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Effects of Chloride Flux Modulators in an in Vitro-model of Brain Edema Formation

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Abstract

Brain edema is a serious consequence of hemispheric stroke and traumatic brain injury and contributes significantly to patient mortality. In the present study, we measured water contents in hippocampal slices as an in vitro-model of edema formation. Excitotoxic conditions induced by N-methyl-D-aspartate (NMDA, 300 μ M), as well as ischemia induced by oxygen-glucose deprivation (OGD) caused cellular edema formation as indicated by an increase of slice water contents. In the presence of furosemide, an inhibitor of the Na,K,Cl-cotransporter, NMDA-induced edema were reduced by 64% while OGD-induced edema were unaffected. The same observation, i.e. reduction of excitotoxic edema formation but no effect on ischemia-induced edema, was made with chloride transport inhibitors such as DIDS and niflumic acid. Under ischemic conditions, modulation of GABA_A receptors by bicuculline, a GABA antagonist, or by diazepam, a GABAergic agonist, did not significantly affect edema formation. Further experiments demonstrated that low chloride conditions prevented NMDA-induced, but not OGD-induced water influx. Omission of calcium ions had no effect. Our results show that NMDA-induced edema formation is highly dependent on chloride influx as it was prevented by low-chloride conditions and by various compounds that interfere with chloride influx. In contrast, OGD-induced edema observed in brain slices were not affected by modulators of chloride fluxes. The results are discussed with reference to ionic changes occurring during tissue ischemia.

Section: Neurophysiology, Neuropharmacology and other forms of Intercellular Communication.

Keywords

edema; furosemide; DIDS; niflumic acid; NMDA receptor; oxygen-glucose deprivation

1. Introduction

Brain edema formation, which is observed after large hemispheric stroke or traumatic brain injury, is one of the most dangerous consequences of acute ischemia and excitotoxicity in the brain (Steiner et al., 2001; Unterberg et al., 2004). While water contents in healthy brains are

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Abbreviations:

DIDS

4,4'-diisothiocyanostilbene-2,2'-disulfonic acid

NMDA

N-methyl-D-aspartate

OGD

oxygen-glucose deprivation.

closely regulated by a variety of homeostatic mechanisms (Kimelberg, 2004), breakdown of these mechanisms in ischemia, including severe dysregulations of ionic distributions, cause swelling of the brain and increased intracranial pressure which is one of the premier factors determining survival in patients (Reilly, 2001). Early brain swelling is known to be due to cellular ("cytotoxic") edema while vasogenic edema develop in a more delayed fashion (over hours). Among the brain regions, the hippocampus has been found to be highly susceptible to ischemia; pyramidal cells of the CA1 region are most sensitive (Schmidt-Kastner and Freund, 1991). Astrocytes are well known to undergo swelling when excitotoxic concentrations of glutamate are present (Kimelberg, 2005; Seifert et al., 2006), and they contribute strongly to ensuing changes of intracranial pressure. Inward transport of sodium, calcium, chloride and water is known to cause edema and subsequent toxicity in neurons; potassium uptake additionally contributes to astrocytic swelling (Hansen, 1985; Somjen, 2002).

Hippocampal slices have recently been introduced as an *in vitro*-model of brain edema formation induced by ischemia (MacGregor et al., 2003). Water contents of slices can be determined by a simple differential weighing procedure and, with short incubation times of less than one hour, reflect cellular edema formation. Preparation and superfusion of hippocampal slices *per se* already causes a minor swelling of slices over 30-60 min which is accompanied by sodium and calcium uptake (Siklos et al., 1997). During *in vitro*-ischemia (oxygen-glucose deprivation, OGD), an increase of sodium and calcium uptake was observed to which voltage-operated cation channels as well as glutamate receptors of the AMPA and NMDA subtypes contributed (LoPachin et al., 2001; MacGregor et al., 2003). Inhibitors of AMPA and NMDA cation channels, as well as sodium channel blockers and antioxidants, were found to attenuate edema formation in earlier studies (LoPachin et al., 2001; MacGregor et al., 2003). The importance of sodium and calcium influx for ischemia-induced edema formation and cellular injury was also documented in organotypical slice cultures exposed to OGD (Breder et al., 2000; Martinez-Sanchez et al., 2004) and in neuronal cell cultures (Goldberg and Choi, 1993; Czyz et al., 2002).

Compared to the extensive data on the importance of cation movements (see above), the significance of anions such as chloride for brain edema formation has only recently drawn attention (Somjen, 2002; see Discussion for further references). In the present study, we investigated the relevance of chloride influx for edema formation. In addition to OGD, we also used N-methyl-D-aspartate (NMDA) as a stimulator of edema formation in hippocampal slices. NMDA receptors have a major role in ischemia-induced neurotoxicity (Lipton, 1999; Arundine and Tymianski, 2004); they not only depolarize neurons but allow influx of large amounts of calcium ions which are detrimental to the cells. Importantly, it is known that NMDA receptor-mediated neurotoxicity is dependent on extracellular chloride. Studies in neuronal cell cultures (Rothman, 1985; Olney et al., 1986; Choi, 1987) demonstrated that cellular influx of chloride ions is required for cytotoxicity induced by glutamate and NMDA. A small number of follow-up studies supported this hypothesis and have shown protective effects of chloride-free media against NMDA-induced toxicity in organotypic cultures and brain slices (Takahashi et al., 1995; Gröndahl et al., 1998). There are also studies that discuss beneficial or malignant effects of ischemia-induced chloride fluxes through ligand-operated chloride channels such as GABA_A receptors (Erdö et al., 1991; Hasbani et al., 1998; Inglefield & Schwartz-Bloom, 1998; Chen et al., 1999; Galeffi et al., 2004; Babot et al., 2005). In the present study, we tested the effects of ionic manipulations, chloride transport inhibitors, and GABA modulators on NMDA- and ischemia-induced edema formation in hippocampal slices. Our results confirm previous reports on the chloride dependence of NMDA-induced responses but caution against an extrapolation of these findings to ischemia-induced edema.

2. Results

2.1 In vitro model of brain edema formation

Hippocampal slices that were superfused with control buffer for 30 min had average water contents of 77-81 % (Figs. 1-4). Some of this variation was probably due to variations in preparation time which was generally kept below six minutes from decapitation of the animals to superfusion of the slices. To mimic excitotoxicity, slices were exposed to NMDA (300 μ M). In the NMDA experiments, the concentration of magnesium, a blocker of NMDA receptor channels, was lowered from 1.2 to 0.12 mM to enable NMDA receptor activation. NMDA-induced edema formation was reflected in increases of slice water contents by 1.5 to 2.5 % (Figs. 1-4). To mimic ischemic conditions, the slices were exposed to oxygen-glucose deprivation (OGD), i.e. glucose was omitted from the superfusion buffer which was also gassed with nitrogen (MacGregor et al., 2003). Exposure of the slices to OGD increased water content by 2-3 % (Figs. 1-4). As relative changes of water contents were less variable than basal water contents, data were sometimes expressed as relative changes vs. control incubations, provided that the compounds under study did not influence basal water contents (Figs. 2 and 4; also see Methods for details of calculations).

2.2 Effects of Na,K,Cl-cotransporter inhibitors

Fig. 1 illustrates the effects of furosemide (10 μ M), an inhibitor of Na,K,Cl-cotransport, on edema formation induced by NMDA (Fig. 1A) and OGD (Fig. 1B). Furosemide slightly reduced water content under control conditions. In the NMDA series of experiments (N=5), this reduction was significant using paired t-test ($p=0.03$) but not in the ANOVA analysis (Fig. 1A). In the OGD experiments (N=5), this effect was significant both after paired t-test ($p<0.01$) and ANOVA ($p<0.05$). More importantly, furosemide significantly reduced the NMDA effect; the NMDA-induced increase in water content was 2.07 ± 0.39 % in the absence of furosemide and 0.74 ± 0.53 % in its presence, equivalent to an inhibition of edema formation by 64 % ($p<0.01$, t-test). In striking contrast, furosemide did not affect the OGD effect; the increase of water contents was 3.06 ± 0.75 % in the absence and 3.06 ± 0.65 % in the presence of furosemide.

Similar effects as observed with furosemide were observed with bumetanide, another inhibitor of the Na,K,Cl-cotransporter. Bumetanide reduced the NMDA-induced water increase (data not shown) but was inactive in the OGD model. In the presence of 10 μ M bumetanide, OGD increased slice water contents by 2.20 ± 0.92 %; in parallel control experiments, the OGD-induced increase was 2.48 ± 0.74 % (N=5 each; $p>0.5$) (not illustrated).

2.3 Effects of chloride transport inhibitors

DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) and niflumic acid are two broad-spectrum inhibitors of membrane chloride transporters. Neither DIDS (10 μ M) nor niflumic acid (100 μ M) affected slice water contents under basal conditions, so the results are summarized in Fig. 2 as changes of water contents, induced by NMDA or OGD, in the absence vs. in the presence of DIDS and niflumic acid, respectively.. As shown in Fig. 2A, DIDS almost completely (by 97%) blocked NMDA-induced edema. In contrast, it was inactive against edema formation induced by ischemia (-14%; $p>0.2$). Similarly, niflumic acid significantly reduced NMDA-induced edema formation by 67 % ($p<0.01$; Fig. 2B) while slightly increasing ischemia-induced water accumulation (+11%); again, the effect on OGD-induced edema was not significant ($p>0.2$).

2.4 Effects of GABA receptor modulators

Fig. 3 illustrates the effects of GABA_A receptor modulators on edema formation induced by ischemia. Bicuculline is a competitive antagonist of GABA at the GABA_A receptor; at 100

μM , it significantly reduced water contents both under control conditions as well as under ischemia (Fig. 3A). There was no significant effect, however, when OGD-induced increases were compared in the absence ($2.21 \pm 0.46 \%$) or presence ($2.18 \pm 0.56 \%$) of bicuculline ($p > 0.5$; $N=6$ each). Similar effects were seen with diazepam ($5 \mu\text{M}$), a positive modulator of GABA_A receptor function. The presence of diazepam caused a reduction of slice water contents under both control and OGD conditions, but the OGD-induced increases of water contents were similar in the absence ($1.88 \pm 1.12 \%$) or presence ($1.68 \pm 1.20 \%$) of diazepam ($p > 0.5$; $N=6$ each). In a small set of experiments, there was no significant effect of either treatment on NMDA-induced edema formation (data not shown).

2.5 Effects of ionic manipulations

Fig. 4 summarizes the effects of ionic manipulations on NMDA- and OGD-induced edema formation. For the experiments shown in Fig. 4A, sodium chloride in the superfusion buffer was replaced by sodium nitrate; the chloride concentrations was, therefore, reduced from 145.7 mM to 8.7 mM (OGD effect) or from 143.5 to 6.5 mM (NMDA effect, low MgCl_2). Under low-chloride conditions, the NMDA-induced increase of water content ($+1.69 \pm 0.68 \%$) was not only completely suppressed, but actually reversed (Fig. 4A); in our hands, exposure of the slices to NMDA, under low-chloride conditions, induced a reduction of water contents ($-0.78 \pm 1.03 \%$). The reduction was significant when compared to the effect of NMDA alone ($p < 0.01$, Fig. 4A) but not when compared to low-chloride conditions in the absence of NMDA ($p > 0.05$; data not shown). In contrast to these findings, low-chloride conditions did not affect OGD-induced edema formation; the OGD-induced increase in water content was not significantly different in control buffer ($+2.97 \pm 0.54 \%$) and in low-chloride buffer ($+3.80 \pm 1.23 \%$) ($p > 0.1$).

Finally, we tested the relevance of calcium for edema formation. Omission of calcium from the superfusion buffer did not affect water contents under control conditions, exposure to NMDA, or OGD (Fig. 4B).

3. Discussion

The primary goal of these experiments was to test the influence of chloride influx modulators and ionic manipulations on edema formation. We adapted a recently described in vitro-model of edema formation in which oxygen-glucose deprivation (OGD) led to uptake of water in hippocampal slices as an indicator of cellular edema formation (MacGregor et al., 2003). The in vitro-model has the advantage to allow manipulations of extracellular ion levels and tight control of drug concentrations. A drawback of this model is the absence of a blood-brain barrier (see below).

The rationale behind the present experiments was to determine the potential usefulness of anion (chloride) channel blockers for the treatment of brain edema after stroke and traumatic brain injury. In addition to the OGD condition, we used N-methyl-D-aspartate (NMDA) as a novel stimulant of edema formation because the role of NMDA receptor activation is central to neuronal cell death following ischemia and an important role of chloride influx for NMDA toxicity had been described previously (see Introduction). It is important to point out that our slice model strictly measures edema formation, not cellular toxicity. Calcium influx is closely coupled to ischemia- and NMDA-induced neurotoxicity (Lipton, 1999; Arundine and Tymianski, 2004). In the present study, however, we found that extracellular calcium is not required for edema formation induced by NMDA or OGD (Fig. 4B).

Furosemide and bumetanide both are inhibitors of the Na,K,Cl -cotransporter (NKCC); bumetanide is regarded as the more selective inhibitor while furosemide also inhibits the neuronal K,Cl -cotransporter KCC2 (Yan et al., 2001; ÓDonnell et al., 2004). We had

previously reported that furosemide inhibits NMDA-induced phospholipid breakdown (Klein et al., 2003). Others had shown that the NKCC is activated following NMDA receptor activation in neurons (Sun and Murali, 1998), and that it contributes to excitotoxicity and cell swelling observed after glutamate challenge in cortical neuronal cultures (Beck et al., 2003). Interestingly, in the same study, bumetanide (5-10 μ M) was also found to reduce neurotoxicity and cell swelling induced by OGD (Beck et al., 2003). In hippocampal slices subjected to ischemia and reperfusion, NKCC inhibitors were unable to inhibit the early chloride influx but were beneficial during later phases of reperfusion (Pond et al., 2006). Interestingly, in an in vivo-model of brain ischemia, bumetanide was found to reduce brain edema when given i.v. or through a microdialysis probe (Yan et al., 2001; ÓDonnell et al., 2004).

In our model, and in agreement with previous results using excitotoxicity (Beck et al., 2003; Klein et al., 2003), furosemide (10 μ M) reduced NMDA-induced edema formation by 64 % (Fig. 1A); however, it was inactive in the OGD model (Fig. 1B). Bumetanide was also inactive in the OGD model, in apparent contrast with earlier reports in cell cultures (Beck et al., 2003). Possible reasons for the discrepancy between brain slices and cultured neurons include the presence of astroglial cells in the slice preparation; most swelling in intact brain tissue is probably due to astroglial responses (Kimelberg, 2005; Seifert et al., 2006). From our data, we suggest that NKCC inhibitors are not effective in blocking acute, ischemia-induced cellular edema formation. This is in agreement with a recent study measuring chloride levels in hippocampal slices (Pond et al., 2006). It should be noted that our ischemia model did not include a reperfusion period during which NKCC inhibitors may be effective (Pond et al., 2006). Moreover, our model measures short-term cellular edema formation in astrocytes and/or neurons whereas it does not adequately reflect in vivo-conditions. Discrepancies between our results and literature studies in vivo may, for instance, be due to the fact that an intact blood-brain barrier has an important influence on edema formation in vivo but not in brain slices. Indeed, previous work has given evidence that NKCC activity in brain endothelial cells is involved in edema formation (Yan et al., 2001; ÓDonnell et al., 2004). The proposed regulation of endothelial NKCC activity by rapid phosphorylation points to a potential contribution of this transporter in early edema formation in vivo (Abbruscato et al., 2004; Foroutan et al., 2005).

In addition to the NKCC inhibitors, we tested two blockers of anion transport, DIDS and niflumic acid (Jentsch et al., 2002). DIDS is a rather unspecific blocker of anion channels, including chloride channels and the chloride/bicarbonate exchangers; it blocked glial cell swelling and NMDA-induced choline release from hippocampal slices in earlier studies (Ringel et al., 2000; Parkerson and Sontheimer, 2003; Klein et al., 2003). At the rather low concentration of 10 μ M, DIDS did not change basal slice water contents but almost completely prevented NMDA-induced edema formation (by 97%). Surprisingly, it was inactive in OGD-induced edema (Fig. 2A). Niflumic acid is an inhibitor of various chloride conductances including calcium-activated and volume-activated chloride channels (Parkerson and Sontheimer, 2003; Stemmer et al., 2004). In our cellular edema assay, niflumic acid strongly (by 67%) inhibited NMDA-induced edema but again was inactive in OGD-induced edema (Fig. 2B).

The results with chloride transport inhibitors suggested a clear-cut discrepancy between the mechanisms of NMDA- and OGD-induced edema. As experiments with inhibitors may suffer from unselective drug effects, we repeated the experiments under low-chloride conditions (Fig. 4A). When extracellular chloride was reduced to 5-10 mM, NMDA-induced edema formation was not only abolished, but actually seemed to be reversed, with NMDA exposure leading to a reduction of cellular water content. Under OGD conditions, the edema was unchanged or slightly increased (Fig. 4A). These experiments reinforce the notion that inhibition of chloride influx prevents edema induced by excitotoxicity, but not those induced by ischemia. While the

reason for this discrepancy is unknown for the present model, we speculate that the energy state of the tissue may explain our observations. While exposure of slices to NMDA causes depolarization followed by influx of sodium, chloride and water, energy production of the cells is not compromised, and active ion pumps work to keep ionic distributions intact. Under these condition, absence of chloride influx prevents edema formation, probably because sodium and water influx are also inhibited when chloride anions cannot cross the membrane or are pumped back out. As mentioned in the introduction, absence of chloride also prevents certain aspects of NMDA-induced neuronal toxicity.

Under ischemic conditions, however, a general failure of energy production occurs which leads to breakdown of ionic distributions. Cellular edema occur with the influx of sodium, chloride and water; the persistent increase of intracellular chloride leads to a reversal of the chloride gradient such that the intracellular chloride concentration may surpass the extracellular levels (Hansen, 1985; Somjen, 2002). This situation is analogous to the reversal of the chloride gradient in immature tissue when GABA acts as an excitatory transmitter (Ben-Ari, 2002; Payne et al., 2003). Accordingly, blockade of chloride influx will not cause protection from ongoing water influx and edema formation, and opening of ion channels may actually cause an efflux of chloride from the cells; this may explain the reduction of water contents induced by NMDA under low-chloride conditions (Fig. 4A).

The above considerations have motivated experiments and an ongoing clinical trial testing the effects of diazepam, a positive modulator of the GABA_A receptor, in stroke (Schwartz-Bloom and Sah, 2001). While the clinical data are inconclusive so far (Lodder et al., 2006), conditions have been described in which diazepam has neuroprotective activity in cell cultures and in vivo (Schwartz-Bloom and Sah, 2001). We therefore decided to test diazepam in the OGD edema model and to compare its effects to bicuculline, the competitive antagonist at the GABA_A receptor. The two compounds had similar effects in the edema model; they unexpectedly reduced water contents under basal conditions but they did not affect edema formation. While this finding does not exclude neuroprotective activities of GABA_A receptor modulators that are based on other mechanisms (such as hypothermia, reduction of metabolic rate; Schwartz-Bloom and Sah, 2001), it seems unlikely that the GABA_A receptor plays an important role for acute, ischemia-induced cellular edema formation.

In conclusion, our results with the in vitro edema model demonstrate that chloride influx is required for cellular edema formation which is secondary to excitotoxicity; low-chloride conditions, as well as inhibitors of chloride influx prevented edema formation during NMDA exposure. In the clinically more relevant OGD model, however, modulators of chloride fluxes (including GABAergic drugs) had no beneficial effects. This discrepancy, which casts doubt on the usefulness of chloride modulators in the acute phase of brain edema formation, is probably due to the consequences of ischemia on chloride gradients in the brain although more work is required to substantiate this hypothesis.

4. Experimental procedures

4.1 Animals

Male Wistar rats (250-350g; Charles River) were kept under standardised light/dark (12h), temperature (22°C) and humidity (70 %) conditions, with rat chow and water available ad libitum. All animal procedures were in accordance with NIH regulations and were registered with the Institutional Animal Care and Use Committee of TTUHSC.

4.2 Edema formation in hippocampal slices

Male Sprague-Dawley rats were briefly anesthetized in an isoflurane induction chamber and decapitated. Hippocampal slices (400 μ m) were prepared as previously described (Weichel et al., 1999; Klein et al., 2003) and superfused (0.7 ml/min) at 35°C with Tyrode solution of the following composition (mM): NaCl, 137; KCl, 2.7; CaCl₂, 1.8; MgCl₂, 1.2; NaH₂PO₄, 0.2; NaHCO₃, 11.9; glucose, 5.6. Preparation time, i.e. the time between decapitation and superfusion of the slices, was kept below 6 minutes in all experiments to minimize early ischemic swelling. During the equilibration period (30–45 min), all superfusion solutions were continuously gassed with carbogen (95% O₂, 5% CO₂). For excitotoxic conditions, the slices were superfused with N-methyl-D-aspartate (NMDA, 300 μ M); concomitantly, the concentration of magnesium chloride was lowered to 0.12 mM. For *in vitro*-ischemia (OGD, oxygen-glucose deprivation), the slices were superfused with solutions which did not contain glucose and which had been gassed with nitrogen (95% N₂, 5% CO₂) instead of carbogen; air contact of the slices was minimized by a glass cover on the superfusion apparatus.

Chloride flux modulators were added in DMSO 5 minutes before the experiment started; control solutions contained the same amount of DMSO (0.1%) as drug-containing solutions. For low-chloride conditions, sodium chloride in the superfusion buffer was substituted with sodium nitrate. For calcium-free conditions, calcium chloride was omitted from the buffer. Four lanes of slices were superfused in parallel for 30 minutes; all slices in one experiments were from the same animal. At the end of the superfusion period, slices from each lane were collected, superficially dried, transferred to aluminum foil, and immediately weighed to minimize water evaporation (“wet weight”). They were then dried over night at 105°C in a desiccating oven and weighed again (“dry weight”). Total tissue brain water was calculated according to $[(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100$.

4.3 Calculations and statistics

In all experiments, four lanes were superfused in parallel containing hippocampal slices from the same rat (four slices per lane). Single experiments always involved control conditions (lane A), and three lanes with slices that were superfused with drug (lane B), NMDA (or OGD) (lane C), and drug plus NMDA (or OGD) (lane D). Ionic modulations substituted for drugs in the experiments shown in Fig. 4. Total tissue brain water was calculated as above; these data are shown in Figs. 1 and 3 (for calculation, see 4.2). Differences of water contents were calculated by subtracting lane A from lane C (NMDA or OGD effect) and by subtracting lane B from lane D (drug effect, or effect of ionic variation); these data are shown in Figs. 2 and 4.

Data are shown as means $\pm$ S.D. of 4–6 experiments (see Legends for number of experiments). Statistical calculations were performed by GraphPad InStat 3.0, using analysis of variance (ANOVA) of paired data for raw (absolute) data for water contents (Figs. 1 and 3), or paired t-test for the comparison of differential values (changes of water contents vs. controls) (Figs. 2 and 4).

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Fig. 1.

Tissue water contents in hippocampal slices: effects of furosemide. (A) Slices were superfused under control conditions ("Ctrl") or with furosemide ("Furo", 10 μ M). Edema was induced by N-methyl-D-aspartate in the absence ("NMDA", 300 μ M) or presence ("NMDA + Furo") of furosemide (N=5). (B) Slices were superfused under control conditions or with furosemide, as in (A). Edema was induced by oxygen-glucose deprivation ("OGD") in the absence ("OGD") or presence ("OGD + Furo") of furosemide (N=5). Slices were exposed to NMDA or OGD for 30 minutes; when furosemide was present, it was added 5 minutes before OGD. All superfusions were done in the presence of 0.1% DMSO which was used to dissolve furosemide. Water contents were determined at the end of the superfusion by a differential weighing

procedure before and after drying the slices. Statistical significance was evaluated by paired ANOVA. *, $p < 0.05$; **, $p < 0.01$ vs controls (Ctr). #, $p < 0.05$; ##, $p < 0.01$ vs. NMDA or OGD, respectively.

Fig. 2. Changes of tissue water contents in hippocampal slices treated with chloride transport inhibitors. (A) Effects of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid ("DIDS", 10 μ M) (N=4 for NMDA, N=6 for OGD). (B) Effects of niflumic acid ("NFA", 100 μ M) (N=4 each). Procedures were as outlined in the legend of Fig. 1. Data were calculated as differences of water contents. Effects of NMDA or OGD were calculated as effects in the absence of drugs ("NMDA", "OGD") or in the presence of drugs taking effects of the drugs on basal water contents into account (see 4.3 for further explanation). Statistical significance was evaluated by paired t-test. **, $p < 0.01$ vs NMDA.

Fig. 3.

Tissue water contents in hippocampal slices: effects of bicuculline and diazepam on OGD-induced edema. (A) Effects of bicuculline (“Bicu”, 100 μ M) under control conditions (“Ctrl”, “Bicu”) and on tissue edema induced by oxygen-glucose deprivation (“OGD”) (N=6 each). (B) Effects of diazepam (“Diaz”, 5 μ M) under control conditions (“Ctrl”, “Diaz”) and on tissue edema induced by oxygen-glucose deprivation (“OGD”) (N=6 each). Procedures were as outlined in the legend of Fig. 1. Absolute data are given for water contents. Statistical significance was evaluated by paired ANOVA. *, $p < 0.05$; **, $p < 0.01$ vs controls (Ctrl). #, $p < 0.05$; ##, $p < 0.01$ vs. OGD.

Fig. 4. Changes of tissue water contents in hippocampal slices under different ionic conditions. (A) Effects of low chloride conditions (“Low Cl”) on increases of tissue water induced by N-methyl-D-aspartate (“NMDA”) or oxygen-glucose deprivation (“OGD”) (N=6 for NMDA; N=4 for OGD). Low chloride conditions were obtained by isoosmolar replacement of sodium chloride in the superfusion buffer with sodium nitrate. (B) Effects of zero calcium (“Ca-free”) on increases of tissue water induced by N-methyl-D-aspartate (“NMDA”) or oxygen-glucose deprivation (“OGD”) (N=4 for NMDA; N=5 for OGD). For zero calcium conditions, calcium chloride was omitted from the superfusion buffer. Data were calculated as differences of water contents. Effects of NMDA or OGD were calculated as effects under control conditions

(“NMDA”, “OGD”) or in the presence of low-chloride or calcium-free conditions taking effects of ionic modulations on basal water contents into account (see 4.3 for further explanation). Statistical significance was evaluated by paired t-test. **, $p < 0.01$ vs NMDA.