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# A novel mechanism for the anticonvulsant effect of furosemide in rat hippocampus in vitro

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ABSTRACT

Though both in vivo and in vitro studies have demonstrated an anticonvulsant effect of the loop diuretic furosemide, the precise mechanism behind this effect is still debated. The current study investigates the effect of furosemide on Cs-induced epileptiform activity (Cs-FP) evoked in area CA1 of rat hippocampal slices in the presence of $Cs^+$ (5 mM) and ionotropic glutamatergic and GABAergic receptor antagonists. As this model diverges in several respects from other epilepsy models it can offer new insight into the mechanism behind the anticonvulsive effect of furosemide. The present study shows that furosemide suppresses the Cs-FP in a dose-dependent manner with a near complete block at concentrations $\geq$ 1.25 mM. Because furosemide targets several types of ion transporters we examined the effect of more selective antagonists. Bumetanide (20 μM), which selectively inhibits the Na–K–2Cl co-transporter (NKCC1), had no significant effect on the Cs-FP. VU0240551 (10 μM), a selective antagonist of the K–Cl co-transporter (KCC2), reduced the ictal-like phase by 51.73±8.5% without affecting the interictal-like phase of the Cs-FP. DIDS (50 μM), a nonselective antagonist of $Cl^-/HCO_3^-$-exchangers, $Na^+$–$HCO_3^-$-cotransporters, chloride channels and KCC2, suppressed the ictal-like phase by 60.8±8.1% without affecting the interictal-like phase. At 500 μM, DIDS completely suppressed the Cs-FP. Based on these results we propose that the anticonvulsant action of furosemide in the $Cs^+$-model is exerted through blockade of the neuronal KCC2 and $Na^+$-independent $Cl^-/HCO_3^-$-exchanger (AE3) leading to stabilization of the activity-induced intracellular acidification in CA1 pyramidal neurons.



## 1. Introduction

Epilepsy is one of the most common neurological disorders with a prevalence of approximately 1% worldwide. Although most patients are well treated with antiepileptic drugs, approximately one-third do not successfully respond to drug treatments (Kwan and Brodie, 2006). The currently available drugs predominantly exert their anticonvulsant action through inhibition of voltage-dependent $Na^+$ and/or $Ca^{2+}$ channels, inhibition of excitatory synaptic transmission or by enhancing inhibitory synaptic transmission (Löscher, 1998). However, seizure generation is likely to be multifactorial involving not only synaptic and

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intrinsic neuronal properties but also non-synaptic interactions (e.g. gap junction signaling, field effects and ion fluctuations). In vitro experiments have shown that targeting the latter mechanisms often leads to an effective blockade of epileptiform activity (Hochman, 2009; Jefferys, 1995; Velazquez and Carlen, 2000). Non-synaptic interactions thus appear to be an obvious focal point for developing new therapeutic strategies. For this reason, loop diuretics such as furosemide and bumetanide have attracted considerable interest as they have been suggested to reduce the efficacy of non-synaptic interactions.

The ability of loop diuretics to suppress epileptiform activity has been demonstrated in several experimental models, and clinical trials have shown that an anticonvulsant effect can be obtained at concentrations which give negligible side effects (Ahmad et al., 1976; Eftekhari et al., 2013; Gutschmidt et al., 1998; Haglund and Hochman, 2005; Hesdorffer et al., 2001; Hochman et al., 1995; Holtkamp et al., 2003; Kilb et al., 2007; Maa et al., 2011; Margineanu and Klitgaard, 2006). Both bumetanide and furosemide blocks the Na–K–2Cl cotransporter (NKCC1), and in addition, furosemide blocks other proteins such as the K–Cl cotransporter (KCC2), the $Na^+$-independent $Cl^-/HCO_3^-$ exchanger (AE3), some subtypes of $GABA_A$ receptors as well as the carbonic anhydrase (Maa et al., 2011; Staley, 2002). Collectively, these proteins are involved in the regulation of extracellular $K^+$ and intracellular $Cl^-$ homeostasis, cell volume, and pH, all of which are important for maintaining normal brain activity. The polypharmacological profile of furosemide as well as the central homeostatic functions of its targets has made it challenging to outline the exact mechanisms behind its anticonvulsant effect. A prevailing idea, which is supported by several studies, is that loop diuretics act by blocking glial NKCC1 thus preventing the activity-driven shrinkage of the extracellular space (ECS) (Haglund and Hochman, 2005; Hochman, 2012; Hochman et al., 1995). This will lead to a reduction in the efficacy of the non-synaptic interactions which are important for recruiting and synchronizing neuronal networks into an epileptiform discharge (Hochman, 2012; Jefferys, 1995). There is also evidence that furosemide works by reducing the activity-driven increase in extracellular $K^+$ ($[K^+]_o$) (Gutschmidt et al., 1998; Holtkamp et al., 2003; Stringer and Pan, 1997). This could either be through a direct effect on neuronal excitability (Gutschmidt et al., 1998), a reduction in glutamatergic synaptic transmission (Staley, 2002), an enhanced buffering of extracellular $K^+$, via glial inward rectifying $K^+$ ($K_{IR}$) channels (Barbaro et al., 2004), or through blocking GABA-dependent transient increase in $[K^+]_o$ (Viitanen et al., 2010). However, other observations suggest that the anticonvulsant effect is related to an increase in $[K^+]_o$ leading to an enhanced excitability and desynchronization (Hochman et al., 1999; Hochman and Schwartzkroin, 2000; Su et al., 2002).

In the present study we have investigated the effect of furosemide on Cs-induced epileptiform activity. In this model, the evoked epileptiform activity is induced in the presence of $Cs^+$ (5 mM) and ionotropic glutamatergic and GABAergic receptor antagonists by orthodromic stimulation of the Schaffer collateral–commissural fibers (Skov et al., 2005). The epileptiform field potential (Cs-FP) is biphasic with an initial positive (interictal-like) phase followed by a prolonged negative (ictal-like) phase. Preliminary findings have indicated that furosemide effectively blocks the Cs-FP. Because of the presence of $Cs^+$, which inhibits glial $K_{IR}$ channels (Ransom and Sontheimer, 1995), it is unlikely that such effect of furosemide involves changes in glial $K_{IR}$ channel activity, and the co-presence of synaptic blockers excludes the involvement of fast glutamatergic and GABAergic synaptic transmission. Furthermore, as the Cs-FP is only in part sensitive to changes in ECS (Andreasen et al., 2007) it is unexpected that the prevention of the activity-driven shrinkage of ECS, observed during seizure-like activity, can fully account for the anticonvulsive effect of furosemide. Thus, the observation that furosemide can supress epileptiform activity in this model seems, in itself, to challenge several of the aforementioned proposed mechanisms. The aim of the present study was therefore to investigate the mechanisms behind the suppressive effect of furosemide on $Cs^+$-induced epileptiform activity. The study was designed to provide a quantitative assessment of the effect of furosemide (including dose-dependency) on different components of the Cs-FP and to compare furosemide and related drugs with different pharmacological profiles. The results indicate that stabilization of activity-induced intracellular acidification is a key step in the anticonvulsant effect of furosemide.

## 2. Results

### 2.1. *Furosemide blocks Cs-induced epileptiform activity*

Earlier in vitro studies have shown that the effective anticonvulsive concentration of furosemide is between 2.5 and 5 mM (Gutschmidt et al., 1998; Hochman et al., 1995; Hochman et al., 1999; Margineanu and Klitgaard, 2006). We found that 2.5 mM furosemide was a sufficient dose for blocking the orthodromically evoked epileptiform field activity in the presence of $Cs^+$ (Fig. 1A). The effect was relatively fast with a 10–12 min delay from onset of perfusion to maximal inhibition ($n=7$). In accordance with previous reports (Gutschmidt et al., 1998; Hochman et al., 1995; 1999), a transient enhancement of the response occurred in the beginning (~5 min) of the application (Fig. 1C). After 30 min perfusion, washing out furosemide for 44.4±21.5 min (mean±S.D., $n=7$) resulted in only minor reversal of the Cs-FP. To investigate the dose-dependency of the suppressive effect of furosemide, we made a quantitative assessment of the size of the interictal-like and ictal-like phases of the Cs-FP before and after adding concentrations between 0.01–2.5 mM. Data obtained with high concentrations of furosemide (0.63 mM, $n=7$; 1.25 mM, $n=6$; and 2.5 mM, $n=7$; 30 min perfusion) showed a reduced amplitude of the interictal-like phase (Fig. 2A), which was significant at the two highest concentrations. The mean amplitude was 31.1±9.1% of control at 1.25 mM ($p<0.05$), and 9.7±2.7% at 2.5 mM ($p<0.001$; one-way ANOVA/Tukey's test). The duration of the interictal-like phase was variably and insignificantly affected by furosemide though there was a tendency for a reduction at the highest concentrations (Fig. 2B). The ictal-like phase was, however, consistently reduced by any of the high concentrations (Fig. 2C). The area of this component was reduced to 17.7±5.2% of control at 0.63 mM ($p<0.001$; one-way ANOVA/