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RAPID COMMUNICATION

Age- and Dose-Specific Anticonvulsant Action of Bumetanide in Immature Rats

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Summary

GABA exhibits depolarizing action in the immature neurons due to high intracellular activity of chloride ions. It is maintained by cation-chloride cotransporter NKCC1 which is present in immature brain. Bumetanide is a specific inhibitor of this cotransporter. We studied possible anticonvulsant activity of bumetanide in pentylenetetrazol-induced seizures in three age groups of rat pups (7, 12, and 18 days old). Pretreatment with bumetanide (0.2-1 mg/kg i.p.) resulted in dose-dependent decrease of incidence of the tonic phase of generalized tonic-clonic seizures in 12-day-old rats only. No effect was observed in younger and older animals. Higher dose of bumetanide (2.5 mg/kg) did not affect tonic convulsions but, on the contrary, decreased latencies of generalized seizures in 12-day-old animals. Lack of marked anticonvulsant effect is probably due to relative maturity of neurons in the brainstem where the generator of generalized seizures is localized. Age- and dose-specific suppression of the tonic phase needs further analysis.

Key words

Chloride transporter • GABA • Pentylenetetrazol • Ontogeny • Rat

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Main inhibitory system in the brain is using gamma-aminobutyric acid (GABA) as a transmitter. Among the two basic types of GABA receptors ionotropic GABA-A receptors are more numerous than metabotropic GABA-B receptors. When supramolecular complex of GABA-A receptor is activated, anionic channel is opened and chloride ions can flow into neurons. An increased permeability for chloride ions results in hyperpolarization of neuronal membrane, i.e. to inhibitory action (for review see Möhler *et al.* 2005). This is valid in mature neurons where intracellular activity of Cl^- is low and KCC2 cation-chloride cotransporter pumping Cl^- from cells into extracellular space is active. On the other hand, immature neurons have low (if any) activity of this transporter but they possess a NKCC1 transporter working in the opposite direction. Therefore activation of GABA-A receptors in immature neurons results in depolarization instead of hyperpolarization and GABA exerts an excitatory action (Ben-Ari 2002). The two cation-chloride cotransporters develop in opposite directions: NKCC1 activity decreases whereas activity of KCC2 increases with maturation (Payne *et al.* 2003). It was recently demonstrated that an inhibitor of NKCC1 transporter bumetanide exhibits anticonvulsant action in at least two seizure models in immature rats (kainic acid-induced seizures - Dzhala *et al.* 2005; rapid hippocampal kindling - Mazarati *et al.* 2009) as well as *in vitro* hippocampal slices (Dzhala *et al.* 2005) and two intact connected hippocampi preparation (Khalilov *et al.* 2003, Kilb *et al.* 2007). Failure of anticonvulsant activity of bumetanide in some *in vitro* models demonstrated specificity of this action (Kilb *et al.* 2007). Because of this specificity we decided to examine bumetanide effects in another *in vivo* model – pentylenetetrazol(PTZ)-induced seizures. Two types of seizures (minimal clonic and generalized tonic-clonic) with different generators and development can be elicited by this drug (Velíšek *et*

al. 1992).

Experiments were performed on three age groups of male albino rats of the Wistar strain – 7, 12 and 18 days old. The animals were taken from the nest immediately before the start of the experiment and their body temperature was maintained by means of a pad heated to 34 °C (temperature in the nest) during the whole experiment. This experiment was approved by Animal Care and Use Committee of the Institute of Physiology to be in agreement with Animal Protection Law of the Czech Republic as well as with European Community Council directives 86/609/EEC.

Bumetanide was dissolved in dimethylsulfoxide (1 mg in 0.5 ml) and immediately before administration was diluted with 1.5 ml of distilled water. It was injected intraperitoneally in doses of 0.2, 0.5, 1 and 2.5 mg/kg. Control animals received 25 % dimethylsulfoxide in a volume corresponding to the highest dose of bumetanide. Each age and dose group consisted from 8 animals. Twenty minutes later pentylenetetrazol (PTZ) was administered subcutaneously in a dose of 100 mg/kg. Both drugs were supplied by Sigma (St. Louis, MO).

The rats were observed in individual cages for 30 min after PTZ injection. Incidence, pattern and latency of seizures and all other behavioral phenomena were registered. Severity of seizures was quantified with a 5-point scale (Pohl and Mareš 1987). Incidence of generalized tonic-clonic and generalized clonic seizures was statistically evaluated by Fisher's exact test, latencies by one-way ANOVA with *post-hoc* pairwise comparison by Holm-Sidak's test (SigmaStat® SPSS). $P<0.05$ was taken as significant.

The first sign of PTZ action was increased locomotion with frequent sniffing. All but one 18-day-old rats in the group with the 2.5-mg/kg dose of bumetanide exhibited generalized seizures (Fig. 1). A complete sequence of generalized tonic-clonic seizures was observed in all 7-day-old rats with the exception of one animal with the highest dose of bumetanide where the tonic phase was missing. Tonic phase was suppressed by bumetanide in 12-day-old rats. This effect increased with the dose of bumetanide up to 1 mg/kg (7 out of 8 rats exhibited only generalized clonic seizures), the highest dose had less expressed effect. No effects on seizure pattern were observed in 18-day-old rats. Minimal clonic seizures (normally preceding generalized tonic-clonic seizures in 18-day-old and older rats) were only exceptionally present in control as well as pretreated animals.

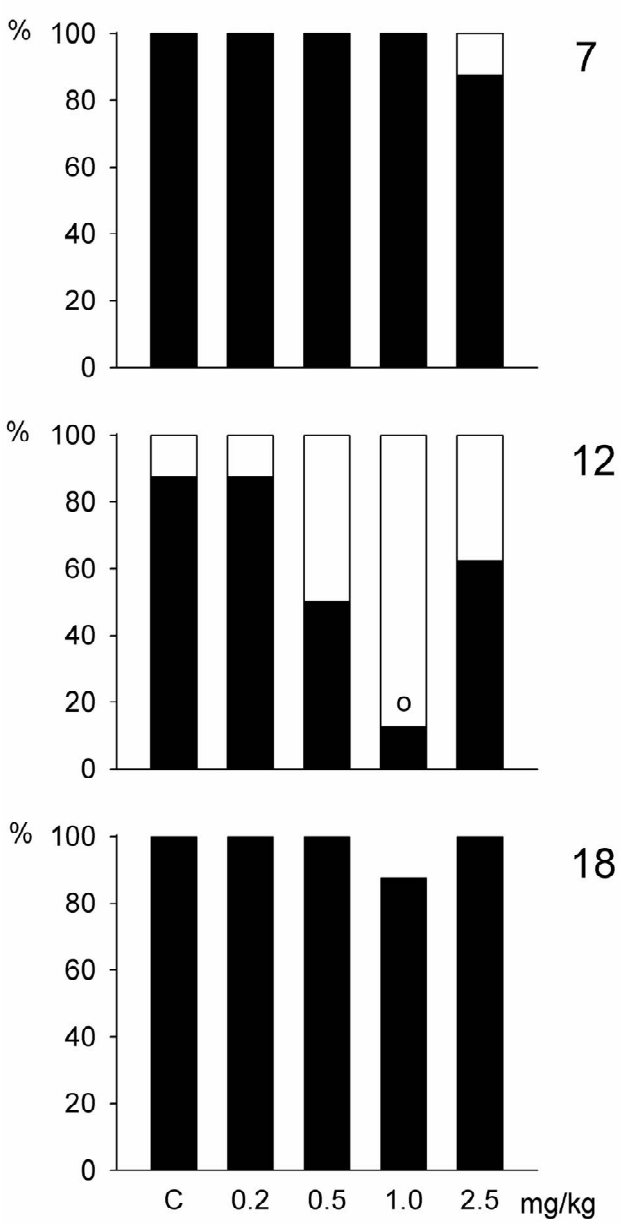

Fig. 1. Incidence of seizures in three age groups of rats; from top to bottom – 7-, 12- and 18-day-old animals. Black parts of columns represent generalized tonic-clonic seizures, white parts – generalized clonic seizures (i.e. tonic phase is missing). Abcissae: doses of bumetanide, C means controls; ordinates: percentage of rats exhibiting seizures. o denotes a significant suppression of the tonic phase.

Seizure severity reflected above mentioned seizure pattern, i.e. presence or absence of the tonic phase (Fig. 2). The only significant decrease in comparison with controls was found after the 1-mg/kg dose in 12-day-old rats.

Latencies of generalized seizures were never prolonged (Fig. 2). On the contrary, the 2.5-mg/kg dose of bumetanide resulted in significant shortening of latencies in 12-day-old animals. Mixed anti- and

proconvulsant effects of bumetanide were demonstrated in whole hippocampus preparation from newborn mice. Bumetanide affected differently not only epileptiform activity in various models but also individual epileptic phenomena in the same model (Kilb *et al.* 2007, Nardou *et al.* 2009).

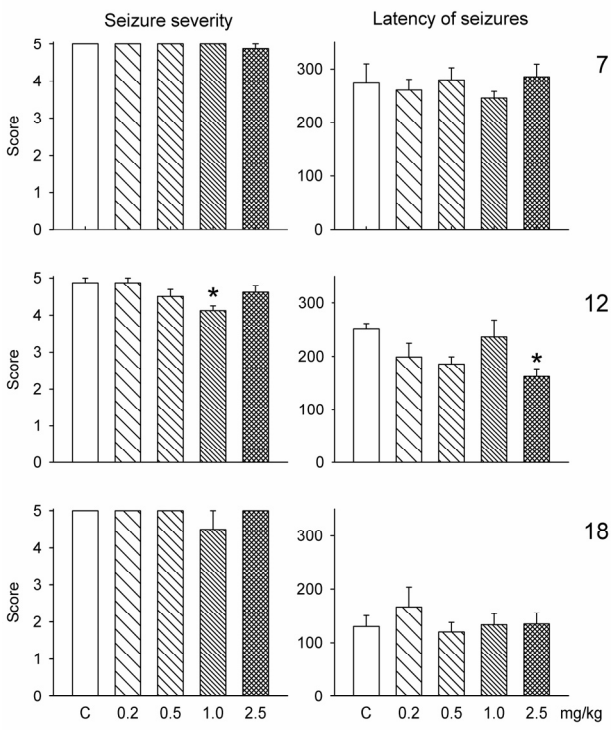

Fig. 2. Severity (left column) and latencies (right column) of seizures in 7-, 12- and 18-day-old rats (from top to bottom) presented as means ± S.E.M. Abscissae: doses of bumetanide; ordinates in the left column: seizure severity scored according to the 5-point scale; in the right column: latencies in seconds. Asterisks denote a significant difference in comparison with the control group.

Our experiments demonstrated only an isolated anticonvulsant effect of bumetanide in 12-day-old rats where the tonic phase of generalized seizures was suppressed. A proconvulsant action – shortening of latencies of seizures – was observed in the same age group with the high dose of bumetanide. Failure of bumetanide in our experiments is probably due to the localization of generators of generalized tonic-clonic seizures. Browning and Nelson (1985) localized the generator of these seizures into the brainstem; descending part of reticular formation, i.e. the most important structure in regulation of muscle tonus, is a hot candidate. Maturation of the brain generally follows caudo-rostral gradient, therefore brainstem should be mature very early. In addition, centers governing breathing and circulation are localized in the brainstem reticular formation and these centers must be mature at latest at a moment of birth. To explain specific suppression of the tonic phase of generalized seizures in 12-day-old but not in younger and older rats detailed data on generation of the tonic and clonic phases are needed. Failure of minimal clonic seizures in 18-day-old rats was surprising but it appeared also in control group with administration of dimethylsulfoxide. Nearly all 18-day-old rats injected with saline or without any pretreatment exhibit regularly this type of seizures (25 out of 27 animals in control groups from recent studies – e.g. Mareš 2008a,b); therefore this effect has to be ascribed to dimethyl-sulfoxide.

Bumetanide is a loop diuretic used in adult and pediatric nephrology (Eades and Christensen 1998); its action in kidney influences water and electrolyte composition in the whole body. A decrease of extracellular space in the brain was described after bumetanide and/or furosemide administration. This change can substantially influence normal as well as pathological (epileptic) activity and should be taken into account in experiments with loop diuretics (Schwartzkroin *et al.* 1998).

According to the literary data bumetanide represents a potential anticonvulsant drug specific not only for neonatal seizures (first clinical case was recently published by Kahle *et al.* 2009) but also for long-lasting epileptic activity which may result in an increased intracellular Cl^- activity as demonstrated *in vitro* in slices from adult rodents (Khalilov *et al.* 2003) and from temporal lobe of epileptic patients (Huberfeld *et al.* 2007). Another possible use of bumetanide in epileptic syndromes in infancy is potentiation of action of anticonvulsant drugs with GABAergic mechanism of action (Dzhala *et al.* 2007). Further studies are necessary to specify possible clinical use of this promising drug.

Conflict of Interest

There is no conflict of interest.

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