JPET #76398

Effect of neocuproine, a copper (I) chelator, on rat bladder function

Cemil Göçmen, Ben Giesselman and William C. de Groat

Department of Pharmacology, School of Medicine, University of Çukurova, Adana, Turkey (C.G.); Department of Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA (B.G., W.C. de G.)

RUNNING TITLE: Neocuproine facilitates bladder activity

Corresponding Author: Cemil Göçmen

Address: University of Cukurova, School of Medicine, Department of Pharmacology, Adana, TR-01330, TURKEY

Telephone: 90-322-3386060/3240

Fax: 90-322-3386572

e-mail: cgocmen@mail.cu.edu.tr

The document statistic:

Number of text pages: 22

Number of tables: 1

Number of figures: 6

Number of references: 42

Number of words in abstract: 253

in introduction: 537

in discussion: 1004

ABBREVIATIONS: NO, nitric oxide; NANC, nonadrenergic-noncholinergic; ATP, adenosine 5'-triphosphate; EFS, electrical field stimulation; Cu, copper; ICI, intercontraction intervals; L-NOARG, L-nitroarginine; CMG, cystometrogram;

ABSTRACT

The effects of a specific copper(I)-chelator, neocuproine (NC), and a selective **c**opper(II)-chelator, cuprizone, on nonadrenergic-noncholinergic transmitter mechanisms in the rat urinary bladder were studied by measuring nerve-evoked contractions of bladder strips and voiding function under urethane anesthesia. After blocking cholinergic and adrenergic transmission with atropine and guanethidine, electrical field stimulation (EFS) induced bimodal contractions of bladder strips. An initial, transient contraction which was blocked by the purinergic antagonist, suramin, was significantly enhanced by NC (20 and 200μM applied sequentially) but not affected by cuprizone. The facilitating effect, which was blocked by suramin and reversible after washout of the drug, did not occur following administration of neocuproine-copper(I) complex (NC-Cu). NC (20μM) significantly increased the second, more sustained contraction; whereas 200μM decreased this response. These effects of NC on the sustained contractions were not elicited by NC-Cu and not blocked by suramin. Nitric oxide synthase inhibitor, L-nitroarginine, did not alter the responses to NC. NC (20μM) elicited a marked increase in basal tone of the strips. This effect was less prominent after the second application of 200μM NC or with NC-Cu treatment or in the presence of suramin. In anesthetized rats, during continous infusion cystometry, intravesical infusion of 50μM NC but not NC-Cu or cuprizone, significantly decreased the intercontraction interval (ICI) without changing contraction amplitude. The ICI returned to normal after washout of NC. Suramin blocked this effect. These results indicate that NC enhances bladder activity by facilitating purinergic excitatory responses and that copper(I)-sensitive mechanisms tonically inhibit purinergic transmission in the bladder.

Introduction

Adenosine 5-triphosphate (ATP) and nitric oxide have been identified as nonadrenergic-noncholinergic (NANC) transmitters in the lower urinary tract (Fujii et al., 1988; Hoyle et al., 1989; de Groat and Yoshimura, 2001; Burnstock, 2002; Benko et al., 2003). Both substances can be released from NANC nerves as well as urothelial cells and may play a role in efferent as well as afferent mechanisms. ATP released from parasympathetic postganglionic nerves can activate postjunctional P2X receptors to elicit atropine resistant contractions of the bladder smooth muscle (Hoyle et al., 1989; Ruggieri et al., 1990; Acevedo et al., 1992, Burnstock, 2002). On the other hand ATP released from urothelial cells can act on $P2X_3$ or $P2X_{2/3}$ excitatory receptors on subepithelial afferent nerves or on urothelial purinergic receptors to modulate sensory mechanisms in the bladder (Namasivayam et al., 1999; de Groat and Yoshimura 2001; Vlaskovska et al., 2001; Rong et al., 2002; Andersson, 2002; Birder et al., 2003). Intravesical administration of ATP induces a facilitation of voiding, which is suppressed by P2X receptor antagonists (Andersson, 2002); whereas $P2X_3$ receptor null mice exhibit hypoactive voiding suggesting that purinergic excitatory mechanisms are essential for normal bladder function (Cockayne et al., 2000).

Nitric oxide (NO) released from afferent nerves or from the urothelium (Birder et al., 1997, 2001) may be involved in inhibitory modulation of sensory mechanisms in the bladder. Intravesical administation of NO donors suppresses detrusor hyperactivity (Ozawa et al., 1999); whereas intravesical administration of oxyhemoglobin, an NO scavenger, stimulates bladder activity (Pandita et al., 2000). Patch clamp studies revealed that NO donors can suppress voltage gated Ca+2 channels in bladder sensory neurons (Yoshimura et al., 2001).

The present study which focused on the effect of copper chelators on bladder function was prompted by recent reports that copper dependent mechanisms can modulate and/or mediate the actions of ATP or NO. For example, copper ions can alter the function of $P2X_4$

purinergic receptors expressed in Xenopus oocytes (Xiong et al., 1999, Acuno-Castillo et al., 2000), raising the possibility that other types of P2X receptors which are expressed in the bladder may also be sensitive to copper ions. Some of the effects of NO in the bladder may also be dependent on copper ions as suggested for nitrergic transmission at other sites in the urogenital system such as the mouse corpus cavernosum (Gocmen et al., 1997, 1998, 2000) and sheep urethra (Garcia-Pascual et al., 2000). In these tissues it has been speculated that the nitrergic transmitter may not be free NO but rather a superoxide-resistant, nitric oxide carrying molecule, such as an S-nitrosothiol. A copper (I)-dependent mechanism has been implicated in the effect of S- nitrosothiols (De Man et al., 1999, 2001; Gocmen et al., 2000).

To investigate the contribution of copper-dependent mechanisms in modulation of purinergic and nitrergic mechanisms in the rat bladder, we studied the effects of a selective Cu(I) chelator, neocuproine (2,9-dimethyl-1,10-phenanthroline) (De Man et al., 1999; Gocmen et al., 2000), and a selective Cu(II) chelator, cuprizone (De Man et al., 1999), on neurally evoked contractions of bladder strips and on voiding function in urethane anesthetized rats. The results indicate that Cu(I) mechanisms tonically suppress purinergic excitatory transmission in the bladder. Preliminary observations have been reported in an abstract (Gocmen et al., 2003).

Materials and Methods

Animals. Female Sprague Dawley rats (200-250 g) were used in this study. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

In Vitro Studies. Halothane anesthetized rats were sacrificed by decapitation. The bladders were harvested, and bladder strips (5 x 1 mm, longitudinal **s**ections) were prepared from the mid portion of bladder body. Strips were subsequently mounted in 5 mL, jacketed organ baths containing Krebs solution (composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.25, $MgSO_4$

1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8 and dextrose 10) maintained at 37^oC and bubbled with a mixture of 95% O_2 and 5% CO_2 (pH:7.4). Tissues were allowed to equilibrate for 1 h, during which the preparation was washed with fresh Krebs solution at 15 min intervals. The responses were recorded with isotonic transducers (Hugo Sachs, B40 type 373). Data were recorded and stored using data acquisition software (Windaq, DATAQ Instruments Inc., Akron, OH). After the equlibrium period, neurally-evoked isotonic contractions were induced using trains of electrical field stimulation (EFS, 10 Hz, 50 V, 0.12 ms duration, 10 s trains) delivered from a Grass S88 stimulator (Astromed) at 2 min intervals through platinum electrodes positioned on the top and the bottom of the organ bath. In experiments in which electrical field stimulation was used, atropine (5 μM) and guanethidine (2 μM) were always present in the bathing medium to block adrenergic and cholinergic transmission. EFS induced bimodal contractions of bladder strips. In control experiments, EFS evoked responses were recorded for a 3 h time period (no drug was added) to examine the stability of the nerve-evoked responses. In other experiments after the control responses to EFS were recorded for approximately 1 hour, neocuproine, a selective Cu(I) chelator, was applied at two concentrations (either 5 and 10μM or 20 and 200 μM) to the same tissue consecutively at intervals ranging from 20 to 25 min. In some experiments, the effects of various agents including, pre-prepared neocuproine-copper (I) complex, a nitric oxide synthase inibitor L-nitroarginine (L-NOARG; 0.5 mM), a selective Cu(II) chelator cuprizone (10 or 20 μM), or a purinergic antagonist suramin (100 μM) were examined on bladder contractions induced by electrical field stimulation in the presence or absence of neocuproine. The neocuproine–copper (I) complex was prepared by reacting glutathione, neocuproine and $CuSO_4$ in molar ratios of 1:2:1, respectively (Dicks et al., 1996). In another series of experiments we studied the effect of exogenous ATP on basal tone of bladder strips. ATP (10, 50 or 100 μM) was applied four times to the same tissue consecutively at intervals ranging from 10 to 15 min.

Exposure time of ATP was 60 s for each application. In some experiments either ATP (10 or 100 μM) or neocuproine (5 or 10 μM) was applied to the bladder strips after prolonged (30 min) ATP treatment (10 or 100 μM) to examine the effects on the EFS-evoked contractions or basal tone in preparations in which the purinergic receptors were desensitized (Driessen et al., 1993; Sahin et al., 2000).

Cystometrogram Recording. Animals were anesthetized with subcutaneous injection of urethane (1.2 gm./kg,: Sigma Chemical Co., St. Louis, MI). The urinary bladder was exposed via a midline abdominal incision and was catheterized. A catheter (PE-50), the bladder end of which was heated to create a collar, was inserted through a small incision in the bladder dome, and a suture was tightened around the collar. The other end was connected via a T-stopcock to a pump for continuous infusion of physiological saline and to a pressure transducer to record bladder pressure. Physiological saline was infused at room temperature into the bladder at a constant rate of 0.04 ml/min to elicit repeated voiding responses. In all experiments, control cystometrograms (CMG) were recorded for about 2 h prior to intravesical drug administration. The parameters evaluated were amplitude of bladder contractions and intercontraction interval (ICI), which is defined as the time between two voiding cycles. Neocuproine (25-200 μM), pre-prepared neocuproine-copper (I) complex, L-NOARG (0.5 mM), cuprizone (20 μM) or suramin (100 μM) were administrated in saline into the bladder for 1 h. CMG parameters were monitored for 1 h and compared with the recordings before drug application.

Drugs. Stock solutions of atropine sulphate; guanethidine; 2,9-dimethyl-1,10-phenanthroline (neocuproine); L-nitroarginine; suramin; adenosine 5-triphosphate (ATP); $CuSO_4$ and glutathione were prepared in distilled water. Cuprizone was dissolved in 50% ethyl alcohol (final concentration in the bath medium was 0.4%). All drugs were administered in saline solutions. Drugs were obtained from Sigma Chemical Co., St. Louis, MI).

Statistical Analysis. All data are expressed as mean ± SE. Data was analyzed by one-way ANOVA followed by Student's paired t-test. A p-value of less than 0.05 is considered significant.

Results

Effect of neocuproine on the EFS induced biphasic contractions of rat urinary bladder strips. After blocking cholinergic and adrenergic responses with atropine and guanethidine EFS (10 Hz, 50 V, 0.12 ms duration) evoked bimodal contractions of bladder smooth muscle strips (Fig.1) consisting of an initial large amplitude transient response followed by a lower amplitude more sustained response which persisted following the termination of the stimulus. Both responses were blocked by tetrodotoxin (1.5 μM). The amplitude of the EFS evoked contractions was constant for a 3 h time period (3.21 ± 0.32 mN at the beginning of the experiment and 3.01 ± 0.42 mN at the end of the experiment, n=4). The initial contraction to EFS was significantly enhanced by two concentrations of neocuproine (3.04 ± 0.33 mN for control and 5.81 ± 0.47 mN at 20 μM; 4.20 ± 0.23 mN for control and 8.85 ± 0.86 mN at 200 μM; Fig.1 and 2 A and B) but not affected by lower concentrations of neocuproine (5 μM and 10μM) or by 10 μM and 20 μM cuprizone or its solvent (4.8 ± 0.9 mN for control; 5.23 ± 0.7 mN for solvent; 5.65 ± 0.95 mN for 10 μM cuprizone and 5.97 ±0.89 mN for 20 μM cuprizone; n=18-20). Neocuproine (20 μM), applied at the end of experiments after cuprizone or its solvent, significantly enhanced the contractile responses (9.23 ± 1.2 mN for 20 μM neocuproine, n=12). The repeated application of 20 μM neocuproine produced a reproducible effect on the initial contraction (3.87 ± 0.52 mN for control and 7.56 ± 0.73 mN at the first application; 3.21 ± 0.33 mN for control and 5.97 ± 0.43 mN at the second application, n=4). This facilitating effect of neocuproine which was reversible in 15-20 min after washout of the drug, did not occur following the application of the pre-prepared neocuproine-copper (I) complex (Fig.2 A and B) and was blocked by the purinergic antagonist, suramin (100 μM,

Fig. 3). The NOS inhibitor, L-NOARG (0.5 mM) did not affect the control contractions or the responses to neocuproine (initial control contraction, 2.3 ± 0.8 mN; 5.6 ± 0.7 mN for 0.5 mM L-NOARG + 20 μM neocuproine; 6.7 ± 1.1 mN for 0.5 mM L-NOARG + 200 μM neocuproine; n=4-6).

The sustained contraction was also affected by neocuproine. The high concentration of neocuproine (200 μM) significantly decreased (33.2 ± 6.3 %) the sustained component of contraction; whereas the low concentration enhanced (242 ± 21.9 %) the sustained component as well as the initial contraction but did not alter the shape of bimodal response (Fig.1). Also the repeated application of 20 μM neocuproine did not affect the the shape of bimodal response (not shown). These effects of the two concentrations of neocuproine on the sustained contraction did not occur following the administration of neocuproine-copper (I) complex (Fig 2 B). Suramin (100 μM) inhibited the facilitatory effect of 20 μM neocuproine on the sustained component, whereas it failed to alter the depressant effect of the higher concentration of neocuproine (Fig 3).

The first application of neocuproine (20 μM) caused a marked increase in the basal tone of the strips (Table 1) which persisted for 22.4 ± 4.2 min (n= 24). A second application of the higher concentration of the drug (200 μM) elicited a considerally smaller effect (Table 1). Also, the enhancement of basal tone by 20 μM neocuproine was significantly less after repeated application of this agent at the same concentration (16.23 ± 5.7 % of the enhancement due to first application; n=4). Neocuproine (200 μM) also induced small amplitude (<1 mN) spontaneous contractions in some strips. Neocuproine-copper (I) complex did not elicit these responses. Pretreatment with suramin (100 μM) prevented the effects of neocuproine (20 μM) (Table 1). Application of ATP (10-100 μM) elicited an increase in basal tone in a concentration-dependent manner (2.43 ± 0.41 mN at 10 μM; 4.36 ± 0.53 mN at 50 μM; 5.30 ± 1.01 at 100 μM). Repeated application of the same concentration of ATP at 15

min intervals produced a significant desensitization, eg., the response to 100 μM ATP declined to 27 ± 5.4% of the control response. Prolonged exposure (30 min) to ATP (10 or 100 μM) also suppressed the EFS-induced contraction in a dose dependent manner (to 40.2 ± 6.9 % of control at 10 μM and to 24.6 ± 4.8 % of control at 100 μM). Neocuproine (5 or 10 μM), which alone had no effect, enhanced the suppression of contractile responses after 10 μM ATP (to 30.8 ± 4.3 % of control at 5 μM and to 24.2 ± 6.7 % of control at 10 μM) or 100 μM ATP (to 19.3 ± 5.6 % of control at 5 μM and to 12.1 ± 7.3 % of control at 10 μM) Neocuproine-copper (I) complex, $CuSO_4$, glutathione or $CuSO_4$+glutathione did not elicit significant changes in basal tone or the response to ATP**.**

Effect of neocuproine on CMG parameters in anesthetized rats

During continuous infusion cystometry under urethane anesthesia, intravesical administration of neocuproine at a concentration of 50 μM significantly decreased the ICI (Fig. 4 and 5A) but did not change the amplitude of bladder contractions. The decrease in the ICI was maintained for 30-40 min during the continuous infusion of neocuproine but returned to normal within 50-60 min after washout of the drug. Repeated administration of neocuproine (50 μM) 30 min after the first application produced a similar effect. Pre-prepared neocuproine-copper (I) complex (Fig. 4 and 5A) did not alter the ICI. Suramin (100 μM) infused with neocuproine application significantly reduced but did not completely block the effect of neocuproine ($p<0.05$, Fig. 5B). However, pretreatment with suramin completely blocked the effect of neocuproine on the ICI (Fig.6). Suramin alone did not alter the ICI. The CMG and/or the effect of neocuproine on the CMG was not affected by infusion of 0.5 mM L-NOARG (ICI, 510 ± 42 sec for control; 567 ± 53 sec for 0.5 mM L-NOARG ; 274 ± 16 sec for 0.5 mM L-NOARG + 50 μM neocuproine) or 20 μM cuprizone (ICI, 523 ± 64 sec for control; 558 ± 51 sec for 20 μM cuprizone). Also, $CuSO_4$, glutathione or $CuSO_4$+glutathione did not alter the ICI.

Discussion

The present experiments revealed that a copper (I) chelator, neocuproine decreased the voiding interval during continuous cystometry in anesthetized rats without altering the amplitude of voiding contractions. This agent also enhanced the electrical field stimulation-induced nonadrenergic-noncholinergic contractions of rat bladder strips. These effects were blocked by a purinergic receptor antagonist, suramin, and did not occur following the administration of an neocuproine-copper (I) complex in which neocuproine was saturated with excess copper ions. These findings suggest that neocuproine can stimulate bladder activity by facilitating purinergic excitatory responses and that copper(I)-sensitive mechanisms play a role in this effect

Neocuproine could modulate purinergic mechanisms at several sites in the bladder including postjunctional purinergic receptors in the smooth muscle, as well as purinergic receptors in afferent nerves and urothelial cells (Namasivayam et al., 1999; de Groat and Yoshimura 2001; Vlaskovska et al., 2001; Rong et al., 2002; Andersson, 2002; Birder et al., 2003, 2004). The initial component of the neurally-evoked contractions of detrusor smooth muscle which persisted in the presence of atropine and guanethidine was suppressed by suramin, a P2X purinergic receptor antagonist as reported by other investigators (Calvert et al., 2001; Benko et al., 2003; Hoyle et al., 1990, Burnstock, 2002). Neocuproine significantly enhanced this initial component and suramin suppressed this effect of neocuproine. The first application of neocuproine also elicited an increase in basal tone of the smooth muscle strips an effect that was suppressed by suramin. These data suggest that neocuproine enhances the postjunctional effect of ATP or enhances the release of ATP from nerves. The finding that the second application of neocuproine (20 or 200 μM) did not cause an enhancement of the basal tone supports this speculation; because, repeated ATP applications elicited a desensitizing effect on the contractile responses (Husted and Nedergaard, 1985) and suppressed EFS-

induced contractions (Sahin et al., 2000). Thus repeated application of neocuproine can induce a desensitizing response similar to that induced by ATP (Namasivayam, 1999). Also, neocuproine application after ATP treatment, enhanced the suppression of the contractile responses to EFS. These results support the data obtained from suramin experiments that neocuproine enhances purinergic mechanisms and when purinergic receptors are desensitized, the neocuproine facilitatory effect is reduced. Further studies are necessary to determine if this effect is due to prejunctional or postjunctional mechanisms.

In addition to purinergic transmission, peptidergic transmission involving substance P or other neurokinins may be involved in nonadrenergic-noncholinergic transmission in the bladder and contribute to the sustained component of the EFS evoked contraction (Benko et al., 2003). The low concentration of neocuproine enhanced the sustained part of the EFS evoked bimodal contraction, whereas the high concentration decreased the contraction. This differential effect of neocuproine at two concentrations was not blocked by suramin raising the possibility that neocuproine influences peptidergic mechanisms in the bladder.

In our study, intravesical application of neocuproine decreased the ICI during continuous cystometry in anesthetised rats and suramin reversed this effect. ATP can be released by the urothelium and $P2X_3$ receptors seem to be critical to the normal physiologic regulation of afferent pathways controlling volume-induced reflexes in the urinary bladder (Namasivayam et al., 1999; Cockayne et al., 2000; Pandita and Andersson, 2002; Vlaskovska et al., 2001; Andersson, 2002). ATP given intravesically induced a pronounced bladder overactivity in unanesthetized rats via action on P2X receptors located on afferent nerve endings in the urothelium and P2X receptors antagonists counteracted this effect (Hoyle et al., 1990; Chen et al., 1995; Namasivayam et al., 1999; Andersson, 2002). Neocuproine may enhance ATP release from urothelium or the actions of ATP on afferent nerves and thereby

induce bladder overactivity and a decrease in the ICI. The finding that suramin prevented this effect of neocuproine supports this possibility.

Pre-prepared neocuproine-Cu complex did not mimick the facilitating effect of neocuproine on EFS-induced contractions and on basal tone of bladder strips or elicit the decrease in the ICI caused by intravesically infused neocuproine. Cuprisone, a copper (II) chelator also did not mimick the effects of neocuproine. These results indicate that a copper (I)-sensitive mechanism has a role in the facilitating effect of neocuproine on bladder activity. It has been suggested that copper may modulate the function of another subtype of purinergic receptor, $P2X_4$ in Xenopus oocytes (Xiong et al., 1999, Acuno-Castillo et al., 2000). The present study indicates that a copper sensitive mechanism may play a role in the modulation of purinergic efferent and afferent pathways in rat bladder.

The present experiments also examined the possibility that neocuproine might influence NO functions in the bladder. It has been suggested that NO can be released from urothelium and it can affect the excitability of adjacent afferent nerves (Andersson and Persson, 1995; Ozawa et al.,1999; Yoshimura et al., 2001; Birder et al., 2002). However, the nitric oxide synthase inhibitor L-NOARG (Bennett et al., 1995) did not affect the responses elicited by intravesical application of neocuproine in vivo or the effect of neocuproine on bladder strips in vitro. This finding indicates that an NO pathway does not play a role in the facilitating effect of neocuproine. Previous studies indicated that NO was involved in the actions of other ions (nickel and manganese) on neurally evoked bladder contractions (Liu & Lin-Shiau, 1997; 1998; Sahin et al., 2000). We considered the possibility that NO released in the bladder is not free NO but a sulfhydryl-containing compound, such as an S-nitrosothiol (Thornbury et al., 1992; Garcia-Pascual et al., 2000), and that neocuproine could affect the responses of S-nitrosothiols by its ability to chelate copper (De Man et al., 1999; Gocmen et

al., 2000). However, this mechanism seems unlikely because the NOS inhibitor, L-NOARG, did not alter the effect of neocuproine on contractile responses to EFS.

In conclusion, our experiments with selective copper chelating agents suggest that neocuproine enhances bladder activity, presumably by facilitating purinergic excitatory responses and that copper(I)-sensitive mechanisms can modulate purinergic transmission in the bladder. NO doesn't mediate the effect of neocuproine on bladder activity. Further studies are needed to determine the mechanisms that underlie the interactions between copper ions and purinergic excitatory responses in the bladder.

References

Acevedo CG, Contreras E, Escalona J, Lewin J and Huidobro-Toro JP (1992) Pharmacological characterization of adenosine A1 and A2 receptors in the bladder: evidence for a modulatory adenosine tone regulating nonadrenergic noncholinergic neurotransmission. *Br J Pharmacol* **107:**120-126.

Acuna-Castillo C, Morales B and Huidobro-Toro JP (2000) Zinc and copper modulate differentially the P2X4 receptor. *J Neurochem* **74:**1529-1537.

Andersson KE (2002) Bladder activation: afferent mechanisms. *Urol* **59:**43-50.

Andersson KE and Persson K (1995) Nitric oxide synthase and the lower urinary tract: possible implications **of** physiology and pathophysiology. *Scand J Urol Nephrol Supply* **175:**43-53.

Benko R, Lazar Z, Porszasz R, Somogyi GT and Bartho L (2003) Effect of experimental diabetes on cholinergic, purinergic and peptidergic motor responses of the isolated rat bladder to electrical field stimulation or capsaicin. *Eur J Pharmacol* **478:**73-80.

Bennett BC, Kruse MN, Roppolo JR, Flood HD, Fraser M and de Groat WC (1995) Neural control of urethral outlet activity in vivo: role of nitric oxide. *J Urol* **153:**2004-2009.

Birder LA, Barrick SR, Roppolo JR, Kanai AJ, de Groat WC, Kiss S and Buffington CA (2003) Feline interstitial cystitis results in mechanical hypersensitivity and altered ATP release from bladder urothelium. *Am J Physiol* **285:** F423-F429.

Birder LA, Kanai AJ and de Groat WC (1997) DMSO: effect on bladder afferent neurons and nitric oxide release. *J Urol* **158:** 1989-1995.

Birder LA, Kanai AJ, de Groat WC, Kiss S, Nealen ML, Burke NE, Dineley KE, Watkins S, Reynolds IJ and Caterina MJ (2001) Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells. *Proc Natl Acad Sci USA* **98:** 13396-13401.

Birder LA, Nealen ML, Kiss S, de Groat WC, Caterina MJ, Wang E, Apodaca G and Kanai AJ (2002) β-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. *J Neurosci* **22:** 8063-8070.

Burnstock G (2002) Purinergic signaling in the lower urinary tract. IN; Abbracchio, MP and Williams M (eds): Handbook of Experimental Pharmacology, Springer Verlag, p. 423

Calvert RC, Thompson CS, Khan MA, Mikhailidis DP, Morgan RJ and Burnstock G (2001) Alterations in cholinergic and purinergic signaling in a model of the obstructed bladder. *Urol* **166:**1530-1533.

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G and Wood JN (1995) A P2X receptor expressed by a subset of sensory neurons. *Nature* **377:** 428-431.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB and Ford AP (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* **407:**1011-1015.

de Groat WC and Yoshimura N (2001) Pharmacology of the lower urinary tract. *Annu Rev Pharmacol Toxicol* **41:**691-721.

De Man JG, Moreels TG, De Winter BY, Herman AG and Pelckmans PA (1999) Neocuproine potentiates the activity of the nitrergic neurotransmitter but inhibits that of S-nitrosothiols. *Eur J Pharmacol* **381:** 151-159.

De Man JG, Moreels TG, De Winter BY, Herman AG and Pelckmans PA (2001) Pre- and postjunctional protective effect of neocuproine on the nitrergic neurotransmitter in the mouse gastric fundus. *Br J Pharmacol* **132:** 277-285.

Dicks AP, Swift HR, Williams DLH, Butler AR, Al-Sa'Doni HH and Cox BG (1996) Identification of Cu^{+} as the effective reagent in nitric oxide formation from S-nitrosothiols (RSNO). *J Chem Soc Perkin Trans* **2:** 481-487.

Driessen B, Bultmann R, von Kugelgen I and Starke K (1993) Effect of opioid receptor subtype-selective agonists on purinergic and adrenergic components of neurogenic contractions of mouse vas deferens. *Br Journal Pharmacol* **108:** 443-447.

Fujii K (1988) Evidence for adenosine triphosphate as an excitatory transmitter in guinea pig, rabbit and pig urinary bladder. *J Physiol* **404:** 39-52.

Garcia-Pascual A, Labadia A, Costa G and Triguero D (2000) Effects of superoxide anion generators and thiol modulators on nitrergic transmission and relaxation to exogenous nitric oxide in the sheep urethra. *Br J Pharmacol* **129:**53-62.

Gocmen C, Giesselman B and de Groat WC (2003) Effect of neocuproine, a copper (I) chelator on rat bladder function. Soc Neuroscience Abstracts 33rd meeeting, New Orleans, USA.

Gocmen C, Gokturk HS, Ertug PU, Onder S, Dikmen A and Baysal F (2000) Effect of neocuproine, a selective Cu(I) chelator, on nitrergic relaxations in the mouse corpus cavernosum. *Eur J Pharmacol* **406:**293-300.

Gocmen C, Uçar P, Şingirik E, Dikmen A and Baysal F (1997) An in vitro study of nonadrenergic – noncholinergic activity on the nitrergic relaxations in the mouse corpus cavernosum. *Urol Res* **25:** 269-275.

Gocmen C, Seçilmiş A, Uçar P, Karataş Y, Önder S, Dikmen A and Baysal F (1998) A possible role of S-nitrosothiols at the nitrergic relaxations in the mouse corpus cavernosum. *Eur J Pharmacol* **361:** 85-92.

Hoyle CHV, Chapple C and Burnstock G (1989) Isolated human bladder evidence for an adenine dinucleotide acting on P2X purinoceptors and for purinergic transmission. *Eur J Pharmacol* **174:** 115-118.

Hoyle CHV, Knight GE and Burnstock G (1990) Suramin antagonizes responses to P2-purinoceptors agonists and purinergic nerve stimulation in the guinea pig urinary bladder and taenia coli. *Br J Pharmacol* **99:** 617-621.

Husted SE and Nedergaard OA (1985) Dual inhibitory action of ATP on adrenergic neuroeffector transmission in rabbit pulmonary artery. *Acta Pharmacol Toxicol* **57:** 204-213.

Liu SH and Lin-Shiau SY (1997) Enhancement by nitric oxide of neurogenic contraction in the mouse urinary bladder. *Naunyn Schmiedebergs Arch Pharmacol* **356:** 850-852.

Liu SH and Lin-Shiau SY (1998) Involvement of nitric oxide in the potentiating of neurogenic contraction by manganese and nickel ions in mouse urinary bladder. *Naunyn Schmiedebergs Arch Pharmacol* **358:**678-681.

Namasivayam S, Eardley I and Morrison JFB (1999) Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat. *BJU International* **84:**854-860.

Ozawa H, Chancellor NB, Jung SY, Yokoyama T, Fraser MO, Yu Y, de Groat WC and Yoshimura N (1999) Effect of intravesical nitric oxide therapy on cyclophosphamide induced cystitis. *J Urol* **162:**191-202.

Pandita RK and Andersson KE (2002) Intravesical adenosine triphosphate stimulates the micturition reflex in awake, freely moving rats. *J Urol* **168:** 1230-1234 .

Pandita RK, Mizusawa H and Andersson KE (2000) Intravesical oxyhemoglobin initiates bladder overactivity in conscious normal rats. *J Urol* **164:** 545-550.

Rong W, Spyer KM and Burnstock G (2002) Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. *J Physiol* ***(Lond.)*** **541:** 591-600.

Ruggieri MR, Whitmore KE and Levin RM (1990) Bladder purinergic receptors. *J Urol* **144:** 176-181.

Sahin AS, Atalik KE and Dogan N (2000) Nonadrenergic noncholinergic responses of the rabbit detrusor smooth muscle and the role of L-arginine/nitric oxide pathway in these responses. *Methods Find Exp Clin Pharmacol* **22:**31-35.

Thornbury KD, Hollywood MA and McHale NG (1992) Mediation by nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in sheep. *J Physiol (Lond.)* **451:** 133-144.

Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford AP and Burnstock G (2001) P2X3 knock-out mice reveal a major sensory role for urothelially released ATP. *J Neuroscience* **21:**5670-5677.

Xiong K, Peoples RW, Montgomery JP, Chiang Y, Stewart RR, Weight FF and Li C (1999) Differential modulation by copper and zinc of P2X2 and P2X4 receptor function. *J Neurophysiol* **81:**2088-2094.

Yoshimura N, Seki S and de Groat WC (2001) Nitric oxide modulates Ca(2+) channels in dorsal root ganglion neurons innervating rat urinary bladder. *J Neurophysiol* **86:**304-311.

FOOTNOTES: This work is supported by NIH grant DK49430.

Legends

Figure 1. Represantative tracings showing the effects of neocuproine (NC, 20 and 200 µM) and neocuproine-copper(I) complex (NC-Cu) on the nerve-evoked bladder strip contractions induced by electrical field stimulation (10 Hz, 50 V, 0.12 ms).

Figure 2. Effects of 20 µM (A) and 200 µM (B) neocuproine (NC) and neocuproine-copper(I) complex (NC-Cu) on the nerve-evoked bladder strip contractions induced by electrical field stimulation (10 Hz, 50 V, 0.12 ms). The bars marked with hatched and cross-hatched bars represent the first and the second phasic responses, respectively. * P‹0.05, *** P‹0.001, significantly different from control (n=6-8).

Figure 3. Effects of 20 µM or 200 µM neocuproine (NC) in presence or absence of suramin (SUR; 100 µM) on the nerve-evoked bladder strip contractions induced by electrical field stimulation (10 Hz, 50 V, 0.12 ms). The bars marked with hatched and cross-hatched bars represent the first and the second phasic responses respectively. *** P‹0.001, significantly different from control (n=6-8).

Figure 4. Continuous cystometrogram tracing during control (A), neocuproine (50 µM) (B and D), washout after first application of neocuproine (C) and neocuproine-copper (I) complex (E).

Figure 5. Effects on the intercontraction interval (ICI) during continuous infusion cystometrograms of neocuproine (NC, 50 µM) or neocuproine copper(I) complex (NC-Cu) **(A)** or and neocuproine (50 µM) plus suramin (100 µM) (NC+SUR) **(B)** on ICI . * P‹0.05, ** P‹0.01, *** P‹0.001, significantly different from control (C); + P‹0.05, significantly different from the second application of NC (n=5-7). w washout.

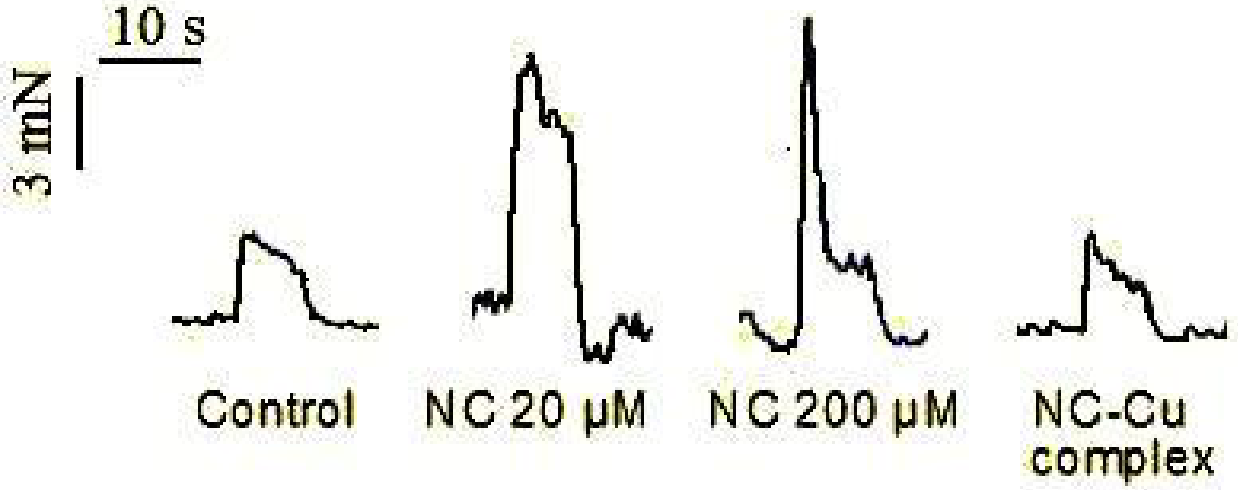
Figure 6. Effects of suramin (SUR; 100 µM, intravesically) or suramin (100 µM) pretreatment plus neocuproine (50 µM, intravesically) (SUR+NC) on the intercontraction interval (ICI) during continuous infusion cystometrograms. (n=5-7). w washout.

Table 1. Effects of neocuproine (NC), neocuproine-copper (I) complex (NC-Cu) or neocuproine+suramin (NC-SUR) on basal tone of rat detrusor smooth muscle. NC was applied at two concentrations (20 and 200 µM) into the same tissue consecutively.The other treatments were applied one time to the different tissues to determine their efects on basal tone. The results are presented as the increase (mN) in basal tone. * indicates a significant difference between the effects of 20 µM neocuproine and the effects of other treatments.

NC (20 µM)	NC (200 µM)	NC-Cu	NC-SUR
23.4 ± 5.2	4.9 ± 2.5*	2.6 ± 1.1*	4.5 ± 1.7*

* P‹0.05.

Figure 1



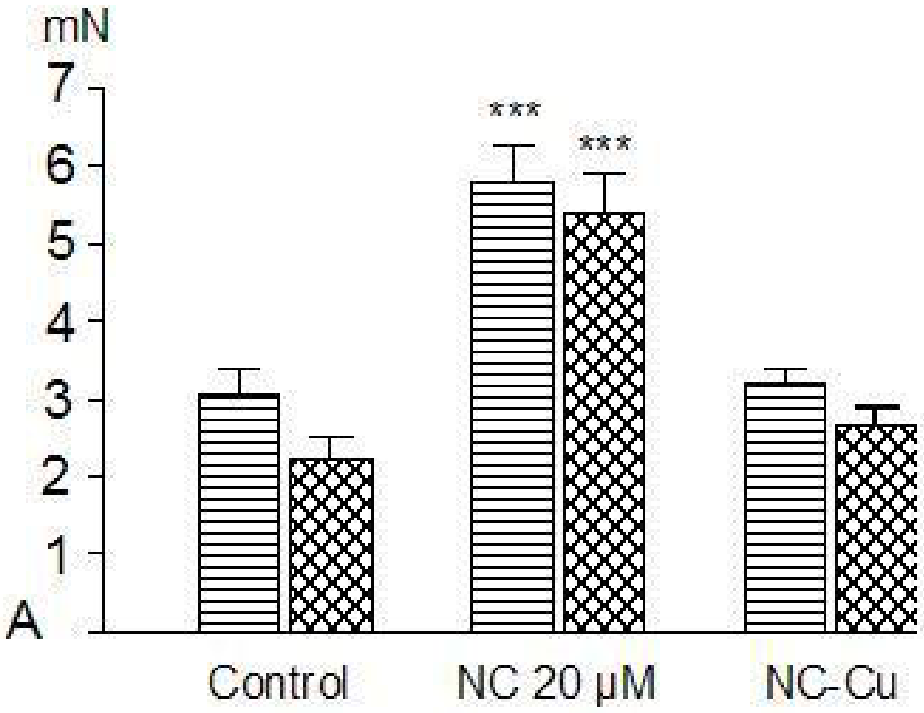
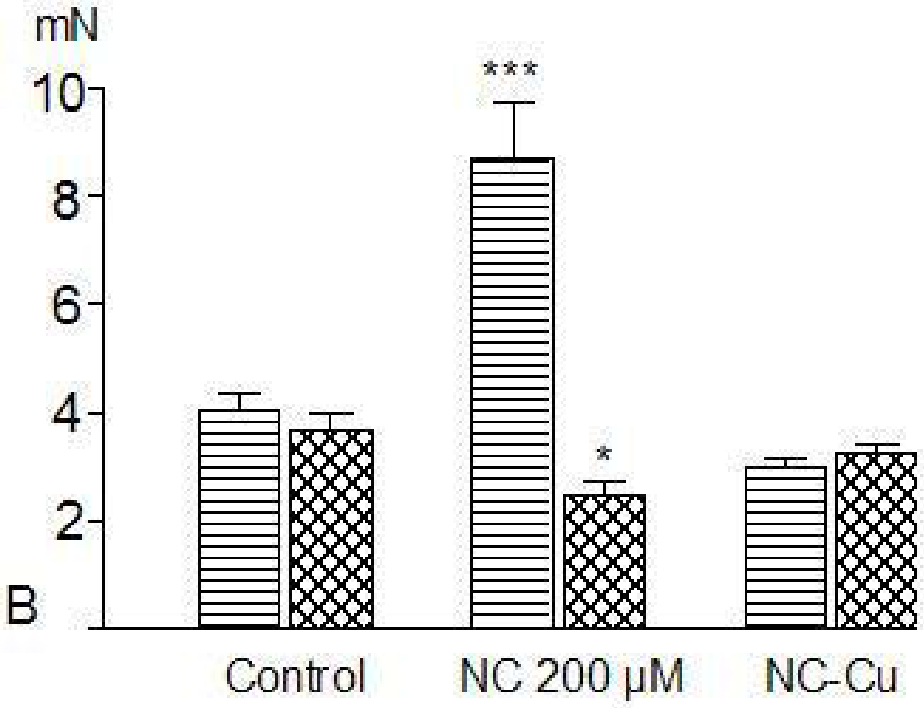

mN
7
6
5
4
3
2
1
A
***
***
Control
NC 20 µM
NC-Cu

Figure 2


mN
10
8
6
4
2
B
***
*
Control
NC 200 µM
NC-Cu

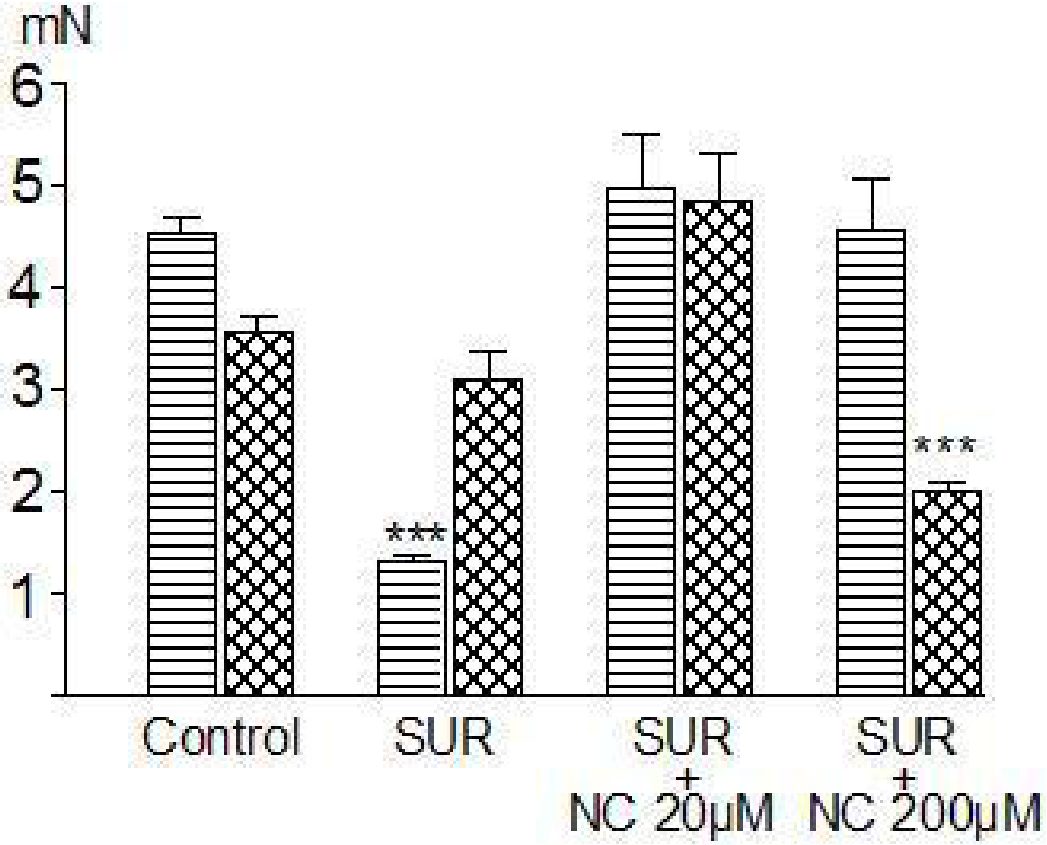


Figure 3

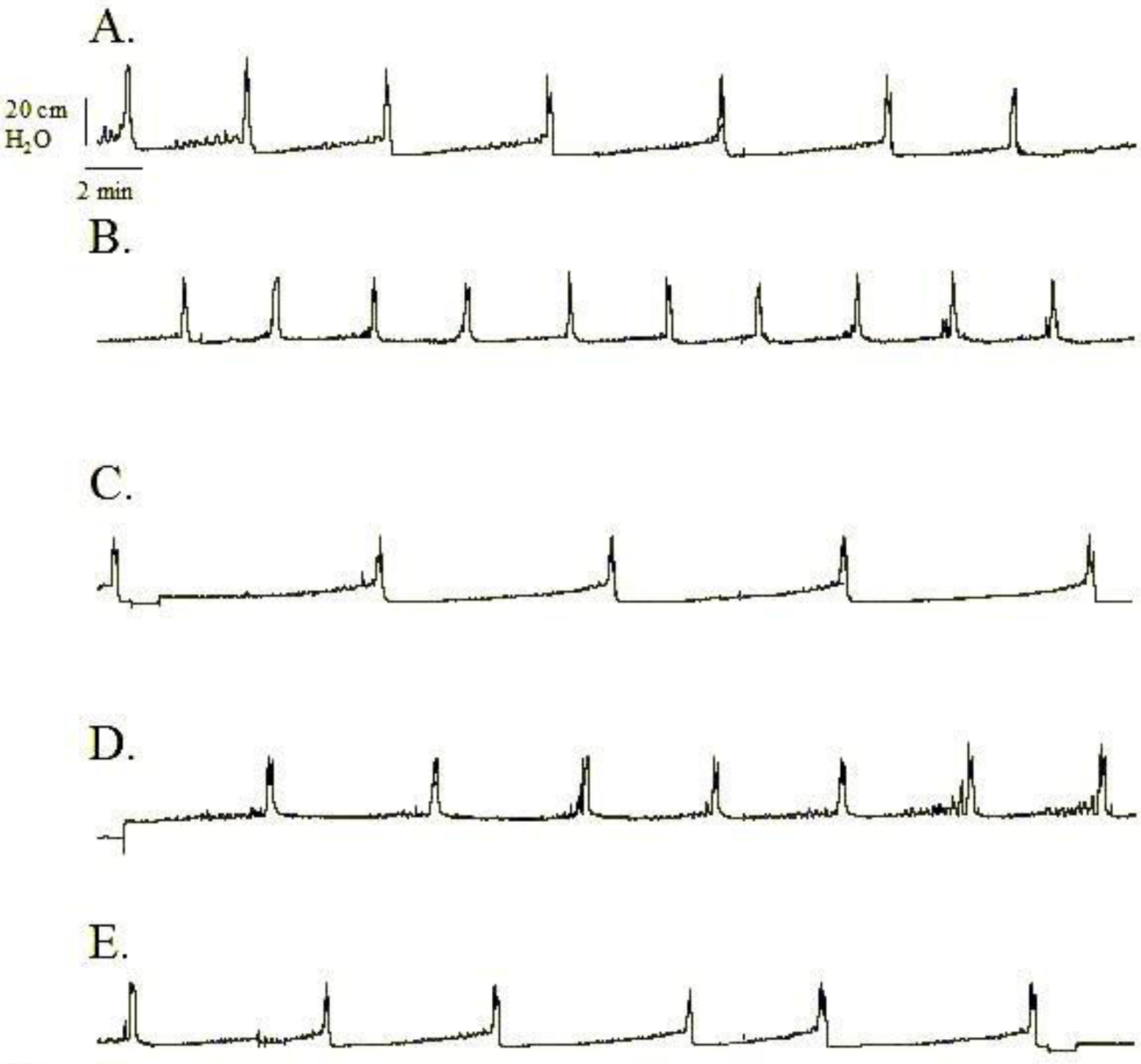
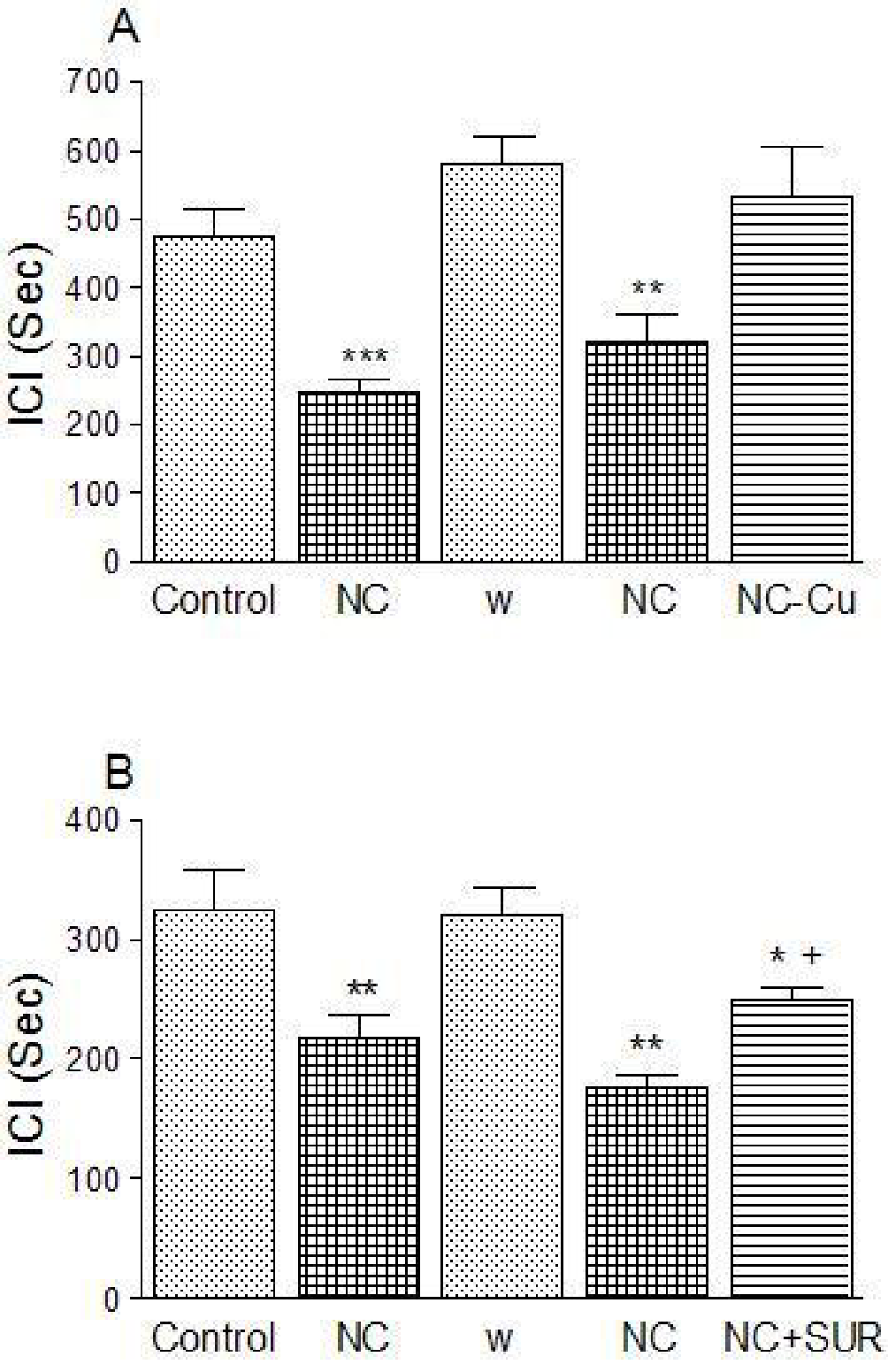


Figure 4

Figure 5


A
ICI (Sec)
700
600
500
400
300
200
100
0
***
**
Control
NC
w
NC
NC-Cu
B
ICI (Sec)
400
300
200
100
0
**
**
* +
Control
NC
w
NC
NC+SUR

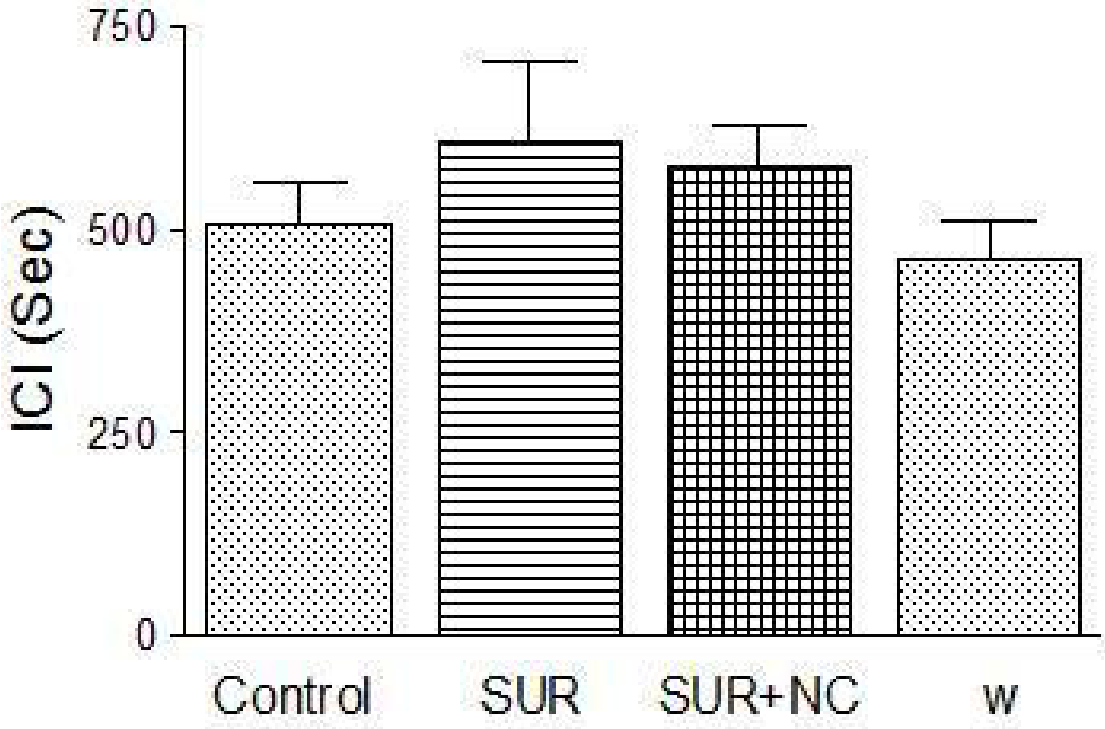


Figure 6