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EFFECTS OF VU0240551, A NOVEL KCC2 ANTAGONIST, AND DIDS ON CHLORIDE HOMEOSTASIS OF NEOCORTICAL NEURONS FROM RATS AND HUMANS

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Abstract—The normal function of $GABA_A$ receptor-mediated inhibition is governed by several factors, including release of GABA, subunit composition and density of the receptors and in particular by the appropriate ionic gradient. In the human epileptogenic neocortex an impaired chloride (Cl^-) gradient has been proposed, due to decreases of potassium-coupled chloride transport (KCC2) and voltage-gated Cl^- channels (ClC). Regarding sodium- and potassium-coupled Cl^- transport (NKCC1) both up- and downregulations have been proposed. We investigated changes of Cl^- homeostasis of human and rat neocortical neurons (layer 2/3) with intracellular recordings and iontophoretic Cl^- loading employing selective compounds. After cessation of iontophoresis, the $IPSP_A$ amplitudes of rat neurons recovered with a time constant (τ_{rec}) of 6.5 s (n = 21). In human neurons, τ_{rec} averaged 17.8 s (n = 36; 23 resections). Application of the novel KCC2 blocker VU0240551 (1 μM) caused in rat neurons a reversible prolongation of τ_{rec} from 5.7 to 8.1 s (n = 11), corresponding to a VU0240551-sensitive Cl^- transport rate ($1/\Delta\tau_{rec}$) of 0.0504 s^{-1}. In human neurons, τ_{rec} increased on application of 1 μM VU0240551, on average from 15.1 to 20.3 s (n = 17). The human neurons comprised two subgroups with different τ_{rec} when segregated according to a border given by the mean + 2 s.d. of rat neurons. In one group, τ_{rec} averaged 8.7 s (n = 6) and reversibly increased to 14.6 s in the presence of 1 μM VU0240551, corresponding to a Cl^- transport rate of 0.0504 s^{-1}. The other group had an average τ_{rec} of 18.5 s which increased in the presence of 1 μM VU0240551 to 23.3 s (n = 11), indicating a much smaller rate (0.0151 s^{-1}). Addition of DIDS, a presumed blocker of anion exchanger (AE), increased the τ_{rec} of rat neurons from 7.5 to 8.8 s (n = 6) corresponding to a DIDS-sensitive rate of 0.0185 s^{-1}. In human neurons, DIDS increased τ_{rec} from 23.3 to 50.7 s (n = 7), corresponding to a DIDS-sensitive rate of 0.0200 s^{-1}. These data suggest a greatly reduced KCC2-mediated transport rate in most of the human neurons. The two subgroups observed in human tissue indicate a considerable variability of Cl^- transport within a given tissue from almost normal to greatly impeded, predominated by a decline of KCC2 whereas AE is unaltered.

Key words: chloride regulation, TLE, VU0240551, anion exchanger, GABA, neocortex.

INTRODUCTION

GABA, the key inhibitory transmitter in the central nervous system activates two functionally and molecularly distinct receptors, coined $GABA_A$ and $GABA_B$. The latter are multimers of heptahelical subunits coupling via G-proteins to various effectors (see Gassmann and Bettler, 2012). The former consist of a pentameric arrangement of subunits with 4 transmembrane domains forming a central pore (Schofield et al., 1987) which allows the passage of small anions (Bormann et al., 1987). The ionic gradient prevailing during the $GABA_A$ receptor-mediated inhibitory postsynaptic potential ($IPSP_A$) is governed by a K^+-coupled Cl^- outward transport in the adult hippocampus (Misgeld et al., 1986; Rivera et al., 1999) and neocortex (Thompson et al., 1988a; DeFazio et al., 2000; Zhu et al., 2005) which maintains a low intracellular Cl^- concentration ($[Cl^-]_i$). This transporter shares several similarities with the K^+-coupled Cl^- extrusion of invertebrate neurons (Aickin et al., 1982; Deisz and Lux, 1982). The underlying transporter has been cloned, revealing a neuron-specific isoform termed KCC2 (K^+–Cl^- cotransporter) (Payne et al., 1996; Williams et al., 1999). Despite the presence of KCC2, the reversal potential of $GABA_A$ responses (E_{IPSP-A}) is close to resting membrane potential

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Abbreviations: 9AC, 9-anthracene carboxylic acid; ACSF, artificial cerebrospinal fluid; AE, anion exchanger; AHS, ammon's horn sclerosis; ANOVA, analysis of variance; CDC-ACSF, ACSF containing CNQX, D-APV and CGP 55845A; CGP 55845A, $GABA_B$ receptor antagonist; CNQX, antagonist for AMPA/kainate type receptors; D-APV, antagonist for NMDA type receptors; DIDS, (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), blocker of AE; E_{Cl}, reversal potentials for Cl^-; E_{IPSP-A}, reversal potential of the $GABA_A$ receptor mediated inhibitory postsynaptic potential; E_m, membrane potential; EGTA, ethylene glycol tetraacetic acid; $IPSP_A$, inhibitory postsynaptic potential mediated by $GABA_A$ receptors; KCC2, K^+–Cl^- cotransporter; mACSF, modified artificial cerebrospinal fluid; MWRT, Mann–Whitney rank test; NKCC1, Na^+–K^+–$2Cl^-$ cotransporter; TLE, temporal lobe epilepsy; TT, Student's t-test; VU024, KCC2 type transport blocker (*N*-(4-methyl-2-thiazolyl)-2-[(6-phenyl-3-pyridazinyl)thio)]-acetamide (VU0240551); WSRT, Wilcoxon signed rank test.

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(E_m; Thompson et al., 1988a; Deisz and Prince, 1989), due to a partial bicarbonate permeability of the $GABA_A$ receptors causing a deviation between the reversal potential for Cl^- (E_{Cl}) and the E_{IPSP-A} (Kaila et al., 1993). During early postnatal stages, however, a Na^+–K^+–$2Cl^-$ transporter (NKCC1) predominates (Luhmann and Prince, 1991; Dzhala et al., 2005; Achilles et al., 2007), which mediates inward transport of Cl^- and might set the E_{Cl} to less negative values, i.e. promotes depolarizing $GABA_A$ receptor-mediated responses (for review see Ben-Ari, 2002). Other established anion pathways in central neurons e.g. the Na^+/HCO_3^- exchanger (Pedersen et al., 1998), Cl^-/HCO_3^- exchanger (AE; Havenga et al., 1994) or voltage-activated Cl^--channels (ClC; Clark et al., 1998) have rarely been investigated.

Impairment of GABAergic inhibition has long been implicated in the generation of epileptiform activity (Gutnick et al., 1982). Yet, studies on human tissue *in vitro* revealed apparently normal synaptic inhibition mediated by $GABA_A$ receptors (Gibbs et al., 1996). More recently, evidence accumulated indicating an impaired Cl^- homeostasis in human epileptogenic tissues (Deisz et al., 1998; Cohen et al., 2002; Huberfeld et al., 2007; Deisz et al., 2011). Two contending mechanisms emerged from these studies which may account for the depolarizing responses mediated by $GABA_A$ receptors: Firstly, an upregulation of NKCC1 (Huberfeld et al., 2007; Palma et al., 2006), similar to the situation prevailing during early postnatal stages (Luhmann and Prince, 1991; Rivera et al., 1999). Secondly, decreases of KCC2 (and other Cl^- pathways; Deisz et al., 2011) may cause a passive Cl^- distribution across the membrane and the partial HCO_3^- permeability of the $GABA_A$ receptors (Kaila et al., 1993) governs the depolarization.

To further evaluate the components of Cl^- homeostasis in neocortical neurons, we investigated the effects of VU0240551 ((*N*-(4-methyl-2-thiazolyl)-2-[(6-phenyl-3-pyridazinyl)thio)]-acetamide; abbreviated here VU024), a novel KCC2 antagonist (Delpire et al., 2009; Lindsley et al., 2009), and of DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) on human and rat neocortical neurons (layer 2/3). The effects of DIDS have been studied on various cells. DIDS binds to the band 3 protein of erythrocytes (an AE homolog) and causes a 90% decrease of sulfate efflux (at 25 μM, Cabantchik et al., 1978). DIDS appears to be selective for the AE type of Cl^- transporters. The Na^+/K^+-dependent Cl^- uptake of squid axons is unaffected by DIDS (50 μM) but blocked by the loop diuretics furosemide and bumetanide (Russell, 1983). In addition, voltage-activated Cl^- currents (ClC) of *Aplysia* neurons are unaffected by extracellular DIDS at concentrations below 1 mM (Chesnoy-Marchais, 1983). In embryonic motoneurons, 50 μM DIDS antagonizes a fraction of Cl^- accumulation, complementing the bumetanide-sensitive NKCC1 (Gonzalez-Islas et al., 2009). The pH regulating mechanisms of cultured cortical neurons are reduced by 30% with 300 μM DIDS (Pedersen et al., 1998). At high concentrations (1 mM), DIDS reduced ClC of dissociated superior cervical ganglia by 35% (Clark et al., 1998).

Previous data indicated a marked depression of the Cl^- transport rates via KCC2, NKCC1 and ClC in human neocortical neurons (Deisz et al., 2011). The rates via KCC2 and AE were inferred rather indirectly from a combination of methods. Here we set out to evaluate the relative contribution of KCC2 and AE type pathways to Cl^- transport in rat and human neocortical neurons using the novel KCC2 antagonist VU024 (Lindsley et al., 2009) and DIDS.

EXPERIMENTAL PROCEDURES

The methods have been described previously (Teichgräber et al., 2009; Deisz et al., 2011). In brief, human neocortical tissues were collected in the operating theater and transported to the laboratory in cold (about 5 °C) modified artificial cerebrospinal fluid (mACSF, see below). Each patient provided informed consent and the experiments were approved by the local ethics committee adhering to the Declaration of Helsinki. We investigated tissues from 26 patients (14 female, 12 male). The patients were on average 35.5 ± 11.9 years old and had epilepsy for 20.0 ± 14.1 years (see Table 1 for details).

Preparation and storage of brain slices

Blocks of human tissues were mounted with histoacryl glue (B. Braun, Melsungen, Germany) on the platform of a vibratome (HM 650V, MICROM International, Walldorf, Germany) and cut into slices (400 μm) in normal artificial cerebrospinal fluid (ACSF, 6 ± 1 °C, see below). Parallel experiments were carried out on neocortical slices from male rats (Wistar, age: 30–60 d, Janvier). Rats were anesthetized with diethylether, decapitated and a block containing the sensorimotor cortex was removed, immersed in cold (about 5 °C, 1 min) ACSF, glued to the platform of the vibratome and cut into slices. The subsequent storage and handling of slices was identical for both species (Deisz et al., 2011). Slices were stored in ACSF at room temperature until used. Individual slices from either species were transferred to a submerged type recording chamber continuously perfused with ACSF. The bath was maintained at constant temperature (32 °C) with a temperature control unit (SCTC 20 E, npi Tamm, Germany).

Solutions

The normal ACSF contained (in mM): 124 NaCl, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D(+)-glucose (pH 7.4) and was continuously equilibrated with carbogen (95%O_2/5%CO_2; pH 7.4). The mACSF, used for tissue transport, contained (in mM): 70 NaCl, 2.5 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 D(+)-glucose and 75 sucrose. All substances were of analytical grade (Merck, Darmstadt, Germany). In some experiments $GABA_A$ receptor-mediated responses were pharmacologically isolated by adding 10 μM CNQX (antagonist for AMPA/kainate type receptors), 20 μM D-APV (antagonist for NMDA type receptors) and 1 μM CGP 55845A ($GABA_B$ receptor