Inhibitory transmitter action of calcitonin gene-related peptide in guinea-pig ureter via activation of glibenclamide-sensitive K channels

¹Paolo Santicioli & Carlo Alberto Maggi

Pharmacology Department, A. Menarini Pharmaceuticals, Via Sette Santi 3, 50131, Florence, Italy

1 In single sucrose gap, electrical field stimulation (EFS, 1–5 Hz) produced graded hyperpolarization of the membrane of the guinea-pig ureter smooth muscle, which was blocked by tetrodotoxin (0.3 μ M) or *in vitro* capsaicin desensitization (3 μ M for 15 min). Capsaicin itself produced a transient hyperpolarization of the membrane on its first application.

2 Superfusion with human α calcitonin gene-related peptide (CGRP, 30–300 nM) likewise produced a transient hyperpolarization of the membrane, mimicking the neurogenic inhibitory junction potential (i.j.p.). The hyperpolarization by CGRP was unaffected by tetrodotoxin, indicating a postjunctional site of action.

3 Both the EFS-evoked i.j.p. and the CGRP-induced hyperpolarization were inhibited by the CGRP receptor antagonist, CGRP(8–37) (0.3–3 μ M) which did not affect the hyperpolarization produced by the K_{ATP} channel opener, cromakalim (0.3 μ M).

4 The K_{ATP} channel blocker, glibenclamide (1 μ M) blocked both the EFS-evoked i.j.p. and the CGRP-induced hyperpolarization.

5 When evoked in a low K medium (1.2 mM, KCl being replaced by an equimolar amount of NaCl), the EFS-evoked i.j.p. and the CGRP-induced hyperpolarization were both markedly enhanced, consistent with the idea that opening of K channels underlies both responses.

6 The present findings provide direct electrophysiological evidence for a neurotransmitter role of CGRP, released from the peripheral endings of capsaicin-sensitive primary afferent neurones, in the guinea-pig ureter. The action of both exogenous and endogenous CGRP involves the activation of glibenclamide-sensitive (K_{ATP}) potassium channels.

Keywords: Guinea-pig ureter; calcitonin gene-related peptide (CGRP); efferent function of sensory nerves; potassium channel openers; glibenclamide

Introduction

Calcitonin gene-related peptide (CGRP), a 37 amino acid neuropeptide, is widely distributed in the mammalian peripheral nervous system, principally stored in the peripheral endings of capsaicin-sensitive primary afferent neurones (Yamamoto & Tohyama, 1989 for review). Nerve fibres containing CGRP-like immunoreactivity (CGRP-LI) are widely distributed to various viscera, including the upper urinary tract (Su *et al.*, 1986); the application of depolarizing stimuli produces the release of CGRP-LI from afferent neurones in the mammalian ureter (Santicioli *et al.*, 1988; Maggi *et al.*, 1990; 1992b). The suggestion has been advanced that CGRP acts as an inhibitory neuromuscular transmitter in the guinea-pig ureter, where it may suppress latent pacemakers and prevent antiperistaltic waves of contraction to produce backflow of urine toward the kidney (Maggi & Giuliani, 1991; Maggi *et al.*, 1992a). The inhibitory action of CGRP on the evoked motility of the guinea-pig ureter is produced through the stimulation of specific receptors densely distributed over the smooth muscle layers (Sann *et al.*, 1993) and is selectively blocked by a CGRP receptor antagonist, the C-terminal fragment CGRP(8–37) (Maggi & Giuliani, 1991).

In a recent study (Maggi *et al.*, 1994) we presented evidence that activation of glibenclamide-sensitive potassium channels (K_{ATP} channels, Cook & Quast, 1990 for review) provides a powerful mechanism for regulation of motility of the ureter by producing membrane hyperpolarization and preventing the activation of latent pacemakers by depolarizing stimuli. Since CGRP has been shown to activate K_{ATP}

channels in arterial smooth muscle (Standen *et al.*, 1989), it appears of interest to know whether this effector mechanism may underlie the action of CGRP in the ureter.

By using a single sucrose gap technique, we have now obtained electrophysiological evidence that endogenous CGRP produces inhibitory junction potentials (i.j.ps) in the guinea-pig ureter and that opening of glibenclamide-sensitive K channels is the effector mechanism for this type of neuromuscular peptidergic transmission.

Methods

Male albino guinea-pigs, weighing 250–300 g were stunned and bled. A segment of distal ureter (1.5 cm in length) was excised and placed in oxygenated and warmed (95% O_2 and 5% CO_2 , pH 7.4 at 37°C) Krebs solution. The composition of Krebs solution was (mmol l⁻¹): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11. A single sucrose gap, modified as described in detail by Artemenko *et al.* (1982) and Hoyle (1987) was used to investigate changes in membrane potential in response to electrical stimulation. The ureters were superfused with oxygenated Krebs solution at a rate of 1 ml min⁻¹. The temperature of the solution was kept constant at 35 $\pm$ 0.5°C.

Depolarizing square wave pulses (1–5 Hz for 10 s, 0.15–0.2 ms pulse width, 30–40 V) were delivered by means of a GRASS S 88 stimulator coupled with a GRASS SIU5 stimulus isolation unit. Tetrodotoxin (0.3 μ M) was applied for 10 min before EFS. *In vitro* capsaicin-desensitization was produced by superfusion with 3 μ M capsaicin for 15 min followed by 15 min washout. In each preparation, two

¹ Author for correspondence.

stimulation cycles at various frequencies were performed before and after drug treatment. The hyperpolarization by CGRP was evoked by 15 s superfusion with the stated CGRP concentration. In some experiments, a low-K (1.2 mM) Krebs solution was prepared by replacing KCl with an equimolar amount of NaCl.

Statistical analysis of data was performed by means of Student's 2 tailed *t* test for paired data. A probability <0.05 was considered statistically significant.

Drugs used were: human α CGRP and human α CGRP (8–37) from Peninsula; glibenclamide and capsaicin from Sigma; tetrodotoxin from Sankyo.

Results

Electrical field stimulation (EFS) produced by applying train of pulses at a frequency of 1–5 Hz (pulse width 0.15–0.2 ms, 30–40 V) for 10 s produced graded hyperpolarizing responses which started within 4–9 s and peaked 15–20 s from the onset of stimulation (Figure 1). The peak amplitude and rate of hyperpolarization (mV s^{-1}) were linearly correlated to the frequency of stimulation. The hyperpolarization produced by EFS could be evoked for several consecutive stimulation cycles at 15–30 min intervals from each other. The evoked hyperpolarization was abolished by $0.3 \mu\text{M}$ tetrodotoxin (Figure 1) indicating its dependence upon transmitter release from intramural nerves (inhibitory junction potentials, i.j.p.). The parameters of i.j.p. activated by EFS are given in Table 1.

Application of capsaicin ($3 \mu\text{M}$) evoked a prompt and transient hyperpolarization ($1.32 \pm 0.31 \text{ mV}$, peak at $26 \pm 3 \text{ s}$ from capsaicin application, $n = 4$) followed by a transient depolarization ($1.43 \pm 0.2 \text{ mV}$, peak at $171 \pm 24 \text{ s}$ from capsaicin application, $n = 4$). A second application of $3 \mu\text{M}$ capsaicin, 15 min later failed to affect membrane potential indicating complete desensitization. In capsaicin-desensitized ureters, EFS was unable to evoke i.j.ps, indicating their dependence from transmitter(s) release from the peripheral endings of capsaicin-sensitive afferents (Figure 1).

Since CGRP is a powerful inhibitor of motility of the ureter, we next examined whether exogenous CGRP mimics the hyperpolarization produced by EFS. Superfusion with human α CGRP (CGRP, 30–300 nM for 15 s, Figure 2) produced a concentration-dependent hyperpolarization mimicking the nerve-mediated i.j.ps. The hyperpolarizing effect of CGRP (100 nM) observed in the presence of tetrodotoxin ($0.3 \mu\text{M}$) was unchanged as compared to controls (0.93 ± 0.21 and $0.88 \pm 0.25 \text{ mV}$, NS, $n = 4$), indicating a postjunctional site of action.

The CGRP receptor antagonist, CGRP(8–37) (0.3 – $3 \mu\text{M}$) had no effect on its own, indicating total lack of agonist activity but prevented, in a concentration-dependent manner, the hyperpolarization produced by exogenously applied CGRP (Figure 2) and the EFS-evoked i.j.ps (Figure 3). The degree of inhibition produced by CGRP(8–37) was inversely related to the concentration of the agonist (in the case of exogenous CGRP, Figure 2) or to the frequency of stimulation (in the case of the EFS-evoked i.j.p., Figure 3), consistent with the competitive nature of its antagonism at CGRP receptors (Chiba *et al.*, 1989; Maggi *et al.*, 1991).

In control experiments, CGRP(8–37) ($3 \mu\text{M}$) did not affect the hyperpolarization produced by $0.3 \mu\text{M}$ cromakalim (0.68 ± 0.07 and $0.65 \pm 0.11 \text{ mV}$, $n = 3$). On the other hand, the K_{ATP} channel blocker, glibenclamide (0.3 – $1 \mu\text{M}$), which blocks the hyperpolarization induced by cromakalim in the guinea-pig ureter (Maggi *et al.*, 1994), suppressed the hyperpolarization produced by CGRP (Figure 2) and the EFS-evoked i.j.p. (Figure 3), providing evidence for the involvement of K_{ATP} channels in the hyperpolarization produced by endogenous CGRP.

Further evidence that CGRP acts as a K channel opener in the ureter, was obtained by examining the effect of a low-K

Figure 1 (a) Frequency-dependent inhibitory junction potentials (i.j.p.) evoked by electrical field stimulation (EFS) in the smooth muscle of the guinea-pig ureter and their inhibition by tetrodotoxin or capsaicin-pretreatment. (b) EFS-evoked i.j.p. in the guinea-pig ureter (controls, ■, $n = 10$) and the effect of tetrodotoxin ($0.3 \mu\text{M}$, □, $n = 6$) or *in vitro* capsaicin-pretreatment (○, $n = 4$). *Significantly different from control, $P < 0.05$.

Table 1 Parameters of the inhibitory junction potential produced by electrical field stimulation in the guinea-pig ureter

Parameter	Frequency of stimulation		
	1 Hz	2 Hz	5 Hz
Latency (s)	7.36 ± 0.29	5.90 ± 1.59	4.73 ± 0.28
T_{max} (s)	17.1 ± 0.58	18.5 ± 1.05	19.8 ± 0.90
$T_{50\%}$ (s)	14.7 ± 1.5	17.0 ± 1.7	22.6 ± 2.22
Amplitude (mV)	0.46 ± 0.06	0.61 ± 0.07	0.83 ± 0.10
Rate (mV s^{-1})	0.031 ± 0.003	0.033 ± 0.004	0.043 ± 0.006

Each value is mean $\pm$ s.e. mean of ten experiments

T_{max} = time to achieve maximal hyperpolarization

$T_{50\%}$ = time to recover 50% of hyperpolarization

Rate = rate of hyperpolarization.

Krebs solution on the hyperpolarization produced by exogenous CGRP and on the CGRP-mediated i.j.p. produced by EFS. In fact the hyperpolarization produced by cromakalim, leading to K outflow via glibenclamide-sensitive K_{ATP} channels, was enhanced when the transmembrane K chemical gradient was increased. Data shown in Figures 2 and 3 indicate that the EFS-evoked i.j.ps and the hyperpolarization produced by exogenous CGRP were both markedly potentiated when elicited in a low-K Krebs solution.

Figure 2 Calcitonin gene-related peptide (CGRP)-induced hyperpolarization of the smooth muscle of the guinea-pig ureter and its modification by the CGRP receptor antagonist human α CGRP(8-37) (a and b), glibenclamide (c and d) or by a low-K Krebs solution (e and f). (a) Control (■), $n = 7$; hCGRP(8-37) 1 μ M, (□), $n = 4$ and 3 μ M, (○), $n = 3$. (c) Control (■), $n = 8$; glibenclamide 0.3 μ M (□), $n = 4$, 1 μ M (○), $n = 4$. (e) Control (■), $n = 4$; low-K Krebs solution (□), $n = 4$. In each panel * indicates values which are significantly different from control, $P < 0.05$. In (b), (d) and (f) the responses to 100 nm CGRP is shown before and after hCGRP(8-37), glibenclamide or in a low K Krebs solution. Horizontal bars mark the application of 100 nm CGRP.

Discussion

The dense plexus of CGRP-containing nerves present in the guinea-pig ureter represents the peripheral terminals of primary afferent neurones sensitive to the actions of capsaicin (Su *et al.*, 1986). These primary afferent neurones are thought to exert a dual, sensory and 'efferent' function, the latter being accomplished through the release of sensory neuropeptides (such as CGRP) in an efferent manner (Maggi & Meli, 1988, for review). A marked release of CGRP occurs in the guinea-pig ureter in response to various depolarizing stimuli, including capsaicin itself, electrical stimulation of intramural nerves and bradykinin (Santicioli *et al.*, 1988; Maggi *et al.*, 1990; 1992b). Under physiological conditions, the mammalian ureter does not display any spontaneous electrical or mechanical activity, being normally excited, through electromechanical coupling, by waves of excitation arising from the renal pelvis pacemaker (Weiss, 1992, for review). Latent pacemakers in the ureter may however be excited in non-physiological conditions (e.g. stones, bacterial infections) to produce antiperistaltic waves of contractions which induce urine backflow toward the kidney (Weiss, 1992). CGRP does not affect the quiescent ureter but produces a powerful suppression of evoked motility by activating specific receptors expressed in the smooth muscle layers (Maggi & Giuliani, 1991; Sann *et al.*, 1993). In a previous study we demonstrated the ability of endogenous CGRP, released from primary afferent nerves to suppress background motility of ureter sustained by chemical excitation of latent pacemakers (Maggi & Giuliani, 1991). By using the competitive CGRP receptor

antagonist, CGRP(8-37) (Chiba *et al.*, 1989; Dennis *et al.*, 1989; Maggi *et al.*, 1991), we have now obtained evidence that CGRP receptors are junctionally activated to mediate a neurogenic hyperpolarization of ureter smooth muscle produced by stimulation of peripheral endings of capsaicin-sensitive afferents.

Although CGRP receptors have not yet been cloned, indirect evidence suggests that they belong to the superfamily of G-protein coupled receptors (Poyner, 1992, for review). Exogenous CGRP has been reported to activate a class of ATP-sensitive K channels (K_{ATP}) in e.g. arterial smooth muscle (Standen *et al.*, 1989) which are also activated by the K channel opener, cromakalim and blocked by the sulphonyl-urea-type antidiabetic agent, glibenclamide. The ATP-sensitive K channels have a role in regulating insulin release from pancreatic β cells and appear also to be present in neurones, cardiac cells and smooth muscles (Cook & Quast, 1990; Ashcroft & Ashcroft, 1990). However, the association of this effector system with neurotransmission in normal tissues has never been demonstrated. Since the K_{ATP} channel agonist, cromakalim, produces a glibenclamide-sensitive hyperpolarization of the guinea-pig ureter smooth muscle (Maggi *et al.*, 1994), the activation of this mechanism would be a likely explanation for the hyperpolarization produced by CGRP in the ureter.

The present findings indicate that cromakalim- and glibenclamide-sensitive K channels are junctionally activated by endogenous CGRP released from capsaicin-sensitive primary afferents. The membrane hyperpolarization produced by activation of K_{ATP} channels is capable of suppressing the

Figure 3 EFS-induced inhibitory junction potentials (i.j.p.) in the smooth muscle of the guinea-pig ureter and its modification by the calcitonin gene-related peptide (CGRP) receptor antagonist CGRP(8-37) (a and b), glibenclamide (c and d) or by a low-K Krebs solution (e and f). (a) Control (■), $n = 8$; hCGRP(8-37) 0.3 μM (□), $n = 3$ and 1 μM (○), $n = 5$. (c) Control (■), $n = 8$; glibenclamide 0.3 μM (□), $n = 4$; 1 μM (○), $n = 4$. (e) Control (■), $n = 5$; low-K Krebs solution (□), $n = 5$. In each panel * indicates values which are significantly different from control, $P < 0.05$. In (b), (d) and (f) the response to EFS at 2 Hz (train duration 10 s) is shown before and after hCGRP(8-37), glibenclamide or in a low K Krebs solution.

evoked motility sustained by depolarization of latent pacemakers in the ureter (Maggi *et al.*, 1994). This mechanism may provide the basis through which locally released CGRP suppresses latent pacemakers and prevents antiperistalsis in the ureter. From the present findings it also appears that

peptidergic neurotransmission in the peripheral nervous system is not necessarily confined to high frequencies of nerve stimulation (Lundberg & Hokfelt, 1983), since a CGRP-mediated response was readily demonstrated here at low frequency stimulation (10 pulses at 1 Hz).

References

- ARTEMENKO, D.P., BURY, V.A., VLADIMIROVA, I.A. & SHUBA, M.F. (1982). Modification of the single sucrose-gap method. *Physiol. Zhurn.*, **28**, 374–380.
- ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP-sensitive K channels. *Cell. Signalling*, **2**, 197–214.
- CHIBA, T., YAMAGUCHI, A., YAMATANI, T., NAKAMURA, A., MORISHITA, T., INUI, T., FUKASE, M., NODA, T. & FUJITA, T. (1989). CGRP receptor antagonist hCGRP (8–37). *Am. J. Physiol.*, **256**, E331–335.
- COOK, N.S. & QUAIST, U. (1990). Potassium channel pharmacology. In *Potassium Channels*. ed. pp. 181–258. Cook, N.S. Chichester, England: Ellis Horwood.
- DENNIS, T., FOURNIER, A., ST PIERRE, S. & QUIRION, R. (1989). Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissue. Evidence for receptor multiplicity. *J. Pharmacol. Exp. Ther.*, **251**, 718–725.
- HOYLE, C.H.V. (1987). A modified single sucrose gap-junction potentials and electrotonic potentials in gastrointestinal smooth muscle. *J. Pharmacol. Methods*, **18**, 219–226.
- LUNDBERG, J.M. & HOKFELT, T. (1983). Coexistence of peptides and classical neurotransmitters. *Trends Neurosci.*, **6**, 325–333.
- MAGGI, C.A., CHIBA, T. & GIULIANI, S. (1991). Human α -calcitonin gene-related peptide (8–37) as an antagonist of exogenous and endogenous calcitonin gene-related peptide. *Eur. J. Pharmacol.*, **192**, 85–88.
- MAGGI, C.A. & GIULIANI, S. (1991). The neurotransmitter role of CGRP in the rat and guinea-pig ureter: effect of a CGRP antagonist and species-related differences in the action of omega conotoxin on CGRP release from primary afferents. *Neurosci.*, **43**, 261–271.
- MAGGI, C.A., GIULIANI, S., DEL BIANCO, E., GEPPETTI, P., THEODORSSON, E. & SANTICIOLI, P. (1992a). CGRP in the regulation of urinary tract motility. *Ann. New York Acad. Sci.*, **657**, 328–343.
- MAGGI, C.A. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19**, 1–43.
- MAGGI, C.A., SANTICIOLI, P., DEL BIANCO, E. & GIULIANI, S. (1992b). Local motor responses to bradykinin and bacterial chemotactic peptide FMLP in the guinea-pig isolated renal pelvis and ureter. *J. Urology*, **148**, 1944–1950.

- MAGGI, C.A., GIULIANI, S. & SANTICIOLI, P. (1994). Effect of cromakalim and glibenclamide on spontaneous and evoked motility of the guinea-pig isolated renal pelvis and ureter. *Br. J. Pharmacol.*, **111**, 687–694.
- MAGGI, C.A., TRAMONTANA, M., CECCONI, R. & SANTICIOLI, P. (1990). Neurochemical evidence for the involvement of N-type calcium channels in transmitter secretion from peripheral endings of sensory nerves in guinea-pigs. *Neurosci. Lett.*, **114**, 203–206.
- POYNER, D.R. (1992). CGRP: multiple actions, multiple receptors. *Pharmacol. Ther.*, **56**, 23–51.
- SANN, H., ROSSLER, W., HAMMER, K. & PIERAU, F.R.K. (1992). SP and CGRP in the ureter of chicken and guinea-pig: distribution, binding sites and possible functions. *Neuroscience*, **49**, 699–713.
- SANTICIOLI, P., MAGGI, C.A., GEPPETTI, P., DEL BIANCO, E., THEODORSSON, E. & MELI, A. (1988). Release of CGRP-LI from organs of the genitourinary tract in rats. *Neurosci. Lett.*, **92**, 197–201.
- STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, T.M. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K channels in arterial smooth muscle. *Science*, **245**, 177–180.
- SU, H.C., WHARTON, J., POLAK, J.M., MULDERY, P.K., GHATEI, M.A., GIBSON, S.J., TERENGI, G., MORRISON, J.F.B., BALLESTA, J. & BLOOM, S.R. (1986). CGRP immunoreactivity in afferent neurons supplying the urinary tract: combined retrograde tracing and immunohistochemistry. *Neuroscience*, **18**, 727–747.
- WEISS, R.M. (1992). Physiology and pharmacology of renal pelvis and ureter. In *Campbell's Urology*, ed. Walsh, P.C., Retik, A.B., Stamey, T.A. & Vaughan, E.D. Volume I, pp. 113–144. Philadelphia PA: W.B. Saunders Co.
- YAMAMOTO, A.I. & TOHYAMA, M. (1989). CGRP in the nervous tissue. *Prog. Neurobiol.*, **33**, 335–395.

(Received January 19, 1994

Revised May 18, 1994

Accepted June 20, 1994)