and/or glutamate release from C6 glioma cells, the cells were incubated for 30 min prior to measurements with nifedipine (50 μM), an inhibitor of L-type Ca^{2+} channels, or with flunarizine (50 μM), inhibiting L- and T-type Ca^{2+} channels. While the release of NO and glutamate upon stimulation with KCl and bradykinin and

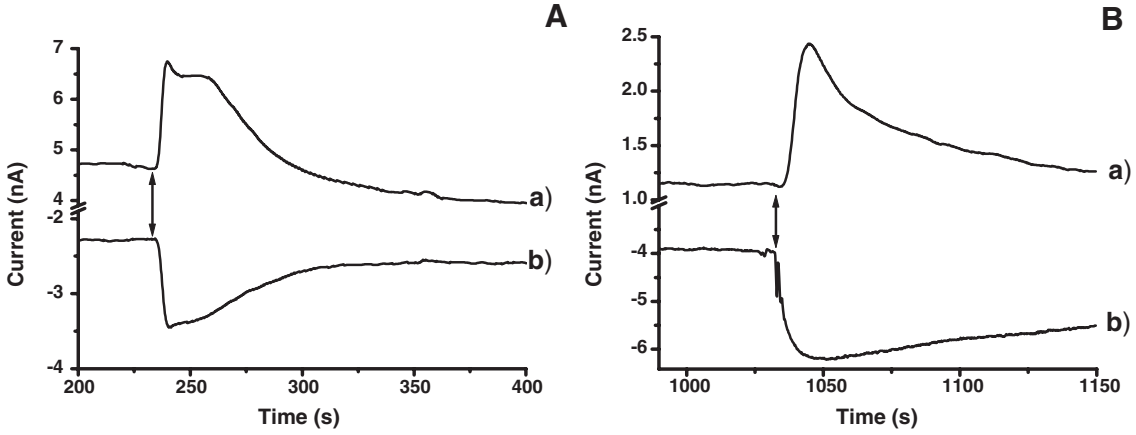

Fig. 6. (A) Bradykinin and KCl stimulated release of (a) NO and (b) glutamate after 4 h incubation of C6 glioma cells with 10 μM rotenone. (B) Stimulation of C6 glioma cells with 50 μM veratridine for glutamate release and 20 μM bradykinin for NO release followed by simultaneous detection of (a) NO and (b) glutamate released from the cells. To rule out any unspecific effect of a depolarization with KCl, the neurotoxin veratridine was used for stimulating glutamate release. Veratridine binds to voltage-sensitive Na^+ channels and prevents their closing. The resulting influx of Na^+ induces a depolarization of the membrane and the release of neurotransmitters [20]. Stimulation of C6-cells with 50 μM veratridine induced a significant increased release of glutamate as compared with KCl stimulation.

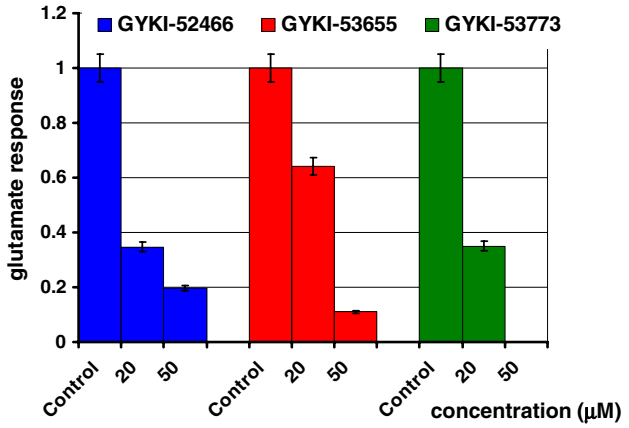

Fig. 7. Normalized glutamate release from HN10 cell after incubation with increasing concentrations of AMPA receptor antagonists GYKI-52466, GYKI-53655 and GYKI-53773 for 30 min. Glutamate release was invoked by stimulation of the cells with K^+ and detected at a potential of −50 mV at the positioned glutamate microsensor.

hence any current response of the NO and glutamate sensors were completely blocked by flunarizine (Fig. 5A), only NO release was blocked by nifedipine (Fig. 5B). The missing inhibitory effect of nifedipine on glutamate release is in accordance with previously reported experiments [33].

In order to further evaluate the applicability of the established in-vitro NO and glutamate detection system we anticipated to investigate its suitability for analyzing oxidative stress in cells. One model for inducing oxidative stress in cells is the disruption of oxidative phosphorylation in the mitochondria of the cells. A drug often used to block the electron flow from NADH to co-enzyme Q in the complex I of the respiratory chain is rotenone. It has been associated with induction of apoptosis [18], oxidative stress [19] and induction of Parkinson disease like effects on dopaminergic cells [20]. It is known that rotenone interferes at the same point of the respiratory chain as 1-methyl-4-phenylpyridinium (MPP^+) does. Thus, it shows the same properties but has the advantage to induce the block in a variety of different cells, while MPP^+ only acts on dopaminergic neurons. Therefore rotenone is seen as a valuable tool for inducing oxidative stress in any cell line of interest. In addition, it is known that oxidative stress will sensitize glutamatergic NMDA-receptors which might increase glutamate release from cells [17]. Thus, we were interested in the possibility to investigate stimulated glutamate and NO release from cells after rotenone treatment. Fig. 6A shows the stimulated release of NO and glutamate after treatment of the cells with 10 μM rotenone for 4 h. A clear increase of both, the current at the NO and the current at the glutamate sensor can be detected.

3.4. Effect of glutamate antagonists on the release of glutamate from HN10 cells

Hypoxic-ischemic neuronal injuries occurring during and after stroke have been attributed to an excess release of glutamate. The neurotransmitter stimulates postsynaptic á-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors leading to a massive increase in intracellular Ca^{2+} and Na^+ concentration [34–36]. In turn, influx of Ca^{2+} produces toxic molecules such as superoxide, hydroxyl radicals, and NO [17,37]. Treatment with an AMPA receptor antagonist reduced the neuronal cell death after such a stress by reducing Ca^{2+} influx into the cells. To evaluate this system using the proposed set-up, a hippocampal neuronal cell line (HN10) was treated with the AMPA receptor antagonists GYKI-52466, GYKI-53655 and GYKI-53773 at different concentrations 30 min prior to stimulation to see if antagonists of AMPA receptors also reduce KCl-stimulated glutamate release. As shown in Fig. 7 the drugs reduced stimulated glutamate release obviously in dependence from the dose of the administered drug. These results are in good agreement with data obtained during in-vivo analysis of ischemia [22,23,38] suggesting that glutamate release and hypoxic-ischemic neuronal cell death could significantly be reduced after treatment of the cells with AMPA receptor antagonists.

4. Conclusion

In this study, we present an in-vitro system for simultaneously analysing stimulated glutamate and NO release from cells. We could demonstrate that this system is sufficiently sensitive to indicate oxidative stress of cells via an increased NO and glutamate release. In addition, we have shown that inhibition of AMPA receptors reduced the activation-induced glutamate release in hippocampal neurons, verifying in vivo results, which demonstrate the beneficial effect of AMPA receptor antagonists on hypoxic ischemic neuronal death. We conclude that the system is capable to analyze stress induction in cells and might be a good model for analyzing the biochemical background as well as treatment of different diseases involving stress induced effects.

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