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Effect of the pyridoindole SMe1EC2 and compounds affecting A_1 and A_{2A} adenosine receptors in rat hippocampus under ischemia *in vitro*

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Abstract:
The effect of the newly synthesized pyridoindole antioxidant SMe1EC2 (1 μmol/l) and drugs activating or inhibiting adenosine receptors was tested under ischemia. Synaptic transmission was recorded extracellularly before and under 6-min ischemia and 20-min reoxygenation in rat hippocampal slices *in vitro*. In untreated slices, ischemia elicited failure of synaptic transmission and excitability expressed by a population spike decay ($t_{0.5}$ = 1.7 ± 0.1 min) and poor recovery of synaptic transmission at the end of reoxygenation, expressed as percentage of PoS amplitude of that at zero minute of ischemia (9.9 ± 3.6%). The compound SMe1EC2 increased recovery of PoS amplitude in reoxygenation (31.2 ± 7.0% of that at the beginning of ischemia) and decreased the number of irreversibly damaged slices in reoxygenation (64%) compared to untreated slices (80%). Co-administration of SMe1EC2 + SCH-58261 (1 μmol/l, A_{2A} adenosine receptor antagonist) resulted in delayed synaptic transmission decay during 6-min ischemia ($t_{0.5}$ = 2.3 ± 0.1 min), increased PoS amplitude recovery during reoxygenation (37.7 ± 12.4% of that at zero minute of ischemia), and in a decreased number of slices with damaged synaptic transmission at the end of reoxygenation (54%), all data compared to untreated controls. Co-administration of pyridoindole with CGS 21680 (1 μmol/l, A_{2A} adenosine receptor agonist) or with DPCPX (100 nmol/l, A_1 adenosine receptor antagonist) eliminated the described effect. Further studies are required to elucidate the putative influence of manipulation with adenosine receptors on the neuroprotective effect of SMe1EC2 under ischemia.

Key words:
ischemia, hippocampus, synaptic transmission, adenosine, pyridoindoles

Abbrevations: aCSF – artificial cerebrospinal fluid, CGS 21680 – 2-p-[(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine, CPA – N^6-cyclopentyladenosine, DPCPX – 1,3-dipropyl-8-cyclopentylxanthine, EPSP – excitatory postsynaptic potential, PoS – field population spike, ROS – reactive oxygen species, SCH-58261 – 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine, SMe1EC2 – 2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3b]indolinium chloride

Introduction

Oxidative stress is one of the mechanisms frequently participating in neuronal damage. Reactive oxygen species (ROS) and consecutive lipid and protein peroxidation play an important role in damage of neuronal tissue due to acute brain ischemia, in chronic

neurodegenerative diseases (e.g. Parkinson's disease, Alzheimer's disease), and also in other conditions associated with the destruction of the central nervous system [3, 22]. Antioxidants, drugs able to minimize oxidative stress, might be used as neuroprotectants to shield the nervous system against toxic effects during the increased ROS and free oxygen radical production.

One of the extensively studied antioxidants is γ-carboline stobadine, designed and synthesized in the early 1980s [18]. It was found to exert multiple pharmacological effects, including free radical scavenging, antioxidant, cardioprotective, α-adrenolytic, cholinolytic, histaminolytic, hypotensive and membrane stabilizing effects. Based on the well-known antioxidant effect of stobadine, attention was focused on designing and synthesizing new, more efficient drugs with even more favorable properties. Certain sites in the pyridoindole stobadine molecule were identified to be responsible for antioxidant and anti-ROS properties. By an appropriate substitution, more than 70 new stobadine derivatives were prepared [19]. One of the new derivatives, substituted with methoxy-group in the aromatic cycle and ethoxycarbonyl-group substituted in the gamma position of pyrimidine nitrogen, i.e. 2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3b]indolinium chloride (SMe1EC2), showed to be a prospective antioxidant and neuroprotectant [21].

Acute and chronic injuries of the nervous system due to ischemia, hypoxia, inflammation or trauma are multifactorial processes and thus for successful treatment it is essential to intervene simultaneously at several levels of these pathological events [10]. One of such approaches is intervention in the generation and scavenging of ROS which got out of control of physiological regulatory mechanisms. On the other hand, there are many other mechanisms involved in ischemic brain injury, e.g. increased release of excitatory amino acids, increased concentration of intracellular Ca^{2+}, acidosis, edema and/or injury of energetic metabolism of ATP. During ischemia, increased concentration of extracellular adenosine was found to be involved in reduction of glutamate release by the activation of adenosine A_1 receptors [9, 14, 16, 17]. Thus, cocktails of neuroprotectants acting at different steps of the ischemic injury cascade may have an advantage over a single agent [10].

The aim of this paper was to investigate the neuroprotective effect of the newly synthesized pyridoindole antioxidant SMe1EC2 on synaptic transmission versus drugs acting on adenosine receptors administered alone and in combination with the pyridoindole during *in vitro* hippocampal ischemia (hypoxia accompanied with decreased glucose concentration) followed by reoxygenation.

Materials and Methods

Animals and preparation of hippocampal slices

Male Wistar rats (weighing 170–240 g), kept under a 12 h : 12 h light-dark cycle), were anesthetized by ether for 1 to 2 min to obtain light anesthesia and than decapitated. Forty rats were used in this study. The brain was removed and the hippocampus was quickly dissected in ice-cold artificial cerebrospinal fluid (aCSF) composed of (in mmol/l) NaCl 124, KCl 3.3, KH_2PO_4 1.25, $MgSO_4$ 2.4, $CaCl_2$ 2.5, $NaHCO_3$ 26, glucose 10 and saturated with 95% O_2 + 5% CO_2, at a pH of 7.4. Coronal slices, 400 μm thick, were cut using a manual tissue chopper and placed by suction pipette into a gas/liquid interface incubation chamber with aCSF at 34°C. Each slice was incubated for at least 80 min under the given conditions before electrophysiological assessment. For synaptic transmission measurement, each slice was placed into a gas/liquid interface recording chamber perfused with gas-saturated aCSF at a rate 0.6 ml/min, the chamber volume being 0.6 ml. The gas mixture was used both to saturate the aCSF and to ventilate the interface chamber.

All procedures involving animals were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology, Slovak Academy of Sciences as well as of the State Veterinary and Food Administration of the Slovak Republic.

Chemicals and drug application

After 10–15 min of stabilization, the tested drugs were added to aCSF 30 min before ischemia and were present in the incubation medium during ischemia, and during reoxygenation of the rat hippocampal slices. The stock solution of the antioxidant SMe1EC2 was prepared in distilled water and then dissolved in aCSF. All adenosine compounds: N^6-cyclopentyladenosine (CPA, A_1 adenosine receptor agonist), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, A_1 adenosine receptor antagonist), 2-p-[-(2-carboxyethyl)-phenethylamino]-

5'-N-ethylcarboxamidoadenosine (CGS 21680, A_{2A} adenosine receptor agonist), and 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine (SCH 58261, A_{2A} adenosine receptor antagonist) were purchased from Sigma-Aldrich Chemie Gmbh, Germany.

In vitro ischemic conditions: glucose and oxygen deprivation

To produce and retain ischemic conditions, O_2 in the original gas mixture was substituted by N_2. Simultaneously the perfusion medium was switched to aCSF saturated with 95% N_2 + 5% CO_2, containing 4 mmol/l glucose. Reoxygenation was attained by restoring the former conditions. The rate of oxygen exchange in the recording chamber was monitored by the miniature Clark 733 oxygen electrode (Diamond Micro Sensors) placed approximately 10 mm next to the slice. The O_2 concentration in the recording chamber atmosphere decreased to less than 5% within 2.5 min of ischemia.

Electrophysiology

Neurons in the CA1 region were trans-synaptically excited by stimulation of Schäffer collaterals. Supramaximal intensity of stimulation was set up for the first population spike (PoS). Neurons were stimulated in the CA3 region using bipolar stainless steel wire electrode by square wave pulses (0.1 ms/0.2 Hz). PoS from the CA1 region were registered in the pyramidal stratum. The responses were recorded using a 3–5 MΩ glass electrode filled with aCSF. Recordings were amplified, visualized on digital oscilloscope (Tektronix 2230), digitalized (Digidata 1322A, Molecular Devices) with sampling rate of 10 kHz and stored on personal computer for off-line analysis.

Synaptic transmission and excitability was assessed by evaluation of the PoS amplitude measured according to Gilbert and Burdette [5]. The excitatory postsynaptic potential (EPSP) and PoS were monitored for 15–20 min before the onset of ischemia to allow for stabilization of the evoked responses. Slices with PoS amplitude smaller than 2.5 mV in the stabilizing period were discarded. The PoS amplitude varied from about 3 to 12 mV in different slices at the beginning of ischemia. To compare the obtained data, the PoS amplitude at zero minute of ischemia was considered to be 100% percent. The changes in the PoS amplitude were calculated as the ratio of the final value at the end of reoxygenation multiplied by 100 and of the initial value measured at zero minute of ischemia.

The synaptic transmission impairment due to ischemia was assessed by measuring a decay of PoS amplitude during ischemia as the half-time ($t_{0.5}$) expressed in minutes and PoS amplitude recovery during reoxygenation expressed as the percentage of response at the zero minute of ischemia. At the end of reoxygenation, slices showing only EPSP or EPSP with less than 15% of initial PoS amplitude at zero minute of ischemia were considered to be irreversibly damaged. The duration of ischemia used (6 min) was necessary to evoke irreversible impairment of synaptic transmision at the end of 20-min reoxygenation in 80% of slices. The drugs tested were applied after a stabilization of the slices in native aCSF.

Statistical analysis

Synaptic transmission and excitability were recorded, stored and analyzed using computer software (AxoScope 9.2, Molecular Devices). The data were statistically evaluated using InStat software ver. 2.05 (graphPad Software). One-way ANOVA followed by Dunnett multiple comparison test was used to evaluate the differences of PoS recovery compared to controls. The χ^2 test was used to determine the significance of the number of irreversibly damaged slices at the end of reoxygenation. The half-time ($t_{0.5}$) of PoS decay during ischemia was derived from the PoS time-decay course using regression analysis. The differences of this parameter were examined using the *t*-test. The *n* values represent the number of slices used in each experimental group, further values are means ± SEM as indicated in the text, legend to Figures and the Table.

Results

Effect of SMe1EC2 compared to selective A_1 adenosine receptor agonist and antagonist on synaptic transmission in rat hippocampal slices exposed to ischemia

After placing the stimulatory and recording electrodes on the rat hippocampal slice, a 10–15-min period was used to stabilize the preparation. We used the field potential recording technique. The slices were exposed to 6-min ischemia followed by 20-min reoxygenation.

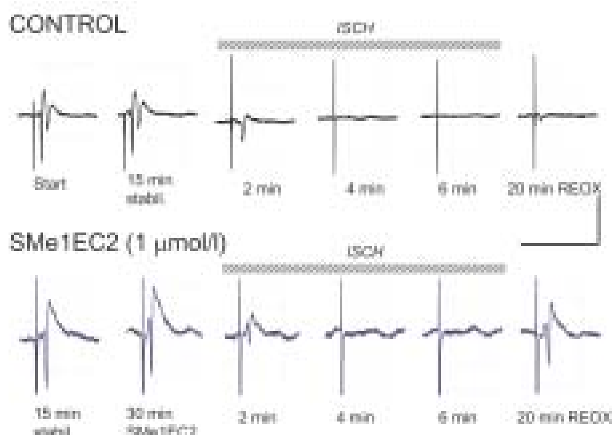

Fig. 1. Synaptic transmission in the rat hippocampus CA1 region during ischemia/reoxygenation in untreated and SMe1EC2-treated slices. Original records of field action potentials in untreated control (upper trace) and SMe1EC2-treated (lower trace) representative rat hippocampal slices. Ischemia is marked by a slashed line. From left to right: synaptic transmission at the end of 15-min stabilization period in control or after 30-min drug application in treated slices just before the onset of ischemia, further at the 2nd, 4th and 6th min of ischemia and at 20th min of reoxygenation. The antioxidant SMe1EC2 (1 µmol/l) was present for 30 min before ischemia, during ischemia as well as during reoxygenation. Synaptic transmission was recovered at the end of reoxygenation in the preparation incubated with the drug tested compared to the irreversibly damaged control slice. Calibration: horizontally 50 ms, vertically 5 mV

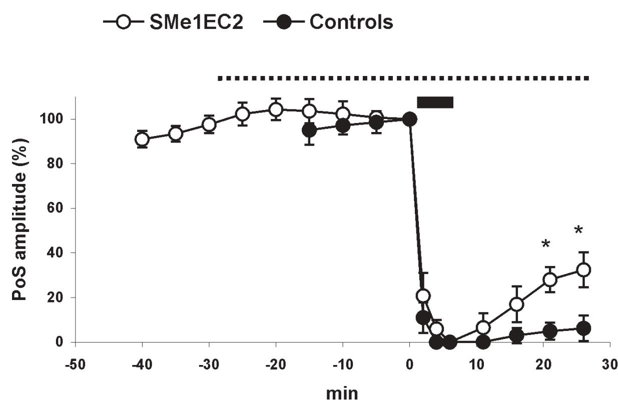

Fig. 2. Effect of the antioxidant SMe1EC2 on synaptic transmission failure and recovery during ischemia/reoxygenation in the rat hippocampal slices. Effect of SMe1EC2 on synaptic transmission in rat hippocampal slices compared to control untreated slices exposed to transient 6-min ischemia. Duration of drug application is marked by a dotted line (SMe1EC2, 1 µmol/l, was present for 30 min before ischemia, during ischemia as well during reoxygenation) and ischemia (start at zero min) is marked by solid line. PoS amplitude at zero min of ischemia from each slice represents 100%. Values are the means ± SEM from untreated (n = 40) and treated slices (n = 39). Recovery of synaptic transmission in the treated group was significantly higher compared to the untreated controls, * $p < 0.05$ (ANOVA test)

Six minutes of ischemia caused a rapid decay of PoS amplitude followed by extinction even of EPSP and this synaptic transmission failure was irreversible after 20 min of reoxygenation in untreated control slices (80% of the total number of slices n = 40). The mean value of PoS amplitude at the end of reoxygenation from all untreated control slices was about 9.9 ± 3.6% of the initial amplitude before ischemic insult (Fig. 1). The neuroprotective effect of the drugs studied was tested against the functional damage induced by transient ischemia at the end of 20-min reoxygenation.

The compound SMe1EC2 (1 µmol/l) exerted a significant protective effect demonstrated by improvement of synaptic transmission; PoS amplitude recovery was 31.2 ± 7.0% of the initial response at zero minute of ischemia at the end of 20-min reoxygenation, the number of slices with an irreversible damage of synaptic transmission decreased to 64% of the total number of slices n = 39 (Fig. 1, 2, Tab. 1).

The selective adenosine A_1 receptor agonist CPA at the concentration of 50 nmol/l neither affected the PoS amplitude during the 30-min perfusion of slices nor did it exert any protective effect on synaptic transmission failure elicited by transient ischemia and on

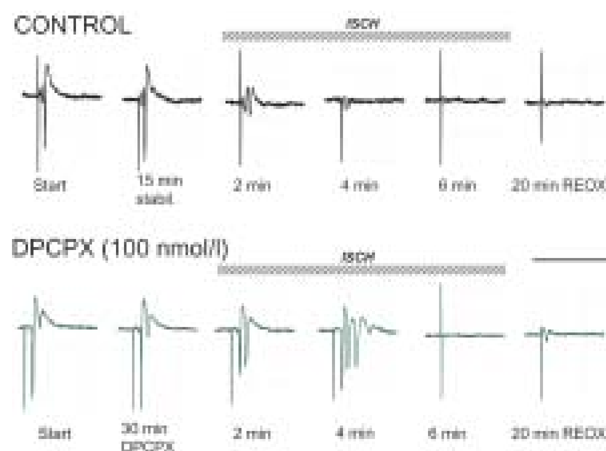

Fig. 3. Effect of the A_1 adenosine receptor antagonist DPCPX on synaptic transmission in the rat hippocampal slices. Original record of field action potentials in untreated control (upper trace) and DPCPX-treated (lower trace) representative rat hippocampal slices. Ischemia is marked by slashed line. From left to right: synaptic transmission at the end of 15-min stabilization period in control or after 30-min drug application in treated slice just before the beginning of ischemia, further at the 2nd, 4th and 6th min of ischemia and at 20th min of reoxygenation. DPCPX (100 nmol/l) was present for 30 min before ischemia, during ischemia, as well as during reoxygenation. Synaptic transmission decay during ischemia was delayed in the presence of DPCPX. In each slice tested (n = 13) an increased number of PoS spikes lasted about several tens of seconds. Synaptic transmission recovery at the end of reoxygenation was not improved compared to untreated control slices. Calibration: horizontally 50 ms, vertically 10 mV

Tab. 1. Effect of SMe1EC2 and compounds acting *via* activation or inhibition of A_1 and A_{2A} adenosine receptors

Group	Drug concentration (µmol/l)	n	PoS decay during ischemia $t_{0.5}$ (min)	PoS amplitude recovery in reoxygenation (%)	Slices with PoS amplitude recovery less than 15 % in reoxygenation (%)
Control	–	40	1.7 ± 0.1	9.9 ± 3.6	80
SMe1EC2	1.00	39	2.1 ± 0.1 ns	31.2 ± 7.0**	64*
CPA	0.05	8	1.6 ± 0.2 ns	10.6 ± 6.9 ns	75
DPCPX	0.10	13	3.6 ± 0.3***	10.6 ± 5.9 ns	77
CGS 21680	1.00	7	1.9 ± 0.3 ns	18.4 ± 14.3 ns	86
SCH-58261	1.00	7	2.0 ± 0.4 ns	3.4 ± 2.9 ns	86
SMe1EC2 + CPA	1.00 + 0.05	8	1.6 ± 0.2 ns	14.1 ± 7.7 ns	63*
SMe1EC2 + DPCPX	1.00 + 0.10	11	2.2 ± 0.2 ns	15.7 ± 11.6 ns	82
SMe1EC2 + CGS 21680	1.00 + 1.00	6	1.2 ± 0.3 ns	5.7 ± 4.7 ns	83
SMe1EC2 + SCH-58261	1.00 + 1.00	13	2.3 ± 0.1*	37.7 ± 12.4*	54***

The summarized effects of the antioxidant SMe1EC2 and four compounds, acting *via* adenosine receptors, on the resistance of rat hippocampal slices exposed to transient ischemia expressed by half-time ($t_{0.5}$) of PoS decay (in minutes) during ischemia, on the recovery of PoS amplitude at the end of reoxygenation, and on the contribution of hippocampal slices with PoS amplitude recovery less than 15% of initial response in each group tested. The *n* values express the number of slices used in each group. Values are the means ± SEM. The difference of PoS amplitude recovery was calculated by ANOVA test, the significance of the number of irreversibly damaged slices was calculated by chi-square test and the difference of the half-time of PoS decay during ischemia was calculated by *t*-test, all compared to untreated control slices. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns – no significant difference

PoS amplitude recovery in reoxygenation (Tab. 1). The concentration of CPA higher by one order of magnitude inhibited synaptic transmission in rat hippocampal slices (n = 6) already during the 15-min perfusion in "normoxic" conditions to less than 25% of the initial response at the beginning of CPA application (not shown), probably due to its presynaptic effect by activation of A_1 adenosine receptors and inhibition of glutamate release. CPA (500 nmol/l) could, therefore, not be further tested in experiments under ischemia.

The compound DPCPX (100 nmol/l), a selective adenosine A_1 receptor antagonist, elicited retarded synaptic transmission discharge during ischemia expressed by increased number of spikes (3–5) and increase in their amplitudes lasting for several tens of seconds in all the slices tested (n = 13) during ischemia (Fig. 4), indicating that DPCPX extended the $t_{0.5}$ of the PoS amplitude decay compared to controls (Tab. 1). No protective effect of DPCPX was found on synaptic transmission and PoS amplitude recovery at the end of 20-min reoxygenation (Fig. 3, Tab. 1).

Effect of A_{2A} adenosine receptor agonist and antagonist on synaptic transmission in rat hippocampal slices exposed to ischemia

Neither the adenosine A_{2A} receptor agonist CGS 21680 (1 µmol/l) nor the adenosine A_{2A} receptor antagonist SCH-58261 (1 µmol/l) exerted any neuroprotective effect on synaptic transmission recovery in rat hippocampal slices after 6-min ischemia at the end of 20-min reoxygenation compared to untreated controls (Tab. 1).

Effect of co-administration of SMe1EC2 with A_1 and A_{2A} adenosine receptor agonists and antagonists on synaptic transmission in rat hippocampal slices exposed to ischemia

Drugs acting *via* adenosine receptors, CPA (50 nmol/l), DPCPX (100 nmol/l), CGS 21680 (1 µmol/l) and SCH-58261 (1 µmol/l), were tested in the presence of the pyridoindole compound SMe1EC2 (1 µmol/l) in slices exposed to transient 6-min ischemia followed by 20-min reoxygenation (Tab. 1). Co-administration

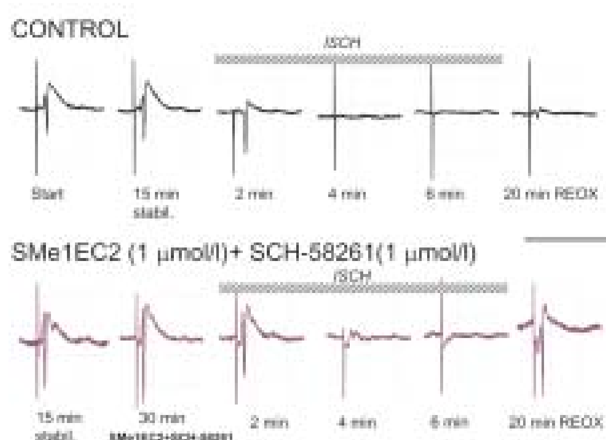

Fig. 4. Effect of co-administration of the pyridoindole SMe1EC2 and the A_{2A} adenosine receptor antagonist SCH-58261 on synaptic transmission in the rat hippocampal slices. Original record of field action potentials in untreated control (upper trace) and SMe1EC2 + SCH-58-treated (lower trace) representative rat hippocampal slices. Ischemia is marked by a slashed line. From left to right: synaptic transmission at the end of 15-min stabilization period in control or after 30-min drug application in treated slice just before the onset of ischemia, further in the 2nd, 4th and 6th min of ischemia and at the 20th min of reoxygenation. Compounds SCH-58261 (1 µmol/l) and SMe1EC2 (1 µmol/l) were present for 30 min before ischemia, during ischemia, as well as during reoxygenation. Synaptic transmission decay during ischemia was delayed in the presence of these drugs and synaptic transmission recovery at the end of reoxygenation was improved compared to untreated control slices. Calibration: horizontally 50 ms, vertically 5 mV

of SMe1EC2 and CPA (A_1 adenosine receptor agonist) induced a significant decrease in irreversibly damaged slices compared to untreated controls. Co-administration of SMe1EC2 + DPCPX (A_1 adenosine receptor antagonist) or with CGS 21680 (A_{2A} adenosine receptor agonist) eliminated the neuroprotective effect of the antioxidant. Co-administration of SCH-58261 and SMe1EC2 resulted in: 1) a significantly delayed ST decay during the ischemic period, 2) a significant increase in PoS amplitude recovery, and 3) an extremely significant decrease in the number of irreversibly damaged slices at the end of 20-min reoxygenation, all compared to untreated control slices (Fig. 4, Tab. 1).

Discussion

One of the purposes of intensive pharmacological research is to obtain drugs with potent neuroprotective effect and to diminish the oxidative stress induced injury, e.g. in stroke, neurotrauma, neurodegeneration, etc. Ischemia evokes a severe reduction of energy supplies and in order to cope with this and to survive, any reduction in energy consuming activity is considered to have a protective effect [6]. Yet an important question is for how long the discharge of synaptic transmission is needed and possible. Based on our experience, complete inhibition of synaptic transmission lasting 3 to 4 min during ischemia results in almost no recovery of synaptic potentials and PoS in neurons in 20-min reoxygenation. Therefore, the effect of drugs on the recovery of neuronal functionality appears to be rather important in neuroprotection.

Reduction of synaptic transmission in hippocampal slices during ischemia was demonstrated in our previous works [4, 20, 23] and also by many other authors [1, 2, 8]. Antioxidants with different structure exerted a protective effect expressed by the increased resistance of hippocampal neurons in reoxygenation. These include for example Trolox (a water soluble analogue of α-tocopherol), α-tocopherol, melatonin, the 21-aminosteroid U-74389G [23], 2,3-dihydromelatonin [4], as well as the pyridoindole SMe1EC2, which surpassed in its antioxidant property such well-established antioxidants as melatonin, Trolox, and others [21]. The inhibitory effect of SMe1EC2 on lipid peroxidation in the nervous system may be involved in the mechanism which retains functional cell membranes. An antiexcitotoxic effect of SMe1EC2 has not been studied yet, nevertheless, we found that the main representative of this pyridoindole group, namely stobadine, did not block [^{3}H]glutamate binding sites [7], and it is unlikely that it would directly diminish excitotoxicity of amino acid neurotransmitters. However, SMe1EC2 at the concentration of 100 µmol/l reduced the PoS amplitude during 15-min "normoxia" to 79%, similarly as did a high concentration of adenosine (100 µmol/l) to 83% [unpublished data]. Thus, some effect on glutamate-elicited neurotransmission cannot be excluded.

Apparently, several mechanisms underlie the block of synaptic function in neurons during ischemic insults and not only an increased generation of ROS. The aim of this study was to find whether the neuroprotective effect of a putative antioxidant will be affected by manipulation of adenosine receptors. On the basis of literature data on neuroprotection due to A_1 adenosine receptor activation or A_{2A} adenosine receptor inhibition [11, 13, 15, 24, 25], our hypothesis was

to improve the neuroprotective effect of the prospective pyridoindole antioxidant SMe1EC2 by its co-administration with selective A_1 and A_{2A} adenosine receptor ligands. SMe1EC1 was the most effective compound in our experimental conditions when the tested drugs were administered singly. Some improvement in hippocampal resistance to oxidative stress occurred only after its co-administration with the A_{2A} adenosine receptor antagonist SCH-58261. It is noteworthy that co-administration of SMe1EC2 with the A_1 adenosine receptor antagonist DPCPX or with the A_{2A} adenosine receptor agonist CGS 21680 eliminated the neuroprotective effect exerted by the singly administered pyridoindole antioxidant.

In conclusion, concerning the multifactorial process in brain ischemic injury, intervention will be needed at many different steps of this impairment. These preliminary data show that manipulation with adenosine receptors may affect the neuroprotective effect of the pyridoindole antioxidant SMe1EC2.

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