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Effect of glutamate and angiotensin II on whole cell currents and release of nitric oxide in the rat subfornical organ

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Summary. Blood-borne angiotensin II (AngII) is known to mediate water-intake by its excitatory effect on neurons in the subfornical organ (SFO). Conversely, nitric oxide (NO) has exclusively inhibitory effects on rat SFO-neurons and on SFO-mediated water-intake. Extracellular and patch-clamp recordings from freshly dissociated rat SFO-neurons showed that glutamate activates AngII-sensitive SFO-neurons by opening ligand-gated cation channels. An immunocytochemical study showed that activation of glutamate receptors increased the concentration of the inhibitory second messenger cGMP in the SFO. A model is proposed suggesting that NO protects SFO-neurons from overexcitability by excitatory neurotransmitters.

Keywords: Angiotensin II – Patch clamp – Glutamate – NMDA – Nitric oxide – Thirst

Introduction

The subfornical organ (SFO) is one of the neural circumventricular organs (CVO) lacking a blood-brain-barrier (McKinley et al., 1990). It has been amply documented that blood-borne angiotensin II (AngII) increases water-intake by activating SFO-neurons (Fitzsimons, 1979).

Furthermore, microapplication of AngII and other excitatory neuromodulators (McKinley et al., 1990) as well as electrical stimulation of the SFO (Smith et al., 1995) increase water-intake in rats. Conversely, it has been shown that direct application of the NO-donor sodium nitroprusside (SNP) reduces AngII-induced water-intake (Nicolaidis and Fitzsimons, 1975) and inhibits the majority of AngII-sensitive SFO-neurons in an *in vitro* slice preparation (Rauch et al., 1997). Endogenous NO, which is produced by the neuronal NO-synthase (NOS) and released within the SFO (Jurzak et al., 1994b; Rauch et al., 1997) exerts its inhibitory effect most likely by stimulating the soluble guanylyl-cyclase, as indicated by the increase in cGMP after stimulation with different NO-donors and by the inhibitory effect of mem-

brane-permeable 8Br-cGMP on neuronal activity (Rauch et al., 1997). In addition, previous studies showed that atrial natriuretic factor (ANF), which acts by stimulating the particulate guanylyl-cyclase (deVente and Steinbusch, 1992) also inhibits the majority of SFO-neurons (Schmid and Simon, 1992), suggesting that the second messenger cGMP has an exclusively inhibitory effect on SFO-neurons, although excitatory effects have also been reported in other regions of the CNS (Pehl and Schmid, 1997).

Aim of the present patch-clamp study was to investigate the effect of the excitatory neurotransmitter glutamate and a peptide with exclusively excitatory effects, AngII, on freshly dissociated rat SFO-neurons, in order to evoke direct cellular responses of individual neurons free from synaptic interactions. The fact that NO-dependent cGMP-production has been observed after stimulation of glutamate receptors with N-Methyl-D-Arginine (NMDA) in other brain areas (deVente and Steinbusch, 1992) stimulated an immunocytochemical study which addressed the question, whether an exclusively excitatory stimulus like NMDA would in addition increase the NO-dependent production of the second messenger cGMP, which caused exclusively inhibitory responses in SFO-neurons.

Effect of glutamate and AngII on freshly dissociated rat SFO-neurons

Patch clamp recordings were made from individual rat SFO-neurons which were prepared from 8–16 days old rats using similar methods as previously described (Schmid and Vijayaraghavan, 1992). SFOs from 3–6 animals were pooled and the hemisected SFOs were triturated mechanically with pasteur pipettes, after incubation for 30 min in collagenase (0.5 mg/ml in oxygenated aCSF, composition see Rauch et al., 1997). Cells were allowed to attach firmly to poly-L-lysine coated petridishes (35 mm i.d.) for at least 30 min in HEPES buffered extracellular solution (ES) supplemented with 10% horse-serum (Schmid and Vijayaraghavan, 1992) and stored at 37°C until use. For recording, the cells were constantly superfused with ES without horse-serum. Recordings were made in a cell-attached mode using an intracellular solution containing (in mM): NaCl 15; KCH_3SO_3 150; HEPES 10; EGTA; 1.1; $CaCl_2$ 0.1, supplemented with 10 mg/ml MgATP and 10 mg/ml GTP. Neurons were identified as cells which were able to generate action potentials either spontaneously or after a brief application of glutamate (10^{-4}M) from a nearby pufferpipette (Fig. 1a). In addition the AngII-sensitivity of neurons was tested by superfusing the cells with AngII (10^{-7}M) for 1–2 min. Neurons which increased their firing rate in response to glutamate and/or AngII were additionally recorded in the whole-cell recording mode. Fig. 1b is a whole-cell recording of the same neuron as shown in Fig. 1a. Application of glutamate at a holding potential of –70 mV caused an inward current in each glutamate sensitive neuron (n=19). In contrast to the only so far available patch-clamp study on SFO-neurons using slow drug applications (Weindl et al., 1992) the glutamate-induced current showed a rapid and a slow inactivation component. This cation-current can cause depolarization of the membrane potential and thus result in the generation of action-potentials (Watkins and Evans, 1981).

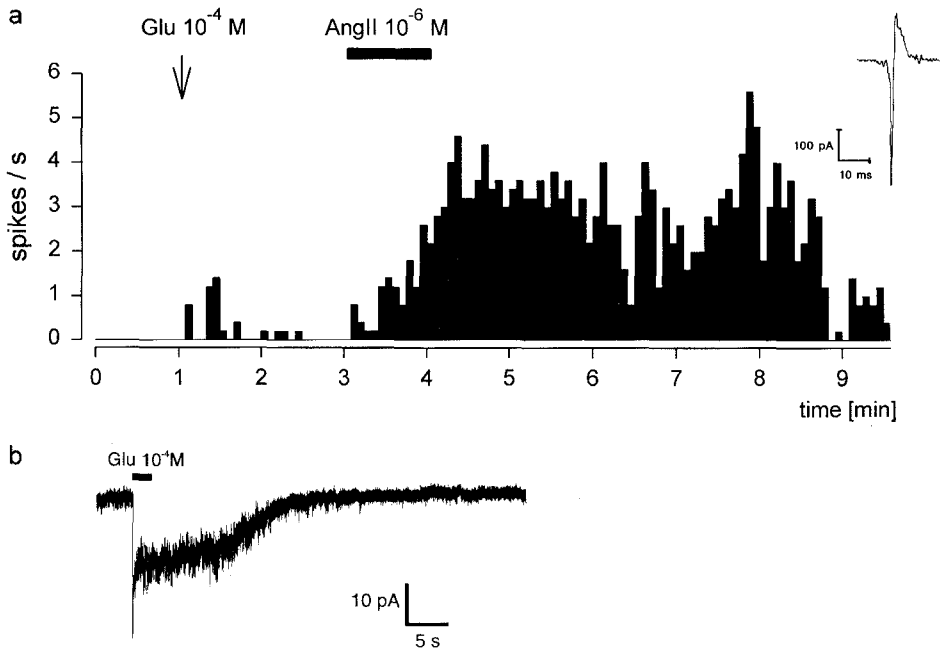

Fig. 1. **a** Cell-attached recording from a freshly dissociated rat SFO -neuron. Application of glutamate from a nearby puffer pipette (for 0.5 sec, arrow) and superfusion with AngII for one min evoke firing of action potentials, one of which is shown in the inset. **b** Original recording of a glutamate activated inward current recorded at –70mV in the whole-cell voltage clamp mode. A fast and a slow desensitizing current component can be distinguished after a rapid glutamate application

A similar transduction mechanism can be excluded for the excitatory effect of AngII on SFO-neurons, because under those conditions which allowed recording of glutamate currents, AngII application (10^{-7}–10^{-6}M) never caused a measurable inward current (n = 55). This results is in contrast to the proposed activation of a voltage-independent nonselective cation conductance by AngII of neurons in the supraoptic nucleus (Yang et al., 1992).

The question whether AngII activates SFO-neurons by modulation of voltage gated currents as shown in other tissues was addressed using the pulse protocol shown in Fig. 2a. Cells were held at –70mV and, after a brief (10 ms) hyperpolarization to –90mV, currents were activated by a family of voltage steps (10 mV steps to +30mV, 80 ms long). The rapidly inactivating inward current component (Fig. 2a) evoked at potentials above –40mV, is completely and reversibly blocked by tetrodotoxin (TTX, 10^{-6}M, superfused for 1 min) and thus represents a sodium current. The outward current consist of a rapidly (measured between 1 and 2) and a slowly (measured between 3 and 4) inactivating current component, which were strongly reduced by 4-aminopyridine (4-AP, 10^{-4}M) and tetraethyl-ammonium (TEA, 10^{-4}M), respectively, although TEA showed less specificity and reduced the rapidly inactivating current component as well.

Patch-clamp recordings from glia cells, which did not generate action potentials in the cell-attached mode, showed no rapidly inactivating inward

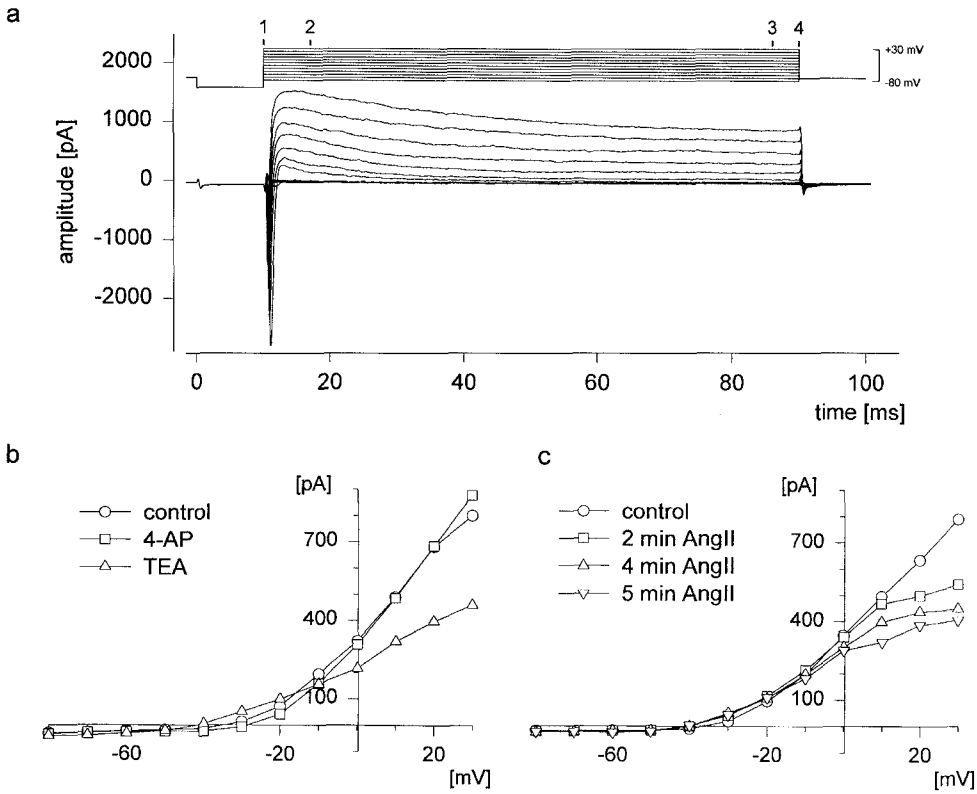

Fig. 2. **a** Inward and outward currents of a freshly dissociated rat SFO-neuron from a 14 day old rat activated by voltage steps between –80 and +30 mV. The inward currents measured between 1 and 2 could be blocked by tetrodotoxin (TTX) 10^{-6} M completely and reversibly. **b** Current-voltage-relationship of the maximum outward current (same cell as a) measured at the end of each voltage step (between 3 and 4) in the absence and in the presence of TEA (10^{-4}M) and 4-AP (10^{-4}M) for 5 min each. **c** Superfusion of the same neuron with AngII (10^{-6}M) for 2, 4, and 5 min resulted in a progressive decrease predominantly of the TEA sensitive outward K^+-current-component, measured at the end of each voltage step

current and the amplitudes of the outward currents were less than 10% compared with the amplitudes of the respective neuronal currents. Although effects of AngII on glia cells can not be excluded (Jurzak et al., 1994a) such an effect is not necessary for the activation of individual SFO-neurons as shown in Fig. 1a. Superfusion of AngII-sensitive neurons with AngII (10^{-6}M) reduced the outward current, but had no effect on the inward (Na^+) current. Figure 2c shows the effect of AngII, superfused for 2, 4 and 5 min on the slowly inactivating outward-current component (measured between 3 and 4). The fast inactivating outward current was less reduced by AngII. These results suggest that the excitatory effect of AngII on SFO-neurons might be the result of an inhibitory effect of AngII on TEA-sensitive K^+-currents, which results in membrane-depolarization and the generation of action potentials.

An inhibitory effect on K^+-currents has also been proposed by Brauneis et al., 1991; Ferguson and Bains, 1996; Nagatomo et al., 1995, and Shapiro et al.,

1994 as the most likely transduction mechanism for AngII, although stimulatory effects on K^+-currents (Kang et al., 1993), excitatory (McCarthy et al., 1993; Cohen et al., 1988) and inhibitory effects on Ca^{2+}-currents (Shapiro et al., 1994; Bacal and Kunze, 1994), an activation on Ca^{2+}-dependent K^+-currents (Stockand and Sansom, 1994) and activation of chloride channels (Bescond et al., 1994) have also been described.

The electrophysiological studies show that individual SFO-neurons can be directly activated by different excitatory stimuli suggesting that these cells bear receptors for glutamate as well AngII. Although glutamate activates SFO-neurons by opening ligand-gated nonselective cation-currents and AngII most likely by decreasing a potassium current, both substances cause an increase in intracellular Ca^{2+}-concentration (Jurzak et al., 1994a; Watkins and Evans, 1981), which is a precondition for the activation of NOS (see below).

Stimulation of a NO-dependent production of cGMP in the SFO

Recently we have demonstrated that stimulation of the NOS or application of NO-donors inhibit the majority of SFO-neurons and cause an increase in cGMP production (Rauch et al., 1997). NOS can also be activated by stimulation of different types of glutamate-receptors (Garthwaite and Boulton, 1995). Therefore we tested the hypothesis whether activation of NMDA-receptors in the rat SFO would also cause a NO-mediated increase in cGMP-production.

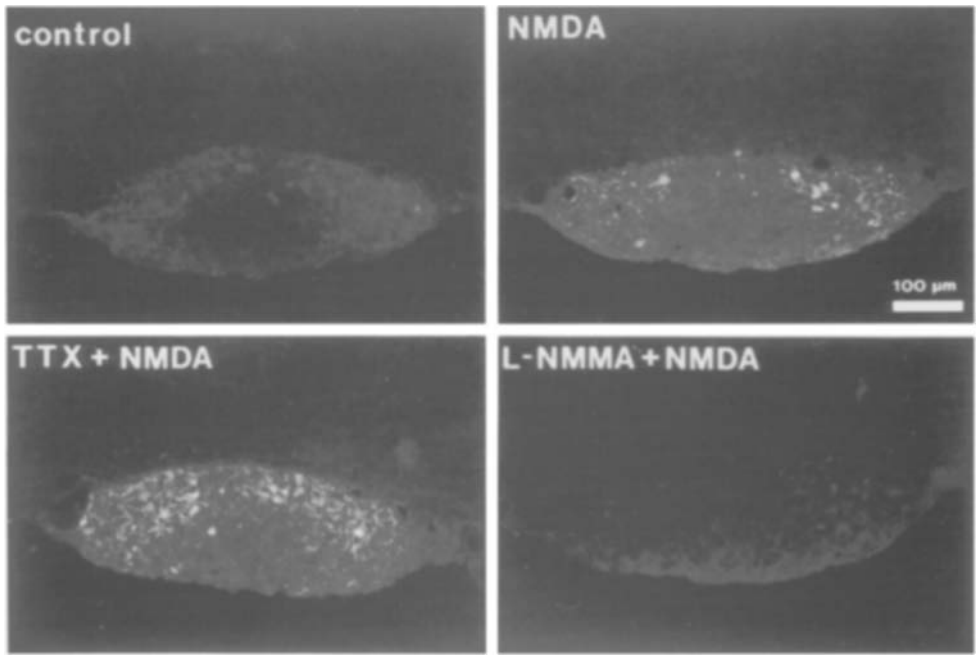

Fig. 3. Immunohistochemical staining for cGMP of medial sections of 4 different SFOs treated *in vitro* with NMDA (10^{-4}M, 10 min) in the presence or in the absence of TTX (10^{-6}M; 10 min) and L-NMMA (10^{-3}M, 40 min)

Figure 3 shows the cGMP-production of the medial section of a rat SFO stimulated *in vitro* for 10 min with NMDA (10^{-4} M; method see Rauch et al., 1997). The increase in cGMP was not dependent on the neuronal activity of SFO-neurons, because preincubation of the SFO in TTX, which completely prevents the generation of action potentials, had no effect on the cGMP-production. However, preincubation of the SFO with a blocker of the NOS (L-NMMA) resulted in a complete loss of cGMP-production, indicating that the entire cGMP production in the SFO after activation of NMDA-receptors is NO-dependent. In contrast, stimulation of the particulate NOS in the SFO by an effective concentration of atrial natriuretic factor (ANF, 10^{-7} M, 10 min, Schmid and Simon, 1992) resulted in a cGMP accumulation, which was only slightly reduced by L-NMMA.

Conclusions

These results suggest that the excitatory neurotransmitter glutamate stimulates the neuronal NOS and thus the release of NO. This stimulation does not require the generation of action potentials, but probably the influx of Ca^{2+}, due to the known Ca^{2+}-dependence of the neuronal NOS. Furthermore, an increase in intracellular Ca^{2+}-concentration has been observed in cultured rat SFO-neurons and glia cells using Fura2 measurements (Jurzak et al., 1994a; Gebke, pers. communication) following glutamate as well as AngII-application. Because NO and the membrane permeable analog 8Br-cGMP have exclusively inhibitory effects on SFO-neurons and because NO-producing and NO-responsive cells are usually codistributed, but not identical (Rauch et al., 1997), one can conclude that the excitatory transmitter glutamate will trigger the production of the inhibitory second messenger cGMP in nearby neurons. Thus NO might function as an "auto-control mechanism" in the SFO to prevent overexcitability of neurons to excitatory neurotransmitters. The question whether AngII and other blood-borne signals also stimulate NO-release in the SFO, which would result in a reduced effectiveness of their excitatory response, will be addressed in future experiments.

References

Bacal K, Kunze DL (1994) Dual effects of angiotensin-II on calcium currents in neonatal rat nodose neurons. J Neurosci 14: 7159–7167

Bescond J, Bois P, Petitjacques J, Lenfant J (1994) Characterization of an angiotensin-II-activated chloride current in rabbit sinoatrial cells. J Membr Biol 140: 153–161

Brauneis U, Vassilev PM, Quinn SJ, Williams GH, Tillotson DL (1991) ANG II blocks potassium currents in zona glomerulosa cells from rat, bovine, and human adrenals. Am J Physiol 260: E772–E779

Cohen CJ, McCarthy RT, Barrett PQ, Rasmussen H (1988) Ca channels in adrenal glomerulosa cells: K^+ and angiotensin II increase T-type Ca-channel current. Proc Natl Acad Sci USA 85: 2412–2416

deVente J, Steinbusch HW (1992) On the stimulation of soluble and particulate guanylate cyclase in the rat brain and the involvement of nitric oxide as studied by cGMP immunocytochemistry. Acta Histochemica 92: 13–38

Ferguson AV, Bains JS (1996) Electrophysiology of the circumventricular organs. Front Neuroendocrinol 17: 440–475
Fitzsimons JT (1979) The physiology of thirst and sodium appetite. Cambridge University Press, Cambridge
Garthwaite J, Boulton CL (1995) Nitric oxide signaling in the central nervous system. Annu Rev Physiol 57: 683–706
Jurzak M, Müller AR, Schmid HA, Gerstberger R (1994a) Primary culture of circumventricular organs from the rat brain lamina terminalis. Brain Res 662: 198–208
Jurzak M, Schmid HA, Gerstberger R (1994b) NADPH-diaphorase staining and NO-synthase immunoreactivity in circumventricular organs of the rat. In: Pleschka K, Gerstberger R (eds) Integrative and cellular aspects of autonomic functions: temperature and osmoregulation. John Libbey Eurotext, Paris, pp 451–459
Kang J, Sumners C, Posner P (1993) Angiotensin-II type-2 receptor-modulated changes in potassium currents in cultured neurons. Am J Physiol 265: C 607–C 616
McCarthy RT, Isales C, Rasmussen (1993) HT-type calcium channels in adrenal glomerulosa cells: GTP-dependent modulation by angiotensin II. Proc Natl Acad Sci USA 90: 3260–3264
McKinley MJ, McAllen RM, Mendelsohn FAO, Allen AM, Chai SY, Oldfield BJ (1990) Circumventricular organs: neuroendocrine interfaces between the brain and the hemal milieu. Front Neuroendocrinol 11: 91–127
Nagatomo T, Inenaga K, Yamashita H (1995) Transient outward current in adult-rat supraoptic neurons with slice patch-clamp technique – inhibition by angiotensin-II. J Physiol (Lond) 485: 87–96
Nicolaidis S, Fitzsimons JT (1975) La dependance de la prise d'eau induit par l'angiotensin II envers la fonction vasomotorice cerebrale locale chez le rat. C R Acad Sci Paris 281: 1417–1420
Pehl U, Schmid HA (1997) Electrophysiological responses of neurons in the rat spinal cord to nitric oxide. Neuroscience 77: 563–573
Rauch M, Schmid HA, deVente J, Simon E (1997) Electrophysiological and immunocytochemical evidence for a cGMP-mediated inhibition of subfornical organ neurons by nitric oxide. J Neurosci 17: 363–371
Schmid HA, Simon E (1992) Effect of angiotensin II and atrial natriuretic factor on neurons in the subfornical organ of ducks and rats *in vitro*. Brain Res 588: 324–328
Schmid HA, Vijayaraghavan S (1992) Inhibition of the nicotinic acetylcholine response by serotonergic and muscarinic agents in chick ciliary ganglion neurones. Neuropharmacol 31: 1001–1008
Shapiro MS, Wollmuth LP, Hille B (1994) Angiotensin-II inhibits calcium and m-current channels in rat sympathetic neurons via g-proteins. Neuron 12: 1319–1329
Smith PM, Beninger RJ, Ferguson AV (1995) Subfornical organ stimulation elicits drinking. Brain Res Bull 38: 209–213
Stockand JD, Sansom SC (1994) Large Ca^{2+}-activated K^{+}-channels responsive to angiotensin-II in cultured human mesangial cells. Am J Physiol 36: C1080–C1086
Watkins JC, Evans RH (1981) Excitatory amino acid transmitters. Ann Rev Pharmacol Toxicol 21: 165–204
Weindl A, Bufler J, Winkler B, Arzberger T, Hatt H (1992) Neurotransmitters and receptors in the subfornical organ. Immunhistochemical and electrophysiological evidence. In: Ermisch A, Landgraf R, Rühle HJ (eds) Circumventricular organs and brain fluid environment. Elsevier, Amsterdam, pp 261–269
Yang CR, Phillips MI, Renaud LP (1992) Angiotensin II receptor activation depolarizes rat supraoptic neurons *in vitro*. Am J Physiol 263: R1333–R1338

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