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Antagonists of the Vasopressin V1 Receptor and of the β_1-Adrenoceptor Inhibit Cytotoxic Brain Edema in Stroke by Effects on Astrocytes – but the Mechanisms Differ

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Abstract: Brain edema is a serious complication in ischemic stroke because even relatively small changes in brain volume can compromise cerebral blood flow or result in compression of vital brain structures on account of the fixed volume of the rigid skull. Literature data indicate that administration of either antagonists of the V1 vasopressin (AVP) receptor or the β_1-adrenergic receptor are able to reduce edema or infarct size when administered after the onset of ischemia, a key advantage for possible clinical use. The present review discusses possible mechanisms, focusing on the role of NKCC1, an astrocytic cotransporter of Na^+, K^+, $2Cl^-$ and water and its activation by highly increased extracellular K^+ concentrations in the development of cytotoxic cell swelling. However, it also mentions that due to a 3/2 ratio between Na^+ release and K^+ uptake by the Na^+,K^+-ATPase driving NKCC1 brain extracellular fluid can become hypertonic, which may facilitate water entry across the blood-brain barrier, essential for development of edema. It shows that brain edema does not develop until during reperfusion, which can be explained by lack of metabolic energy during ischemia. V1 antagonists are likely to protect against cytotoxic edema formation by inhibiting AVP enhancement of NKCC1-mediated uptake of ions and water, whereas β_1-adrenergic antagonists prevent edema formation because β_1-adrenergic stimulation *alone* is responsible for stimulation of the Na^+,K^+-ATPase driving NKCC1, first and foremost due to decrease in extracellular Ca^{2+} concentration. Inhibition of NKCC1 also has adverse effects, e.g. on memory and the treatment should probably be of shortest possible duration.

Keywords: Astrocyte, β_1-adrenoceptor, brain edema, MCAO, memory, Na^+,K^+-ATPase, NKCC1, vasopressin.

1. INTRODUCTION

Ischemic stroke is a major contributor to human death and functional impairment. Besides the direct adverse effects energy deprivation has on brain cells, brain ischemia followed by re-oxygenation often leads to brain edema. A major reason for this is 'cytotoxic' swelling, mainly of astrocytes. However, redistribution of water from extracellular to intracellular fluid cannot on its own cause expansion of total water content in the brain, so in addition water distribution across the blood-brain barrier must also be affected. Brain edema is an important event, because even relatively small changes in total brain volume can compromise cerebral blood flow or result in compression of vital brain structures on account of the fixed volume of the rigid skull. This review deals mainly with the 'cytotoxic' astrocytic swelling and the ability of antagonists of either the V1 vasopressin receptor or the β_1-adrenergic receptor to prevent the swelling, at least in experimental stroke. A major reason for the swelling is the increase in extracellular K^+ concentration occurring during brain ischemia [1, 2] and its effect on the Na^+, K^+, and 2 Cl^- transporter NKCC1, which co-transports not only ions but also water [3, 4]. However, when necessary for understanding of the mechanisms involved in the creation of brain edema hypotonicity-induced cell swelling and aquaporin-mediated water fluxes will also be discussed.

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2. NKCC1 PATHWAY FOR K^+-INDUCED STIMULATION AND K^+ EFFECT ON AQUAPORIN-MEDIATED WATER FLUXES

2.1. K^+effects on NKCC1 and the Aquaporin AQP4

Experiments in cultured astrocytes have shown that extracellular K^+ concentrations ≥15 mM stimulate the Na^+,K^+,Cl^-, water cotransporter NKCC1 [5]. This co-transporter [3, 4, 6] is in the adult brain mainly expressed in astrocytes [7-10] and at the blood-brain barrier [11, 12]. In contrast to cultured astrocytes, cultured neurons show no increase in the rate of K^+ uptake at highly elevated K^+ concentrations [13, 14]. Astrocytes are the cells that show most swelling in brain tissue after exposure to pathologically elevated extracellular K^+ concentrations [15, 16]. In experimental stroke the specific NKCC1 inhibitor bumetanide

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inhibits edema [17, 18]. Moreover, knock-out of NKCC1 in mice reduces brain swelling by one half following 2 hr of MCAO and 24 hr of reperfusion [19]. During exposure to highly elevated K^+ concentrations brain slices show pronounced swelling [20], localized mainly in astrocytes [21], and the swelling increases with increasing extracellular K^+ concentration up to ~50 mM [22]. This is important because extracellular K^+ concentrations of this magnitude (and even higher) rapidly occur during brain ischemia [1, 2], where they stimulate NKCC1. During ischemia there is also a massive decrease in extracellular Ca^{2+} to ~0.1 mM and a more moderate decrease in extracellular Na^+ and Cl^- [2].

Parts of the signaling pathway by which highly elevated K^+ concentrations stimulate NKCC1 are shown in Figs. **1** and **2**. Addition of 8-10 mM K^+ causes an increase in astrocytic uptake of pre-bound Ca^{2+}, which has reached its maximum around 20 mM K^+ (Fig. **1a**). This response is completely inhibited by nimodipine, a dihydropyridine inhibitor of L-channel-mediated Ca^{2+} uptake (Fig. **1b**). The increase in $[Ca^{2+}]_i$ is reinforced by stimulation of the ryanodine receptor by accumulated Ca^{2+} (Fig. **1c**) as shown by the inhibition by the ryanodine receptor inhibitor ryanodine of all but the initial increase in $[Ca^{2+}]_i$ caused by addition of 45 mM KCl [24]. The latter Fig. also shows that the K^+-induced increase in Ca^{2+} uptake is reflected by a nifedipine-inhibited increase $[Ca^{2+}]_i$, although at least at highly elevated extracellular K^+ concentrations a minor $[Ca^{2+}]_i$ increase is resistant to this dihydropyridine. These observations are consistent with neuroprotective and edema-reducing effect of L-channel inhibitors during ischemia when given early [25-27], because K^+-mediated Ca^{2+} entry initiates a signaling pathway that leads to NKCC1 activation. This pathway is shown in Fig. **2a** until phosphorylation of extracellular regulated kinases 1 and 2 ($ERK_{1/2}$) together with the inhibitors used for its identification, shown in yellow ovals [28]. An important point is Src-activation of the metalloproteinase ADAM 17, releasing a growth factor causing transactivation (phosphorylation) of the epidermal growth factor (EGF) receptor leading to Ras-Raf-MEK-induced phosphorylation of $ERK_{1/2}$. This in turn causes swelling that can be prevented by inhibitors of this pathway (Fig. **2b**). Fig. **2a** also shows that hypo-osmotic swelling activates $ERK_{1/2}$ *via* a different pathway and therefore is not an appropriate model for swelling induced by ischemia/re-oxygenation. Since this pathway bypassed Ras and was not inhibited by bumetanide, it was tentatively concluded that bumetanide inhibited Ras. It is consistent with this conclusion that regulatory volume increase caused by hypertonic medium exposure is inhibited in NIH 3T3 cells by furosemide and bumetanide, but only in cells expressing Ras [29]. The further pathway between ERK phosphorylation and NKCC1 activation was not investigated by Cai *et al*. [28] but NKCC1 is directly regulated by phosphorylation [30, 31], mediated by the specific kinases SPAK (STE20/SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase-1 [32, 33].

Smaller increase in extracellular K^+ concentration (to ~10 mM) do not increase swelling but they stimulate the Na^+,K^+-ATPase, which on its own is the transporter responsible for most extracellular K^+ clearance during normal brain activity [5, 34]. Since excitation causes Na^+ increase in neurons, its neuronal operation needs no stimulation of Na^+ uptake, whereas that in astrocytes does [5, 34, 35]. Experiments in cultured astrocytes have demonstrated activation of a ouabain signaling pathway initiated by small increases in extracellular K^+, which mediates Na^+ uptake by opening of Na^+ channels. In contrast to the pathway mediating the effect of highly elevated K^+ concentrations activation of the IP_3 receptor is necessary in the ouabain pathway [5]. In spite of not leading to cell swelling these small increases in extracellular K^+ concentration augment water fluxes through AQP4 [36]. These authors showed in cultured astrocytes that the water permeability increase occurred *via* increased cAMP and that no significant increase in specific AQP4 water permeability occurred in AQP4-negative cells. They also showed that larger elevation of extracellular K^+ (to 35 mM) abolished the K^+-induced increase in AQP4-mediated water flux. This is consistent with the observation by Peng *et al*. [37] that astrocytes in which AQP4 was knocked out by siRNA treatment show absent or reduced hypotonicity-induced swelling. Na^+,K^+-ATPase activity is also needed to create the ion gradients providing the driving force for NKCC1, which is a secondary active transporter [6]. The regulation of the astrocytic Na^+,K^+-ATPase will be discussed in section 4.

2.2. Summary

Highly elevated extracellular K^+ concentrations ($\geq$ 15 mM) stimulate the ion/water co-transporter NKCC1, which in brain parenchyma is localized in astrocytes. Smaller K^+ increases stimulate Na^+,K^+-ATPase activity and aquaporin-mediated water flux in astrocytes, but do not cause edema. Both transporters are also likely to influence AQP4-mediated water flow across the blood-brain barrier, but this aspect of brain edema is not discussed in detail in this review.

3. VASOPRESSIN (AVP)

3.1. AVP Targets Include Astrocytes

AVP is not only a peptide hormone with a major effect on the kidney but also acts as a neurotransmitter/neuromodulator. Thus i) it is synthesized not only in hypothalamo-neurohypophysial cells, but also in other neurons, whose axon projects to the limbic system, the brainstem and the spinal cord; ii) it can be released from central axons similar to classical neurotransmitters; and iii) specific binding sites, i.e., membrane receptors with high affinity for AVP are present in the central nervous system [38]. It has long been known that AVP receptors are expressed not only on neurons but also on astrocytes [39-41].

AVP acts on different receptors in the kidney and in the brain. Its antidiuretic effect occurs *via* activation of the G_s- and cAMP-linked V2 receptor in the kidney, whereas astrocytes in the brain express the V1 receptor [41, 42]. The $G_{q/11}$-coupled, V1a receptor stimulates phospholipase C, PLC [43] and is thus linked to intracellular Ca^{2+} release, although some of the different effects of V1a stimulation require the presence of extracellular Ca^{2+} [44]. The V1b (also called V3) receptor is also PLC- and Ca^{2+}-linked, but it has a

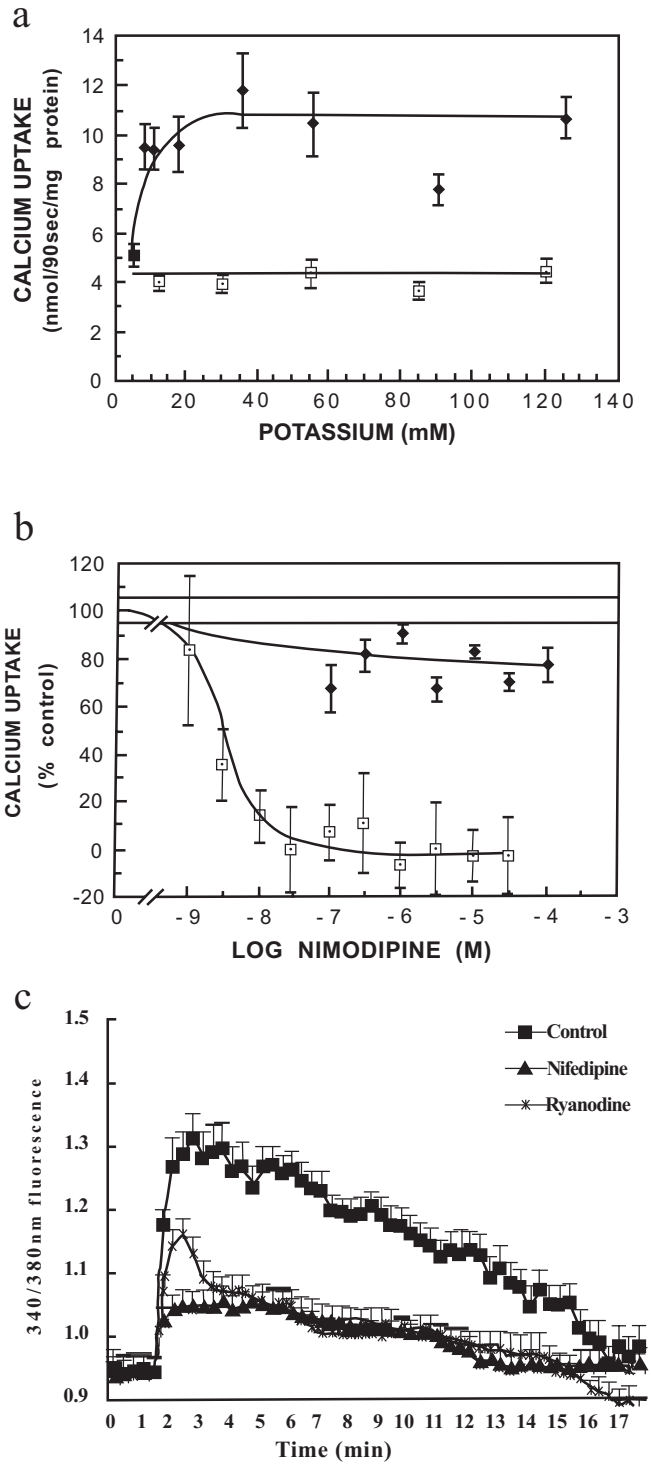

Fig. (1). (**a**) Uptake of $^{45}Ca^{2+}$ (90 sec) into astrocytes in primary cultures as a function of the final extracellular K^+ concentration. Excess K^+ (2.5 to 125 mM KCl) was added either simultaneously with $^{45}Ca^{2+}$ (□) or 60 sec after $^{45}Ca^{2+}$ (◆). The difference between the two conditions suggests that the channel transports pre-bound Ca^{2+}. (**b**) Effect of calcium channel blocker nimodipine on basal unstimulated $^{45}Ca^{2+}$ uptake (5.4 mM potassium, ◆) and potassium-stimulated $^{45}Ca^{2+}$ uptake (55.4 mM potassium, □) into cultured astrocytes. Methodologies for a and b were as in Hertz *et al*. [23]. (**c**) Effects of nifedipine or ryanodine on the increase of $[Ca^{2+}]_i$ by addition 45 mM KCl to normal medium (to a total K^+ concentration of 50 mM), determined as described by Yan *et al*. [24]. After loading with fura-2 AM for 30 min, 45 mM KCl was added with or without nifedipine (100 nM), or ryanodine (1 μM), which at this concentration inhibits the ryanodine receptor. Results are averages from 60 cells on three individual coverslips. S.E.M. values are indicated by vertical bars. *Statistically significant ($p<0.05$) difference from control group at the same time period. From Hertz *et al*. [23] and Yan *et al*., 2013 [24].

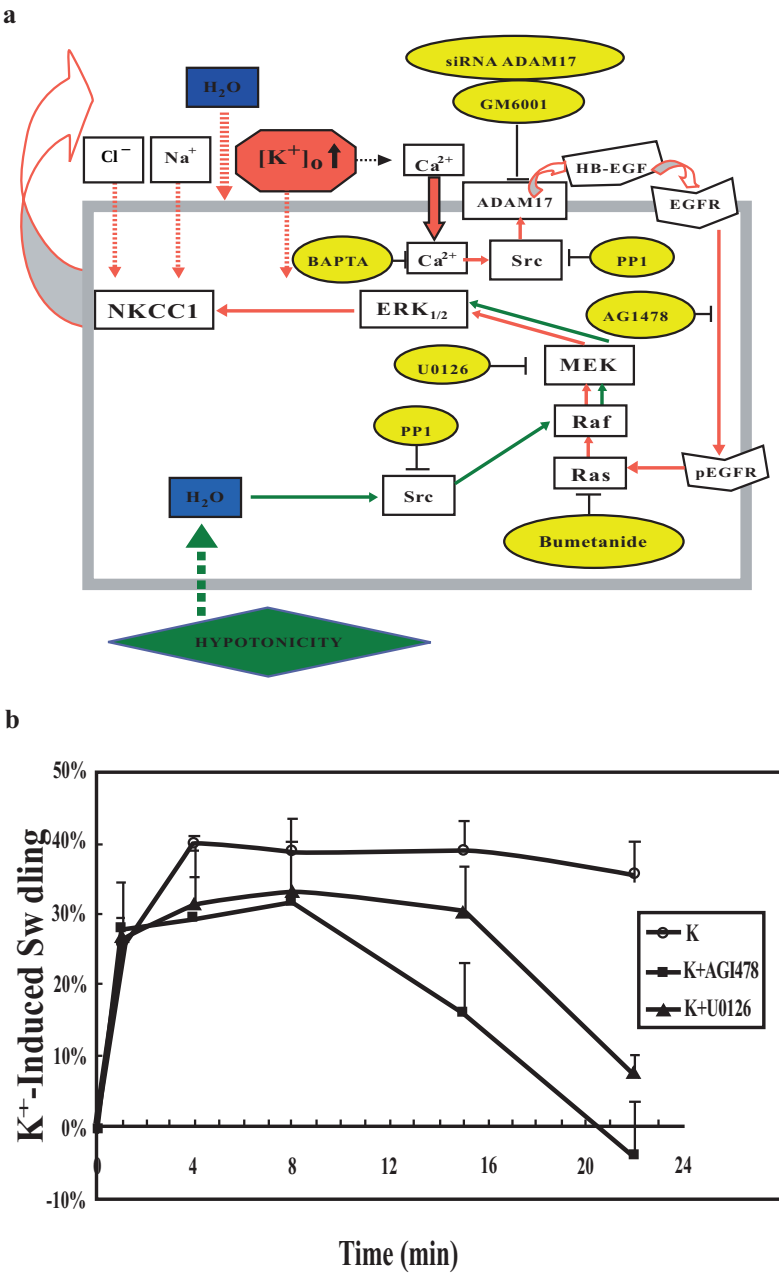

Fig. (2). (**a**) Diagram showing signaling pathways towards $ERK_{1/2}$ phosphorylation activated by elevation of $[K^+]_o$ (red arrows) or hypotonicity (green arrows) and inhibition of these pathways by specific inhibitors (yellow ovals). Elevation of $[K^+]_o$ depolarizes the cell membrane and thereby leads to Ca^{2+} entry through voltage-dependent L-channels. The increase in $[Ca^{2+}]_i$ is necessary for $ERK_{1/2}$ phosphorylation, which is inhibited by BAPTA-AM, and it leads to a Src-dependent (and PP1-inhibited) release of HB-EGF from its membrane-bound precursor by the metalloproteinase ADAM 17 (inhibited by GM6001 and by siRNA against ADAM 17). The released HB-EGF activates (phosphorylates) the EGF receptor (inhibited by AG1478), leading to activation of the MAP kinase cascade, Ras (inhibited by bumetanide), Raf and MEK (inhibited by U0126), with activation of MEK causing $ERK_{1/2}$ phosphorylation. $ERK_{1/2}$ phosphorylation activates (phosphorylates) the cotransporter NKCC1 through pathways that were not studied and are only partly known. This leads to influx of Na^+ and K^+ together with 2 Cl^- and water. Accordingly K^+-induced swelling is contingent upon $ERK_{1/2}$ phosphorylation. In contrast hypotonicity-induced swelling is independent of $ERK_{1/2}$ phosphorylation, since it is not inhibited by U0126, which inhibits swelling induced by high extracellular K^+ concentrations. From Cai *et al*., 2011[28]. (**b**) Effect of high $[K^+]_o$ on cell swelling in astrocytes requires EGF receptor stimulation and $ERK_{1/2}$ phosphorylation. Astrocytes were treated with isotonic phosphate buffered saline containing 60 mM K^+ with concomitant reduction of the Na^+ concentration to maintain iso-osmolarity (○), in some experiments the cells were treated with 1 μM tyrphostin AG1478, the inhibitor of the EGF receptor tyrosine kinase (■) or 10 μM U0126, the inhibitor of MEK (▲) at the same time high K^+ was added. Means ± SEM were calculated for 3–5 individual experiments from the fluorescence ratios at selected times after medium change and converted to change in water space relative to that in the corresponding isotonic media at time zero. Two-way ANOVA using GraphPad showed drug effects which initially were non-significant but rapidly became significant at $P < 0.05$. From Cai *et al*. 2011 [28].

pharmacological profile distinct from that of the human V1a receptor [45].

3.2. Inhibition of V1 Receptors Reduces Edema after Ischemia/Reperfusion

Vakili *et al*. [46] in their study of mice subjected to 60-min middle cerebral artery occlusion (MCAO) followed by 23 hr of reperfusion found that inhibition of V1 receptors reduced infarct volume in a dose-dependent manner by 54% to 70%, and brain edema formation by 67%, while V2 receptor inhibition had no effect. The V1-specific effect has been confirmed by Liu *et al*. [47]. Reduction of infarct size and edema in an embolic focal ischemia model in rats by a V1 receptor antagonist had previously been shown by Shuaib *et al* [48]. Kleindienst *et al*. [49] also found a reduction of brain water content by a V1 antagonist after a 2-hr MCAO followed by 2 hr of reperfusion in the rat. Na^+ and water increase in the brain was prevented regardless whether the antagonist was administered 60 min before or 60 min after MCAO while K^+ loss was inhibited only by pretreatment. The authors attributed all effects to inhibition of AQP4, an inhibition which has been shown experimentally [47, 50], but the difference between the effects on Na^+ and K^+ *must* indicate an additional effect on NKCC1. It has been mentioned that ischemia causes a massive and relatively early increase in extracellular K^+ in the brain by cellular release together with a decrease in extracellular Na^+ and a 90% reduction in extracellular Ca^{2+} [1, 2]. AQP4 may be involved during some of these alterations. However, the ability to prevent cellular increase in Na^+ by administration of V1 receptor antagonist 1 hr after the insult can only be explained by an additional effect on either the Na^+,K^+-ATPase or NKCC1. Unfortunately Kleindienst *et al*. [49] did not measure Cl^- content, which would have been important to distinguish between these two transporters. Ion and water uptake occurs after re-supply of metabolic energy as shown by the finding that 3 hr after MCAO there is no significant edema in the ipsilateral hemisphere, whereas the increase is considerable (and statistically significant) after 8 hr of re-perfusion (Table **1**). This finding differs from a previous report by Matsuoka and Hossmann [51], but blood flow was not completely abolished in their experiments. However it is in good agreement with the observation by Nielsen *et al*. [52] that recirculation usually precedes malignant edema in middle cerebral artery infarcts.

The ischemia/reperfusion-mediated edema described in Table **1** is abolished by intraventricular injection of the NKCC1 inhibitor furosemide (D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International) suggesting that both the Na^+ and water uptake found by Kleindienst *et al*. [49] depend upon this co-transporter. It is important and consistent with the Kleindienst results [49] that edema following complete MCAO occlusion does not occur until after reperfusion, because this should allow administration of a V1 antagonist. That administration of a V1 antagonist can reduce edema following an intracerebral hemorrhagic stroke in mice when administered after the onset of the ischemic insult has also been observed by Manaenko *et al*., [53]. These results are obviously of key clinical importance. The potential advantage of using a V1 antagonist in stroke patients is further supported by observations of increased plasma levels of AVP in patients with ischemic stroke [54]. Moreover, post-ischemic brain edema is exacerbated after exogenous AVP application [55] and ischemia-evoked cerebral edema is attenuated in AVP-deficient rats [56].

3.3. AVP Increases Water Content only in Astrocytes that are Already Swollen

That astrocytes possess a mechanism for volume regulation which depends on both AVP and NKCC1, making it furosemide-sensitive, was first shown by Chen *et al*. [13]. However, before that del Bigio and Fedoroff [57] had demonstrated an AVP-induced swelling in cultured astrocytes exposed to either elevated K^+ concentrations or hypotonic surroundings. Fig. **3** (from [13]) shows that exposure to 60 mM extracellular K^+ causes a significant increase in water content both in cultured mouse astrocytes and neurons, but only the response in astrocytes shows an additional, large increase in the presence of 10^{-12} M AVP. In the absence of an increase in extracellular K^+ AVP had no effect on astrocytic volume, and AVP does not increase the rate of astrocytic K^+ uptake at non-increased K^+ concentrations [13]. Swelling of astrocyte cultures during exposure to a hypo-osmotic medium (reduction of Na^+ concentration) is also greatly enhanced by AVP [58], but these authors did not report whether AVP had any effect under isotonic conditions. That this is not the case in cultured astrocytes was, however, shown by Du *et al*. [59], confirming the results of Chen *et al*. [13]. Like Sarfaraz and Fraser [58] they found an increased swelling in the presence of AVP during exposure to hypotonic media (Fig. **4**).

3.4. AVP Signaling

AVP has consistently been found to increase $[Ca^{2+}]_i$ in astrocytes [14, 60], pituicytes, a bona fide astrocyte-like cell

Table 1. Brain water content in MCAO model with and without reperfusion.

	No Reperfusion		8 hr Reperfusion	
	Left Hemisphere	**Right Hemisphere**	**Left Hemisphere**	**Right Hemisphere**
Control	77.99±0.12 (n=2)	77.61±0.09 (n=2)	77.34±0.18 (n=3)	77.32±0.14 (n=3)
Ischemia 3 h	77.31±0.13 (n=3)	78.14±0.32 (n=3)	77.97±0.17 (n=8)	81.28±0.34* (n=8)

Contents of dry matter (as percent) were calculated as the ratio between wet and dry weights, and water content determined as 100% minus % dry matter. In control animals no change occurred with or without reperfusion. In animals with MCAO in the right hemisphere a small apparent increase in water content in this hemisphere after 3 hr of ischemia was not statistically significant, whereas the much larger increase after re-perfusion marked with *was significant (P<0.05). No changes occurred in control animals. From D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International.

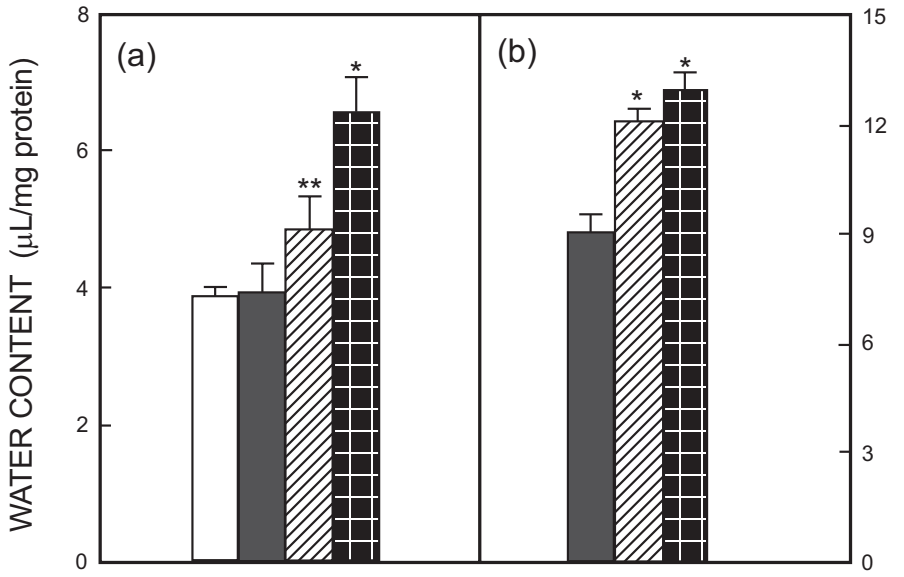

Fig. (3). Changes of water content (determined as [^{14}C] urea space) in astrocytes (a) and neurons (b) in primary cultures by an elevated K^+ concentration and/or addition of AVP. The following incubation conditions were used during a 30-min period: control (5 mM potassium) (□); AVP 10^{-12} M added to control (■); 60 mM potassium (▨); 60 mM potassium plus 10^{-12} M AVP (▦). SEM values are indicated by vertical bars. *Significantly (p<0.05) different from the control; **Significantly different from value obtained at the same K^+ concentration (60 mM) in the presence of AVP. From Chen *et al*., 1992 [13].

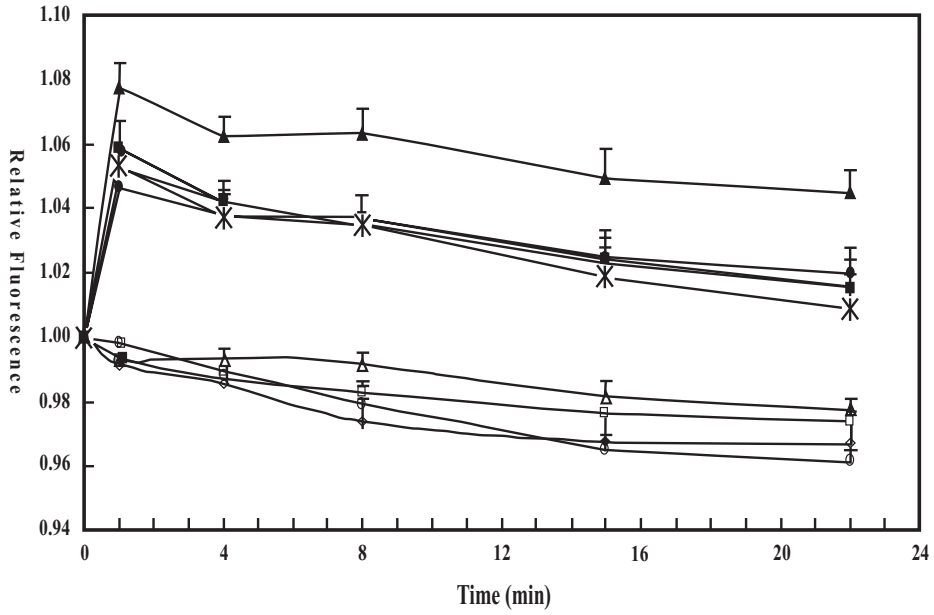

Fig. (4). Fluorescence of calcein relative to the initial fluorescence during incubation in isotonic drug-free medium as an indication of tonicity- and/or AVP-induced changes in astrocytes. After the cells had been loaded with calcein, they were incubated for 2.5 min in 200 µl isotonic solution in the absence of any drug. Subsequently either 200 µl isotonic medium or 200 µl de-ionized water was added to each well, without any drug, together with 10 nM AVP; together with 1 µM AG1478 (inhibitor of EGF receptor); together with AVP and AG1478; or together with AVP and 10 µM U0126 (inhibitor of MAP kinase/ERK kinase (MEK) and thus of $ERK_{1/2}$ phosphorylation), and the incubation was continued for another 22.5 min. Fluorescence was recorded at 15 s intervals and is shown at selected times as means±S.E.M with n = 5-7. Results obtained in isotonic media are shown by open symbols and results obtained in hypotonic media by filled symbols. Squares indicate no addition of drugs, triangles addition of vasopressin, circles addition of vasopressin plus AG1478, diamonds addition of AG1478 alone, and stars addition of AVP plus U0126. Note that all cultures in the isotonic groups are significantly different from all in the hypotonic groups but not from each other, indicating no drug effects under isotonic conditions. Among the cultures in the hypotonic groups those treated with AVP alone was significantly different from the 3 other groups (except at 22min), and no significant difference was found among the 3 other groups. Thus both AG1478 and U0126 completely inhibit the effect of AVP under hypotonic conditions. A small decrease in fluorescence with time may be a methodological artifact or indicate slight cell shrinkage. From Du *et al*., 2008 [59].

[41] and non neuronal cells from dorsal root ganglia [61]. The highest potency (threshold 10^{-10} M) was reported by Chen *et al*. [14] and Moriya *et al*. [61], whereas the other authors found a threshold effect at 10^{-8} M. However this effect can, on its own not explain the effect of AVP on astrocyte swelling induced by exposure to high extracellular K^+ concentrations or hypotonic medium, since it occurs under normal conditions. All authors found the response to be independent of the presence of extracellular Ca^{2+} and thus caused solely by intracellular release of Ca^{2+}.

The ability of AVP to increase hypotonic astrocyte swelling depends on a pathway, which like that activated by elevated K^+ concentrations (Fig. **2a**) leads to phosphorylation of $ERK_{1/2}$ *via* metalloproteinase-mediated release of a growth factor that stimulates EGF receptors and leads to phosphorylation of $ERK_{1/2}$ [59]. It is noteworthy that the metalloproteinase involved in both the K^+-activated and the AVP-activated pathway is ADAM 17, a metalloproteinase not involved in transactivation of astrocytes by any other transmitter studied [37]. It is therefore feasible, but not proven, that AVP increases swelling by high extracellular K^+ concentrations simply by additional stimulation of the pathway involved. AVP alone may cause too little stimulation to activate NKCC1, although it does stimulate $ERK_{1/2}$ phosphorylation, inhibited by the same inhibitors in cells incubated in isotonic medium [59]. This does not explain why AVP also increases hypotonicity-induced swelling. However, this effect can probably be explained by the demonstration by Gunnarson *et al*. [62] that the AQP4 residue serine 111 is a molecular target for dynamic, short term regulation of water permeability and that Ca^{2+}-dependent phosphorylation of this residue augments water flux. The role of serine 111 was confirmed by Song and Gunnarson [36] by water permeability measurements with the mutant AQP4 S111A as well as *in vitro* phosphorylation experiments on AQP4 S111. Although the study by Gunnarson *et al*. [62] used glutamate-induced $[Ca^{2+}]_i$ increase and phosphorylation it is likely that $[Ca^{2+}]_i$ increase evoked by AVP will have a similar effect. Thus, not only are the mechanisms for brain edema evoked by exposure to hypotonic media and highly elevated K^+ concentrations different, but the AVP stimulation is also evoked on NKCC1 in one situation (high K^+) and on AQP4 in another (hypotonicity). Both effects are likely to be important for the edema-reducing effect by antagonists of the V1 receptor and an additional effect on blood-brain barrier NKCC1 and AQP4 activity cannot be excluded.

The studies by Gunnarson and coworkers, by Du *et al*. and by Xu *et al*. were all carried out using cultured astrocytes. At least part of the findings are however in agreement with a study by Niermann *et al*. [63] in which neuronal stimulation increased extracellular K^+ concentration to ~ 9 mM. Although this study was performed in animals too young (15 days old) to reflect all function of the mature brain and the ionic composition of the extracellular fluid was abnormal (drastic reduction in Cl^- content) it did show that neuronal excitation increased water flux. A rapid onset and high capacity of this flux suggested that it was mediated through the AQP4-containing astrocytic syncytium spanning the entire cortex. AVP and receptor V1 agonists facilitated the flux whereas the effect of AVP was blocked by a V1 antagonist. The antagonist even reduced the flux in the absence of AVP suggesting endogenous AVP function. V2 agonists or antagonists had no effect. The effect of a minor increase in extracellular K^+ concentration is important. It may seem peculiar that no stimulation is exerted at normal K^+ concentrations but that a minor increase has profound effects. However, this situation is similar to that of K^+ effects on glycogenolysis, which both in brain tissue [64] and in cultured astrocytes [65] is stimulated by minor K^+ increases. The studies in the cultured cells showed that the glycogenolytic effect of the small increases in K^+ is due to K^+/Na^+,K^+-ATPase-mediated stimulation of the signaling pathway for endogenous (and non-endogenous) ouabains.

Whether the AVP effects are mainly exerted on V1a or V1b receptors has not been discussed. Previous conclusions by Chen *et al*. [14] that the effects on NKCC1 are exerted on V1b receptors were based exclusively on their independence of extracellular Ca^{2+}, but this is not a reliable criterion, and should be disregarded. Most studies in cultured astrocytes regard the effects of AVP as V1a-mediated in cerebral cortex, whereas V1b receptors are expressed in other regions [66]. Exclusive V1a expression in the presently used astrocytes was confirmed as shown in Fig. **5**, which also suggests that it is the only or major subtype in brain tissue. However V1b expression was found in the pituitary. Its expression in the cultured astrocytes was enhanced after differentiation with dibutyl cyclic AMP (dBcAMP), an indication of its expression in mature cells.

3.5. Summary

Antagonists of the AVP1 receptor are capable of reducing experimental brain edema after MCAO/reperfusion. AVP may enhance the effect of elevated extracellular K^+ concentrations due to the similarities between the signaling pathway activated by the high K^+ concentrations and by AVP, perhaps especially the use of the same metallo-proteinase (ADAM 17) for release of the growth factor causing transactivation of the EGF receptor and ultimately NKCC1 stimulation. The V1 antagonists also inhibit water flux into the brain by an effect on AQP4, but NKCC1 in endothelial cells might also be affected.

4. β_1-ADRENERGIC ANTAGONISTS

4.1. MCAO/Reperfusion-Induced Edema and Infarct are Reduced by β_1-Adrenergic Antagonists

Administration of subtype-specific β_1-adrenergic antagonists before experimental brain ischemia has been shown to provide neuroprotection against transient focal cerebral ischemia in rats [67]. Unfortunately, although the β_1-adrenergic antagonists provided long-term improvement of histological outcome, they had no effect on neurological outcome and spatial memory retention 14 days later when administration began 30 min before the onset of ischemia and continued for 24 hrs [68]. Nevertheless, the same group has shown that administration 30 min after the onset of a 2-hour-long ischemic period drastically reduced infarct size (Fig. **6**) and improved neurological deficit score after 7 days (Table **2**) [69]. Unfortunately they did not measure edema. Similarly Iwata *et al*. [70] found that administration of

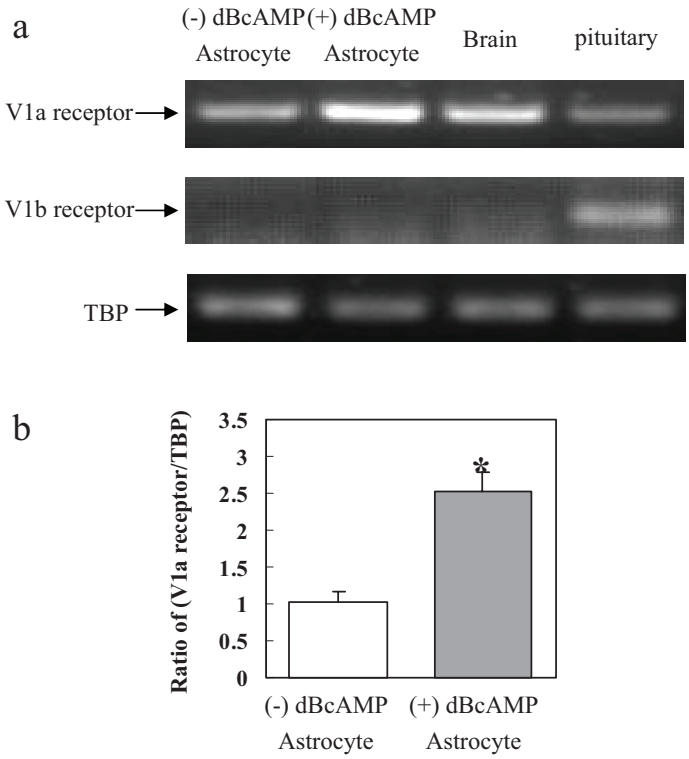

Fig. (5). Expression of mRNA of V1a receptor and V1b receptor in primary cultures of astrocytes, in brain *in vivo* and in the pituitary. Astrocytes were cultured for 3 weeks with or without addition of 0.25 mM dibutyryl cAMP to the medium from the age of 2 weeks. (**a**). A representative experiments showing mRNAs for V1a receptor, V1b receptor and for TBP, used as a house-keeping gene. The first lane represents the PCR product from primary cultures of astrocytes without dBcAMP, the second lane that from primary cultures of astrocytes cultured with dBcAMP, the third lane that from brain of adult mouse, and the last lane that from the pituitary of an adult mouse. The size of PCR product of V1a receptor is 332 bp, that of V1b receptor 110 bp, and that of TBP 236 bp. (**b**) Average mRNA expression was quantitated as scanned ratios between V1a receptor expression in 4 individual experiments and that of TBP. S.E.M. values are indicated by vertical bars. *Statistically significant ($P < 0.05$) difference from astrocytes without dBcAMP. (Previously unpublished experiments by Ting Du and Liang Peng).

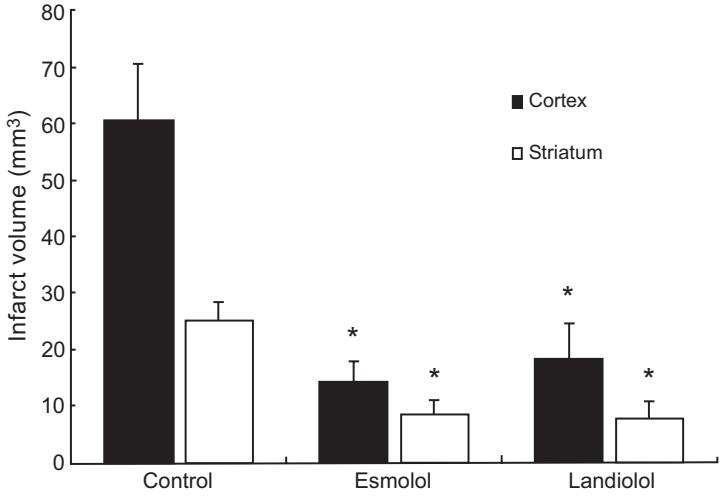

Fig. (6). Infarct volumes (determined by staining with triphenyltetrazolium chloride) 7 days after reperfusion of the cortex and striatum in rats exposed to 2 hr MCAO followed by 24 hr re-perfusion and treated with saline (control), esmolol, or landiolol, shown as means ± SEM. Drug (or saline) treatment was started 30 min after the onset of MCAO and continued during 24 hr of reperfusion. *P<0.05 versus saline treatment group. From Goyagi *et al*., 2010 [69].

Table 2. Neurological deficit scores determined as described by Goyagi *et al.* [69] in control animals (injected with saline) and exposed to 2 hr of MCAO followed by reperfusion and in rats treated with either of the β_1-adrenergic antagonists esmolol or landiolol, beginning 30 min after the onset of ischemia and continued for 24 hr.

	Control (n=8)	Esmolol (n=8)	Landiolol (n=8)
1 day	18 (10-45)	7.5 (0-20)*	0 (0-16)*
4 days	4 (0-24)	0 (0-4)*	0 (0-6)*
7 days	2 (0-13)	0 (0-2)*	0 (0-0)*

Values are expressed as median and range). The neurological deficit scores in the esmolol- or landiolol-treated rats were significantly lower than the saline-treated control rats 1, 4, and 7 days after focal ischemia. *P<0.05 versus control group. From Goyagi *et al*, 2010 [69].

antagonists specifically of the β_1-adrenoceptor beginning 60 min after an 8-min bilateral carotid artery occlusion combined with hypotension reduced neuronal injury after forebrain ischemia, although motor activity was not improved. However, motor deficit index scores were significantly lower and neuronal survival better in rats treated with β_1-adrenoceptor antagonists beginning 30 min before 10 min of spinal cord ischemia and continued for 24 hr [71]. Recently Song *et al*. (D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International) found that intraventricular injection of any of several inhibitors of the β_1-adrenergic pathway as well as the β_1-adrenoceptor antagonist betaxolol abolished cerebral edema in rats exposed to 3 hr of MCAO followed by 8 hr of reperfusion. In contrast a single selective inhibitor of the β_2-adrenergic pathway or the β_2-adrenoceptor antagonist ICI118555 had no effect.

4.2. β_1-Adrenergic Stimulation of NKCC1-Mediated Regulatory Volume Increase

Most cells shrink during exposure to a hypertonic medium and NKCC1 activity is important for a subsequent regulatory volume increase [6]. This also happens in cultured astrocytes, where the regulatory volume increase after addition of 100 mM sucrose to the incubation medium is inhibited by bumetanide, showing the involvement of NKCC1 [72]. The volume regulation is slow in cultured astrocytes under control conditions, but accelerated by the β-adrenergic drug isoproterenol (D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International). The pathway for both β_1- and β_2-mediated signaling is known in astrocytes [73]. In well-differentiated astrocyte cultures this pathway reminds of that shown in Fig. **2a** for the effect of highly elevated K^+ concentrations. Although β-adrenergic receptors are G_s-linked, a G_s to G_i switch mediated by protein kinase A (PKA) leads to an increase in $[Ca^{2+}]_i$ and a subsequent growth factor release, which transactivates the EGF receptor and stimulates $ERK_{1/2}$ phosphorylation [73]. Two important differences between the two signaling pathways are that Src and the metallopropteinase ADAM 17 are not involved in the pathway activated by stimulation of β_1-adrenergic receptors, although Src constitutes part of the β_2-adrenoceptor-mediated pathway. Song *et al*. (D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International) found that inhibitors of G_i, metalloproteinases, EGF receptor phosphorylation and $ERK_{1/2}$ phosphorylation as well as the β_1-adrenergic antagonist betaxolol all abolished the ability of isoproterenol to enhance regulatory volume increase in the astrocytes exposed to a medium supplemented with 100 mM sucrose, whereas an inhibitor of β_2-adrenergic signaling and the β_2-adrenergic antagonist ICI118555 had no effect. Moreover it was also shown that glycogenolysis, which is essential for K^+-stimulated activation of both the Na^+,K^+-ATPase and NKCC1 [5] is needed for β-adrenergic stimulation of the Na^+,K^+-ATPase/NKCC1 transport system during regulatory volume increase. These observations are in excellent agreement with stimulation of the astrocytic, but not the neuronal Na^+,K^+-ATPase with isoproterenol [74]. This ATPase, which provides the driving force for NKCC1 operation (see above) is ubiquitously expressed in both neurons and astrocytes [75, 76].

4.3. Abrogation of Na^+,K^+-ATPase Stimulation by Elevated K^+ Due to Extracellular Ca^{2+} Deficiency Allows Exclusive β_1-Adrenergic Stimulation of NKCC1

The stimulation of regulatory volume increase by β_1-adrenergic stimulation triggered the experiments by Song and her colleagues showing that the same antagonists inhibited edema formation after MCAO/reperfusion. However, there is one important difference between the two situations. During regulatory volume increase the extracellular K^+ concentration is unaltered, whereas it is increased after ischemia. This difference should be important, because a rise in extracellular K^+ concentrations above its normal level provides an important stimulus for the astrocytic Na^+,K^+-ATPase [77, 78]. In cultures of cerebral cortical astrocytes Hajek *et al*. [74] found K_m value for K^+ of 1.9 mM, which is high enough for an increase in extracellular K^+ concentration in the interval 5-12 mM on its own to allow a significant increase in the activity of the enzyme. In cerebral cortical neurons, the affinity was higher (K_m 0.43 mM), showing that there is no stimulation of enzyme activity when the extracellular K^+ concentration is increased beyond control levels. Astrocytes are a major target for noradrenaline released from locus coeruleus [79]. It is likely that under normal, non ischemic conditions most of the K^+ released to the extracellular space during action potential propagation is initially accumulated into astrocytes, and that this uptake stops due to cessation of K^+-stimulated glycogenolysis when extracellular K^+ normalizes [5]. This will allow Kir4.1-mediated K^+ release from astrocytes [80] and re-uptake by the neuronal Na^+,K^+-ATPase. This raises the question why K^+-mediated stimulation of Na^+,K^+-ATPase did not support NKCC1 operation after ischemia when energy became available after reperfusion.

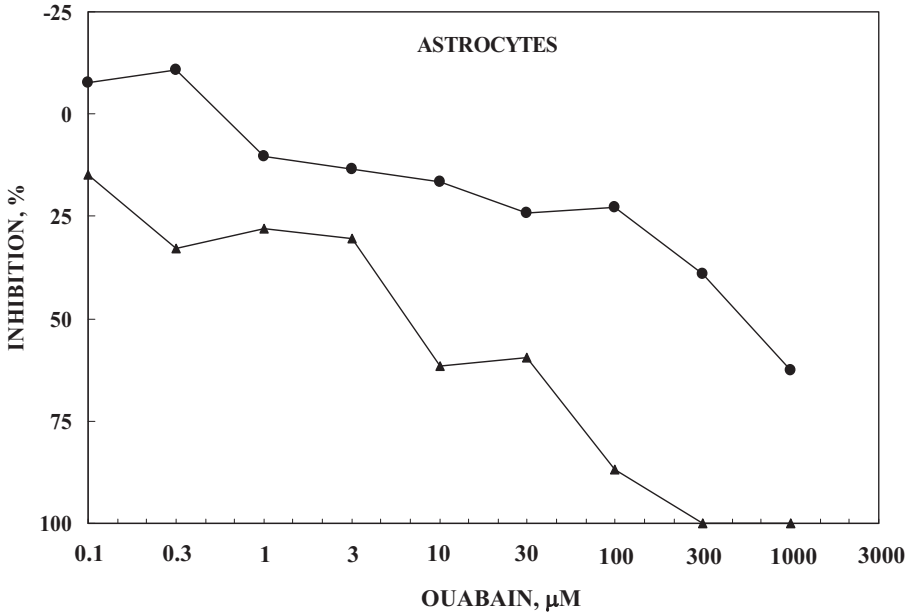

Fig. (7). Ouabain induced inhibition of net influx of ^{42}K into primary cultures of mouse astrocytes determined during a 1.00 min incubation, which provides initial uptake rates. The graph marked by triangles was obtained in a slightly modified Dulbecco's medium containing 1.8 mM Ca^{2+} and that marked by circles in a corresponding medium containing no $CaCl_2$. The inhibition was calculated from the difference between uptake rates in the absence of ouabain and that at the ouabain concentration in question. Without ouabain and at all lower ouabain concentrations the S.E.M. values of these measurements were below 5%, but at the highest ouabain concentration they were slightly higher, although always below 10%. For both graphs 6 cultures were routinely used from at least two different batches. The apparent stimulation at low ouabain concentrations in the absence of Ca^{2+} was not significant, but might suggest a ouabain-mediated stimulation of uptake, normally occurring at low nanomolar concentrations, as described by Xu *et al.* [5]. No similar increase was seen in neurons, which also showed a decrease in ouabain potency in the absence of Ca^{2+}, although of somewhat smaller magnitude (not shown). Results from D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International.

The profound decrease in extracellular Ca^{2+} caused by brain ischemia is combined with an increase in $[Ca^{2+}]_i$, causing cell damage and even death [81]. Cellular increase in Ca^{2+} promotes NADH hyperoxidation and electrical dysfunction after anoxia in hippocampal slices [82]. Not only neurons are affected by the Ca^{2+} overload, since cultured astrocytes rapidly die following anoxia and re-perfusion when the gaseous and interstitial ionic changes of transient brain ischemia are simulated, and their death requires external Ca^{2+} [83]. Nevertheless, our own results (D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International) have shown that in astrocyte cultures exposed to 3 hr anoxia the increase in swelling in response to 50 mM K^+ occurred more slowly than normally, but it was not abolished. This may shift the focus to the 90% reduction in extracellular Ca^{2+} concentration shown by Hansen and Nedergaard [2].

It was pointed out above i) that stimulation of the astrocytic Na^+-K^+-ATPase requires ouabain signaling in order to allow entry of Na^+, needed for co-stimulation at the intracellular Na^+-sensitive site, and ii) that NKCC1 function requires Na^+-K^+-ATPase activity. It is therefore of interest that Ca^{2+} deficiency in the medium (0 Ca^{2+} added, but no chelating agent) leads to a large decrease in potency of ouabain-induced inhibition of ^{42}K uptake in cultured astrocytes, expressed as percentage of the uninhibited rate in a medium containing1.8 mM $CaCl_2$ (Fig. **7**) (D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International). Since no Ca^{2+} chelator was added, the Ca^{2+}-deficient medium probably contained traces of Ca^{2+}released from the cells. The K^+ concentration in the medium was at control level (5.4 mM) but addition of ^{42}K provided a small increase of ~0.3 mM. In both types of media a minor part of the uptake is inhibited by the lower ouabain concentrations, with relatively little dependence on percentage changes in ouabain concentration, and a major part of the uptake is inhibited by higher ouabain concentrations with a greater effect of relative changes in ouabain concentration. This biphasic effect can be explained by binding to two different Na^+,K^+-ATPase subunits [84]. However, at all concentrations the potency of ouabain is ≥ 10 times lower in the absence of added Ca^{2+} to the medium. Accordingly, the normally occurring endogenous ouabain concentrations may not suffice to stimulate the Na^+,K^+-ATPase when the extracellular Ca^{2+} concentration is drastically reduced. The apparent slight stimulation at low concentration of ouabain (100 and 300 μM) seen in Fig. **7** suggests that in the Ca^{2+}-deficient medium ouabain may be stimulatory at concentrations which are normally inhibitory, although in a medium containing sufficient Ca^{2+} stimulatory ouabain concentrations are much lower [5].

The large decrease in ouabain potency in the absence of added Ca^{2+} in the medium is consistent with an abolishment

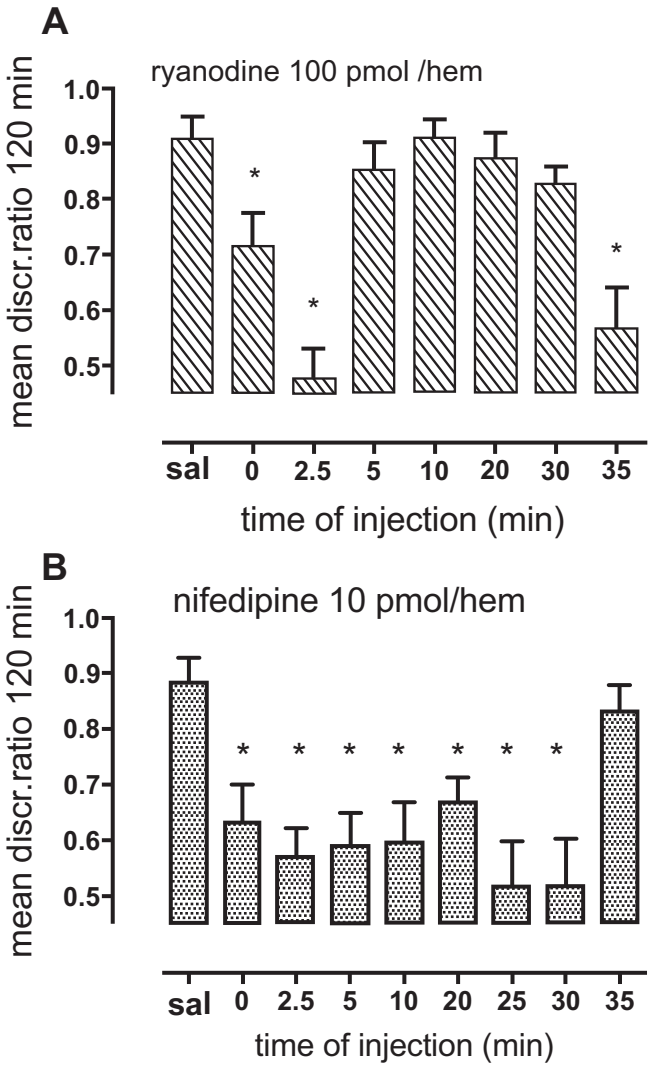

Fig. (8). Effects of the ryanodine receptor antagonist ryanodine and the L-channel inhibitor nifedipine, compared to injection of saline only, on learning in day-old chickens. As described in the text the DR between pecking at a bead of a previously neutral color and a bead of a color that during training was aversive is an indication of learning, with a high DR (~0.9) indicating normal learning and a low DR (close to 0.5) indicating failure to learn. **A**: Intrahippocampal injection of ryanodine inhibits learning when injected at two different time periods, 2.5 and 35 min after training. **B**: Intraventricular injection of nifedipine inhibits learning at several injection times. It does not inhibit learning when injected at 35 min, although ryanodine was inhibitory at this time. This difference is consistent with L-channel activation and Ca^{2+} entry (Fig. **1**) occurring slightly earlier than the effect of accumulated Ca^{2+} on the ryanodine receptor. Previously unpublished results by M.E. Gibbs.

of K^+-mediated uptake into cultured astrocytes in the absence of Ca^{2+} in the incubation medium (D. Song, J. Xu, L. Hertz, W. Walz and L. Peng, in press, BioMed Research International). It is also in agreement with the observation by Wang *et al*. [35] that a transmitter-induced rise in $[Ca^{2+}]_i$ triggers an increase in ouabain-sensitive K^+ uptake in cultured astrocytes, which was abolished by the Na^+/Ca^{2+} exchange inhibitors SEAO4000 or SN-6. The same astrocyte-specific transmitter also evoked a transient decrease in extracellular K^+ concentration in hippocampal slices. Finally an increased utilization of glucose in the striatum in freely behaving rats in the presence of elevated extracellular K^+ concentrations [85] confirms a previously observed stimulation of oxygen consumption rate in brain slices [86, 87]. In the slices the threshold concentration of K^+ evoking this effect (20 mM) coincides with that causing swelling [22] and the stimulation is inhibited by ethacrynic acid [88], suggesting that it is a metabolic manifestation of the normally occurring NKCC1-stimulated K^+ uptake. In the present context it is important that the metabolic stimulation was abolished when Ca^{2+} was excluded from the medium. *In vivo* the stimulation probably also reflects stimulation of Na^+,K^+-ATPase activity, which in the slices may be supported exclusively by glycolysis, the rate of which in brain slices is greatly enhanced as indicated by a large lactate release [86].

Since brain swelling also requires additional fluid uptake across the blood-brain barrier, which similarly expresses NKCC1 [12], it is possible that Ca^{2+} deficiency at the blood-

brain barrier also may have played a role for the prevention of edema after MCAO/reperfusion. The NKCC1 in endothelial cells is expressed at their luminal side [89, 90], facilitating uptake from the circulation. Ca^{2+} deficiency also reduces the potency of ouabain in neuronal cultures, but to a lesser degree. Consistent with neuronal Na^+ uptake during excitation abolishing the need for additional Ca^{2+} entry there is no evidence suggesting stimulation at the lowest concentrations (L. Hertz and W. Walz, unpublished).

4.4. Which Ionic Consequences can be Expected after Complete NKCC1 Inhibition?

Even when NKCC1 is completely inhibited the large increase in extracellular K^+ is likely to be re-accumulated, but exclusively into neurons and considerably more slowly than normally. This is both because no NKCC1 activity contributes to the uptake and because the neuronal Na^+,K^+-ATPase has a lower V_{max} than the astrocytic enzyme and is not stimulated by elevated K^+ [74]. However, it may be stimulated by excess intraneuronal Na^+. Hossmann *et al* [1] have shown that uptake of the increased extracellular K^+ concentration after a non-complete MCAO under normal conditions is completed within one hr, and it would be very important also to obtain information how rapidly excess extracellular K^+ is normalized during inhibition of β_1-adrenergic activity. NKCC1 inhibition beyond that point in time may be damaging brain function rather than protecting it. Normalization of Ca^{2+} distribution between extra- and intracellular spaces is equally important. Although it is known that extracellular Ca^{2+} normalizes more slowly than K^+ after shortlasting anoxia [91], similar information is lacking after a longer insult, let alone in a situation where Na^+,K^+-ATPase function is inhibited. Such information would also be crucial.

4.5. Adverse Effect of NKCC1 Inhibition: Impairment of Learning

Frieder and Allweis [92] showed that ethacrynic acid, a less specific inhibitor of NKCC1 than bumetanide, inhibits memory formation in a rat model. Since nifedipine inhibits the Ca^{2+} uptake which after reinforcement by Ca^{2+} release by stimulation of the ryanodine receptor leads to the initiation of the pathway that ultimately stimulates NKCC1 (Figs. **1**, **2**) we tested effects of nifedipine and the ryanodine receptor antagonist ryanodine on learning. In Figs. **8a** and **b** the discrimination ratio (DR) between pecking at beads of a neutral color and a color previously associated with an aversive taste during earlier 10-sec training is an indication of learning by day-old chicks [93]. Ethacrynic acid also inhibits learning in this model [94]. Fig. **8a** shows that injection of a high, inhibitory concentration of ryanodine into the hippocampus at two different times, 2.5 and 35 min inhibits learning, as indicated by almost similar pecking at beads of the two colors (DR close to 0.5), whereas ryanodine injection at other times have little or no effect (DR 0.8-0.9). These two time periods are known to be very sensitive to inhibitors of learning, and glutamate release causing excitation with cellular release of K+ occurs immediately before these times [95, 96]. That the effect of ryanodine is associated with deficient Ca^{2+} entry leading to stimulation of NKCC1 is supported by the effect of nifedipine to inhibit learning. This is seen in Fig. **8b**, which shows that in contrast to earlier administration of nifedipine injection at 35 min does not inhibit learning. That this is slightly earlier than the shown sensitivity to ryanodine is consistent with the uptake of Ca^{2+} preceding Ca^{2+} release from the ryanodine receptor. It is likely, but not proven that the shown effects, like many others [97], are exerted on astrocytes. An effect on astrocytes is supported by a memory-enhancing effect of thrombin at the same times and its inhibition by fluoroacetate, an astrocyte-specific toxin [98]. However, even if this should not be the case, Fig. **8** demonstrates that inhibition of the first steps of the pathway leading to NKCC1-mediated uptake of ions and water inhibits memory. Although this effect is likely to be reversible it strongly suggests that inhibition of NKCC1 function after MCAO/reperfusion should be of as short duration as possible.

5. CONCLUDING REMARKS

The present review has focused on the role of cytotoxic swelling primarily or exclusively in astrocytes and the ways in which antagonists of the V1 receptor or the β_1-adrenergic receptor can counteract edema formation. Since the β_1-adrenergic receptor antagonists in principle can completely prevent the development of cytotoxic edema they may be the more powerful agents, whereas V1 receptor antagonists only prevent the additive effect of AVP on cell swelling. However, AVP inhibition of AQP may be very important to inhibit water uptake across the blood-brain barrier as discussed below. Like other recent review papers it points out that a volume shift from the extracellular to intracellular space in itself does not cause brain swelling, which depends upon water influx across the blood-brain barrier. Mechanisms leading to this influx have recently been discussed by Khanna *et al*. [99]. We would like also to emphasize that due to the 3/2 ratio between Na^+-K^+-ATPase-mediated Na^+ efflux and K^+ uptake [100, 101] the intense operation of the Na^+-K^+-ATPase/NKCC1 system during removal of extracellular K^+ concentrations as high as 80 mM might lead to considerable hypertonicity in brain extracellular fluid that would facilitate AQP4 mediated water uptake across the blood-brain barrier. A small increase in osmolality during a non-complete MCAO has been demonstrated by Matsuoka and Hossmann [51]. The same group [102] also concluded that early edema following a non-complete ischemia is cytotoxic and develops at flow rates below 10-15 ml/100g tissue per min and that the blood-brain barrier remains intact for at least 4 hr. These conclusions seem to be in agreement with the observations cited in this review. Also, although ischemic and traumatic brain injury are widely different diseases it is highly relevant that the successful Lund project for treatment of post-trauma brain edema recommended use of a β_1-adrenergic antagonist and that AVP should be omitted unless absolutely necessary [37, 103]. However, the effect of β_1-adrenergic antagonists may seem at odds with the finding by de Raedt *et al*. [104] that stroke patients already under treatment with β_1-adrenergic inhibitors have no improved outcome after stroke. In this connection it may be important that the human hypothalamus and hippocampus have a much lower β_1-/β_2-adrenoceptor ratio than the corresponding tissues in the rat [105, 106]. On the other hand a higher level of total β-adrenergic receptor binding was

found in the human than in the rat hippocampus in the regions investigated [106]. Also, Russo-Neustadt and Cotman observed high levels of β_1 receptors in layers I and II, low levels in layers III–V, and intermediate levels in layer VI of the human orbitofrontal cortex, while confirming the low β_1-/β_2-adrenoceptor ratio in hypothalamus [107]. Since β_1-adrenergic receptor expression has been determined with certainty in freshly isolated astrocytes (from the mouse) but not in corresponding neurons [108], the species difference may mainly or exclusively apply to astrocytes. Finally, human white matter contains no β_1-adrenergic receptors [107, 109], suggesting a difference between gray and white matter astrocytes and inability of β_1-adrenergic inhibitors to counteract white matter ischemic damage. In addition there may be a question of drug dosage in patients treated chronically with β_1-adrenergic inhibitors. The doses used to completely inhibit brain edema may be much higher than those used for continuous treatment, where they would have intolerable side effects, including learning difficulties. As previously mentioned, clinical effect of V1 or β_1-adrenergic antagonists would probably require use of high doses during the shortest possible time period.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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