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Defective smooth muscle regulation in cGMP kinase I-deficient mice

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Regulation of smooth muscle contractility is essential for many important biological processes such as tissue perfusion, cardiovascular haemostasis and gastrointestinal motility. While an increase in calcium initiates smooth muscle contraction, relaxation can be induced by cGMP or cAMP. cGMP-dependent protein kinase I (cGKI) has been suggested as a major mediator of the relaxant effects of both nucleotides. To study the biological role of cGKI and its postulated cross-activation by cAMP, we inactivated the gene coding for cGKI in mice. Loss of cGKI abolishes nitric oxide (NO)/cGMP-dependent relaxation of smooth muscle, resulting in severe vascular and intestinal dysfunctions. However, cGKI-deficient smooth muscle responded normally to cAMP, indicating that cAMP and cGMP signal via independent pathways, with cGKI being the specific mediator of the NO/cGMP effects in murine smooth muscle.

Keywords: blood pressure/cGMP-dependent protein kinase/gene targeting/nitric oxide/regulation of smooth muscle tone

Introduction

Contraction of smooth muscle defines the lumen in blood vessels, airways, the intestine, uterus and bladder. An abnormal increase in smooth muscle tone has been implicated in the pathogenesis of hypertension, cardiogenic shock and pyloric stenosis (Somlyo and Somlyo, 1994). The principal mechanisms that initiate smooth muscle contraction are the rise of intracellular free Ca^{2+} concentration and/or increased sensitivity of the contractile apparatus towards Ca^{2+} (Somlyo, 1997). Relaxation of smooth muscle can result from activation of receptors coupled to adenylyl cyclase, generating cAMP, or guanylyl cyclase, generating cGMP (Rasmussen *et al.*, 1990; Murad, 1994). Among the various cyclic nucleotide receptors, cGMP-dependent protein kinase (cGK) has been postulated to play a key role in smooth muscle relaxation (Rapoport *et al.*, 1983). Mammals express two different forms of cGK, cGKI (Sandberg *et al.*, 1989; Wernet *et al.*, 1989) and cGKII (Uhler, 1993; Jarchau *et al.*, 1994), but only cGKI is highly expressed in smooth muscle (Waldmann *et al.*, 1986; Keilbach *et al.*, 1992). Many of the biochemical features of cGKI have been studied in detail (Hofmann *et al.*, 1992; Lohmann *et al.*, 1997). *In vitro* studies showed that cGKI lowers agonist-stimulated cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) in smooth muscle and other cells (Felbel *et al.*, 1988; Cornwell and Lincoln, 1989; Geiger *et al.*, 1992; Ruth *et al.*, 1993). Due mainly to the presence of numerous and varied receptors for cGMP in eukaryotic cells, however, it was concluded that cGKI lacks a clearly delineated biological role (Francis and Corbin, 1994). Furthermore, *in vitro* studies (Lincoln *et al.*, 1990; Jiang *et al.*, 1992; Chao *et al.*, 1994) led to the hypothesis of a cross-talk between the cGMP and cAMP signalling cascades at the level of cyclic nucleotide-dependent protein kinases, i.e. cross-activation of cGKI by cAMP, or vice versa. This hypothesis was supported further by biochemical analyses which showed that autophosphorylation of cGKI lowers cyclic nucleotide concentrations needed for activation (Landgraf *et al.*, 1986; Smith *et al.*, 1996). Thus, nitric oxide (NO)/cGMP signalling may be mediated by cGKI, cAMP-dependent protein kinase (cAK) or both (Lincoln *et al.*, 1995). To test this hypothesis and to investigate the physiological role of cGKI, we have established mice bearing homozygous cGKI null mutations.

Results and discussion

Targeted disruption of the murine cGKI gene

The importance of cGKI for smooth muscle relaxation was tested by gene targeting in murine embryonic stem (ES) cells. Two isoforms of cGKI (cGKIα and Iβ) have been identified that are splice variants differing only in their first N-terminal exons (Orstavik *et al.*, 1997). To ensure inactivation of both isoforms, we disrupted an exon that lies within the common region of cGKI encoding part of the ATP-binding domain (Hanks *et al.*, 1988) (Figure 1A and B). The mutation was transmitted at Mendelian ratio (n = 40 pups from five litters), suggesting that embryonic and fetal development of homozygous mutant mice were not impaired. The null mutation was confirmed by Northern and Western blot analysis (Figure 1C and D). Lack of cGKI had no significant effect on the expression of cGKII and cAK (Figure 1D). Functional ablation of cGKI was confirmed by measuring cGMP-stimulated kinase activity in lung extracts from wild-type (±cGMP:

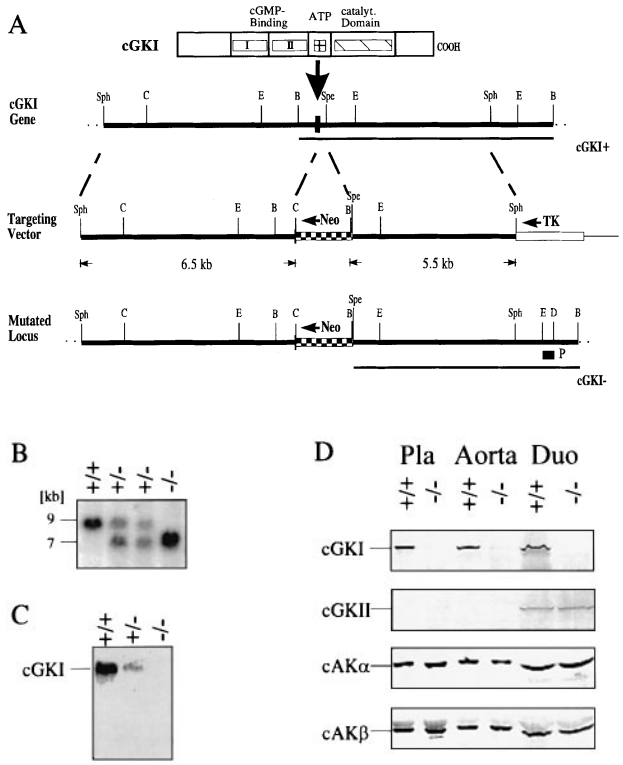


Fig. 1. Targeted disruption of the cGKI gene. (**A**) Top: structure of cGKI. Upper middle: localization, restriction map and organization of the cGKI target locus; exon (filled box) and introns (lines). Lower middle: the targeting vector contains 12 kb of cGKI genomic sequence that flank PGK-*neo* (Neo). The insertion of the Neo cassette deletes a 0.3 kb sequence from the target exon and the following intron. The herpes simplex virus thymidine kinase cassette (TK) was cloned 3′ to the cGKI sequence. Bottom: the mutated locus. Abbreviations: B, *Bam*HI; C, *Cla*I; D, *Dde*I; E, *Eco*RI; Spe, *Spe*I; Sph, *Sph*I; P, Southern probe. (**B**) Southern blot: hybridization of *Bam*HI-digested genomic DNA with probe P results in a 7 kb mutant- and 9 kb wild-type-specific band. (**C**) Northern blot of total RNA extracted from the uterus of cGKI+/+, +/– and –/– mice, hybridized with a cGKI-specific cDNA probe. The integrity and amount of RNA were judged by ethidium bromide staining of the gel (not shown). (**D**) Western blot analysis of the expression of cGKI, cGKII and the catalytic α and β subunits of cAK in platelets (Pla), aorta (Aorta) and duodenum (Duo) isolated from cGKI +/+ and –/– mice.

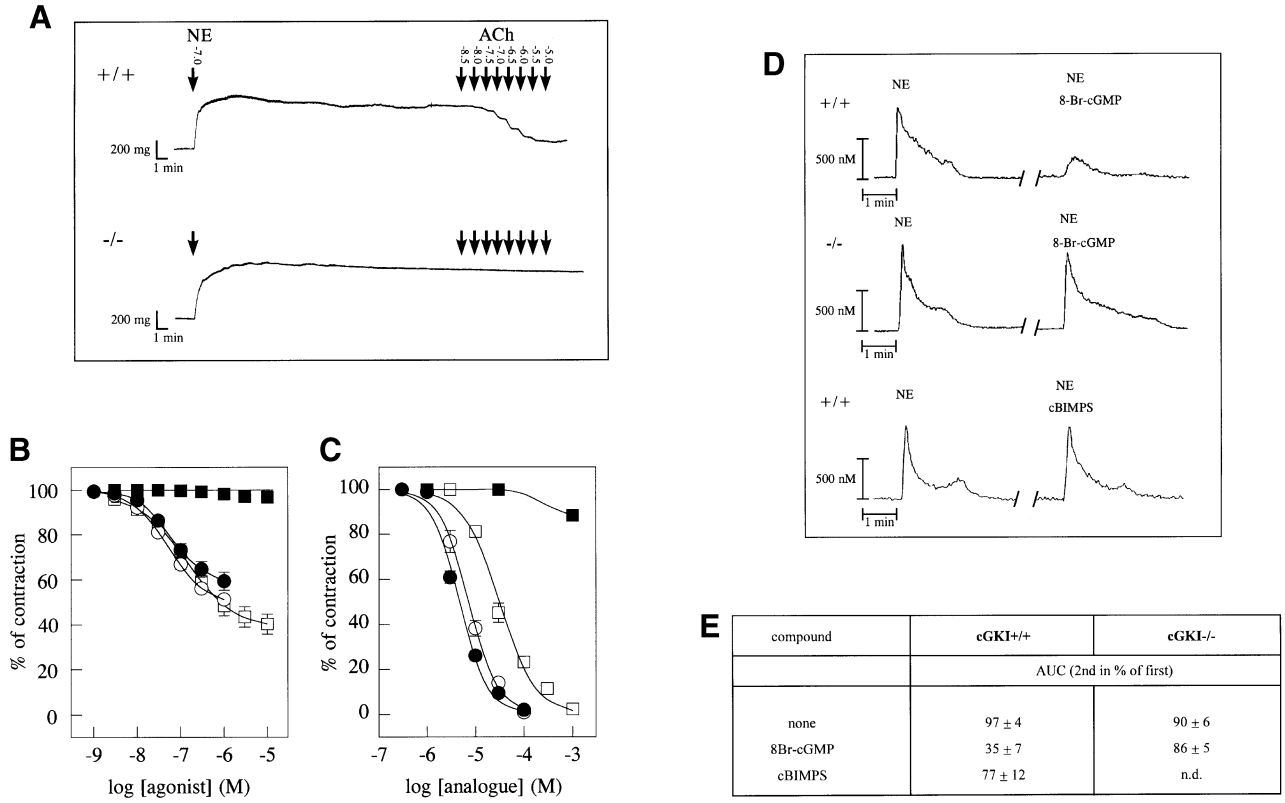
70 ± 6.5–262 ± 12 fmol P_i/min×mg) and cGKI–/– (±cGMP: 80 ± 4.5–90 ± 10.3 fmol P_i/min×mg) mice. cGKI deficiency had no effect on the integrity of the smooth muscle cGMP pathway proximal to the kinase, since incubation with 0.1 μM and 100 μM 2-(*N*,*N*-Diethyl-amino)-diazenolate-2-oxide (DEA-NO) stimulated cGMP production 2- and 90-fold in cGKI+/+ and cGKI–/– aortic rings, with basal values being 0.35 ± 0.05 (5) and 0.28 ± 0.03 (3) pmol/mg wet weight, respectively.

Lack of cGKI impairs NO/cGMP effects in isolated aortic rings

NO is a potent modulator of vascular resistance and blood pressure (Furchgott and Zawadzki, 1980; Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Fleming and Busse, 1995), and mice lacking endothelial NO synthase (eNOS) are hypertensive (Huang *et al.*, 1995; Sheseley *et al.*, 1996). Since cGKI was postulated to be a major mediator of cGMP effects, we analysed whether or not NO/cGMP induces relaxation in cGKI-deficient vascular smooth muscle. Histological analysis of aortic rings did not reveal gross abnormalities in the cGKI–/– mice (not shown). Wild-type and cGKI–/– aortic rings developed similar tension in the presence of 0.1 μM norepinephrine (NE) (Figure 2A). Acetylcholine (ACh), which induces NO synthesis in endothelial cells (Furchgott and Zawadzki, 1980), reduced the tension of wild-type aortic rings by 59.7 ± 4.5%, with an EC_{50} of 171 ± 14 nM. Similar values have been reported by others (Huang *et al.*, 1995). However, ACh elicited only a 3.4 ± 1.4% relaxation in the cGKI–/– aortic rings (Figure 2B). The unresponsiveness of the cGKI-deficient aortas to ACh was not caused by a defect in endothelial NO synthesis, since the cGMP analogue 8-bromo-cGMP (8-Br-cGMP) relaxed the aortic rings of control mice lacking functional endothelium, with an EC_{50} of 30 μM, but not that of cGKI–/– mice (Figure 2C). This demonstrates that ablation of cGKI disrupts the NO/cGMP signalling pathway in vascular smooth muscle. Previous studies *in vitro* suggested that cAMP relaxes vascular smooth muscle by cross-activation of cGKI (Lincoln *et al.*, 1990; Jiang *et al.*, 1992). To test the physiological relevance of this mechanism, we superfused aortic rings with CGS-21680, an agonist on adenosine A2 receptors. Surprisingly, CGS-21680 relaxed wild-type as well as cGKI–/– aortas by 56%, with IC_{50} values of 56 ± 6 nM (n = 8) and 60 ± 2 nM (n = 6), respectively (Figure 2B). In agreement with this result, the cAMP analogue cBIMPS relaxed aortic rings from both mutant and normal animals, with an EC_{50} of ~6 μM (Figure 2C). Thus, the cAMP-induced relaxation of murine vascular smooth muscle is not dependent on cGKI, indicating that cGMP and cAMP signal via different effectors in murine aortic smooth muscle cells.

Analysis of cGMP and cAMP effects in isolated vascular smooth muscle cells

To study the molecular mechanism of cGMP- and cAMP-induced vasorelaxation, we analysed the effect of cGMP and cAMP on NE-stimulated $[Ca^{2+}]_i$ transients in single aortic smooth muscle cells. The basal $[Ca^{2+}]_i$ was similar in cGKI+/+ (110 ± 3 nM, n = 62) and cGKI–/– (111 ± 6 nM, n = 28) aortic cells (Figure 2D). Superfusion of cGKI+/+ and cGKI–/– cells with 10 μM NE elicited a transient increase in $[Ca^{2+}]_i$ to 901 ± 47 nM (n = 62) and 875 ± 87 nM (n = 28), respectively. The other parameters of the NE-induced $[Ca^{2+}]_i$ transients, such as the area under the curve, latency to rise in $[Ca^{2+}]_i$, decay time and duration of transient, were identical in both cell types. To analyse the effects of cGMP and cAMP on agonist-induced transients, we elicited in the same cells a second $[Ca^{2+}]_i$ transient. Under control conditions, both cell types showed the same response, that was not significantly different from the first transient (Figure 2D and E). Pre-incubation of the cells with 8-Br-cGMP attenuated the $[Ca^{2+}]_i$ transient in wild-type cells to 35%, but had no effect on the $[Ca^{2+}]_i$ in cGKI–/– cells (Figure 2E). In wild-type cells, the decay time and duration were shortened to a similar degree (Figure 2D), while the latency of the rise in $[Ca^{2+}]_i$ was increased >2.5-fold by 8Br-cGMP (n = 20). In contrast to cGMP, cBIMPS did not significantly affect the $[Ca^{2+}]_i$ transients in wild-type cells (Figure 2D and E), clearly showing that the cGMP and cAMP



compound	cGKI+/+	cGKI-/-
	AUC (2nd in % of first)	
none	97 ± 4	90 ± 6
8Br-cGMP	35 ± 7	86 ± 5
cBIMPS	77 ± 12	n.d.

Fig. 2. Analysis of contractility and of cellular Ca^{2+} concentration in vascular smooth muscle. (A, B and C) Effects of ACh, adenosine, cGMP and cAMP on isolated aortic rings. (**A**) Representative original registration of aortic segments isolated from cGKI+/+ (top) and cGKI–/– (bottom) mice. The aortas were pre-contracted with 100 nM NE. After reaching a steady state, 3 nM to 10 μM ACh was added to the bath. Numbers above the arrows indicate the logarithm of concentration in mol/l. (B and C) Concentration–response curves to (**B**) ACh (□, ■) and the adenosine receptor A2 agonist CGS-21680 (○, ●) and (**C**) 8-Br-cGMP (□, ■) and cBIMPS (○, ●). Aortic rings developed the same tension in the presence of 0.1 μM NE: 7.5 ± 0.5 (cGKI+/+, ○, □) and 6.7 ± 0.5 mM (cGKI –/– ●, ■). Values are the means ± SEM for 12 aortic strips from 3–5 different animals of each genotype. Curves are fits using a logarithmic function. (D and E) Effect of cGMP and cAMP on NE-induced $[Ca^{2+}]_i$ transients in isolated aortic smooth muscle cells. (**D**) Representative original registration of $[Ca^{2+}]_i$ in cells isolated from cGKI+/+ (top and bottom) and cGKI–/– (middle) mice. The first and second $[Ca^{2+}]_i$ transients were elicited by superfusion with 10 μM NE for 1 min. After washout for 20 min, the cells were pre-incubated for 10 min with 1 mM 8-Br-cGMP or 10 μM cBIMPS followed by NE for 1 min. (**E**) Summary of experiments in (D). The $[Ca^{2+}]_i$ transients were quantified as area under the curve (AUC). Values are expressed as a percentage of the first peak and are the means ± SEM of 12–20 cells.

signalling cascades differ fundamentally in murine aortic cells.

cGKI-deficient mice are hypertensive

The physiological significance of the *in vitro* data was studied by analysing the blood pressure regulation *in vivo*. In anaesthetized animals, the mean arterial blood pressure (MAP) (Figure 3) and the heart rate (not shown) were similar in cGKI–/– and cGKI+/+ mice. However, in the unrestrained, conscious mice, the MAP was significantly ($P < 0.001$) higher in the cGKI–/– mice (134 ± 2 mmHg) than in wild-type (118 ± 2 mmHg) littermates. In wild-type mice, intraperitoneal injection of the NOS inhibitor N^G-nitro-L-arginine (L-NNA) induced a 20 mmHg rise in MAP to 138 ± 5 mmHg. This MAP was similar to the basal MAP of the cGKI–/– and eNOS-deficient mice (Huang *et al.*, 1995; Sheseley *et al.*, 1996), indicating that the loss of cGKI also impairs the NO-induced vascular relaxation *in vivo*. The consecutive administration of DEA-NO induced an 18 mmHg drop of MAP in the wild-type mice, demonstrating the specificity of the pharmacological block of NOS. In sharp contrast, the hypertensive effects of L-NNA and the hypotensive effects of DEA-NO were essentially absent in the cGKI–/– mice. cGKI deficiency

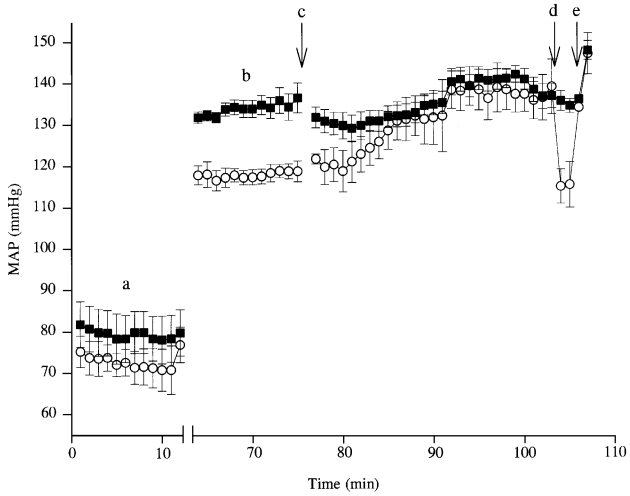


Fig. 3. Mean arterial blood pressure (MAP) of six cGKI+/+ (○) and cGKI –/– (■) mice (age 32.2 ± 1.8 days) was measured continuously starting during inhalational anaesthesia (a), and after 60 min recovery (break) in conscious, unrestrained mice (b–e). (c), (d) and (e) indicate the administration of L-NNA (12 μg/g i.p.), DEA-NO (75 ng/g i.v.) and phenylephrine (200 ng/g i.v.), respectively.

did not result in an impaired response of the vascular system to hormonally induced vasoconstriction. The injection of the α-adrenoreceptor agonist phenylephrine increased the MAP to >148 mmHg in both wild-type and cGKI–/– mice (Figure 3). These findings clearly show that ablation of the cGKI gene abolishes the NO-dependent modulation of blood pressure under physiological conditions and leads to hypertension.

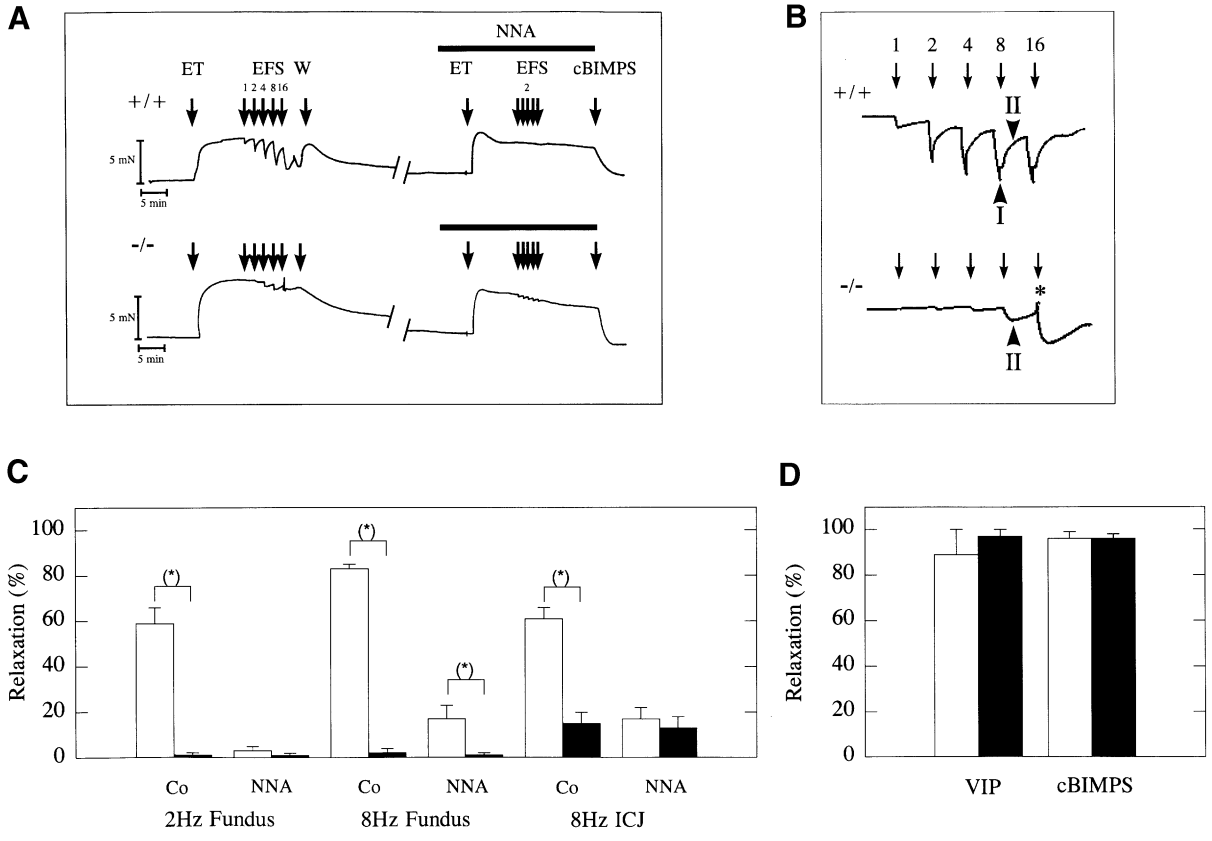
The same experiments were repeated in older animals (42.8 ± 2.1 days). Again, the NO-induced drop in blood pressure was abolished in these older cGKI–/– mice. However, the baseline MAP of the cGKI–/– mice did not differ significantly from that of the wild-type animals (not shown). Although the exact reasons for the apparent normalization of the blood pressure are not known at present, it is conceivable that defects in other organ systems influence the blood pressure.

Intestinal abnormalities in cGKI–/– mice

The experiments conducted so far clearly show that cGKI mediates the NO/cGMP-dependent regulation of vascular muscle tone. NO-induced relaxation also plays a major role in the gastrointestinal tract, where NO is released by inhibitory non-adrenergic non-cholinergic (NANC) neurons (Desai *et al.*, 1991; Burns *et al.*, 1996). Other inhibitory NANC transmitters are ATP and vasoactive intestinal peptide (VIP) (Sanders and Ward, 1992). Recent findings suggest an interplay between NO and VIP, in which VIP stimulates cAMP and NO/cGMP production in intestinal smooth muscle (Jin *et al.*, 1996). Since cGKI is highly expressed in intestinal smooth muscle, we sought to determine the role of cGKI in this setting. Autopsies of cGKI–/– mice revealed a gross intestinal distension, especially of the caecum, and signs of pyloric stenosis, and the histological analysis of the cGKI–/– mice showed a marked hypertrophy of the gastric fundus and pylorus muscle (not shown). The NOS and VIP immunoreactivity did not differ between the mutant and wild-type mice (not shown), indicating a normal development of the inhibitory enteric nerve system.

Analysis of intestinal muscle strips

Isolated gastric fundus muscle strips responded to endothelin-1 with a stable contraction, and to electrical field stimulation (EFS) of intramural nerves with a frequency-dependent relaxation in wild-type but not in cGKI–/– muscle (Figure 4A). The relaxation consisted of a rapid and transient 'first' phase followed by a 'second' phase of slower onset that lasted longer (Figure 4B). The first phase was absent in the cGKI–/– fundus strips (Figure 4A and B). However, at 4–16 Hz, we observed long-lasting relaxations of the mutant muscles, similar to the second phase relaxation seen in the wild-type (Figure 4B). At all stimulation frequencies, the EFS response was significantly reduced ($P < 0.05$) as compared with the wild-type (Figure 4). Furthermore, the 8–16 Hz stimulation elicited a frequency-dependent paradox 'on' contraction that was not seen in wild-type mice (Figure 4B). Pre-incubation of control muscles with L-NNA (300 μM) severely inhibited the relaxant effect—especially the first phase—at 2 and 8 Hz EFS (Figure 4A and C), clearly demonstrating that the major component of EFS-induced relaxation was NO dependent. The residual relaxation observed in the cGKI–/– fundus was not significantly affected by L-NNA. As expected, superfusion of the muscle strips with 100 μM SIN-1 or 100 μM 8-Br-cGMP,



a membrane-permeable analogue of cGMP, completely relaxed the wild-type muscles but had only a marginal effect on the cGKI-deficient muscles (not shown). The analysis of the pylorus (not shown) and of the ileocaecal junction (ICJ) (Figure 4C) gave identical results. However, the relaxant effects of VIP and of the cAMP analogue cBIMPS were not affected by the loss of cGKI (Figure 4D). Apparently, cGKI specifically mediates the NO/cGMP signalling, but is not required for the cAMP signalling pathway in mouse intestinal smooth muscle.

In vivo analysis of intestinal motility

From the above results, it was conceivable that cGKI–/– mice should have severe disturbances in gastrointestinal motility *in vivo*. Therefore, we analysed intestinal motility of three litter-matched cGKI+/+ and cGKI–/– mice by X-rays studies (Figure 4). Immediately after feeding barium sulfate, the stomach was filled with the X-ray-dense contrast material (Figure 4E and H). In the wild-type mice, we observed regular bowel movements and peristaltic waves. Ten minutes after feeding, contrast material was detected in the duodenum, and at 30 min most of the barium was found in the small intestine of the wild-type mice (Figure 4F). In contrast, the passage of contrast material into the duodenum was delayed in the cGKI–/– mice (Figure 4I). Instead of regular peristalsis, spastic contractions of long intestinal segments followed by scarce and slow relaxations were observed. The clearest difference between cGKI–/– and normal mice was seen at 180 min (Figure 4G and K). The wild-type stomach was empty, the intestine contained only residual amounts of barium sulfate and barium-contrasted faeces were detected outside the mouse (Figure 4G). However, at that time point, the cGKI–/– stomach still contained contrast and the small intestine had just begun to fill (Figure 4K). These results indicate that cGKI is essential for the proper function of intestinal smooth muscle *in vivo*. Disruption of the NO/cGMP signalling cascade at cGKI leads to severe intestinal malfunction.

The analysis of the cGKI-deficient mice clearly shows

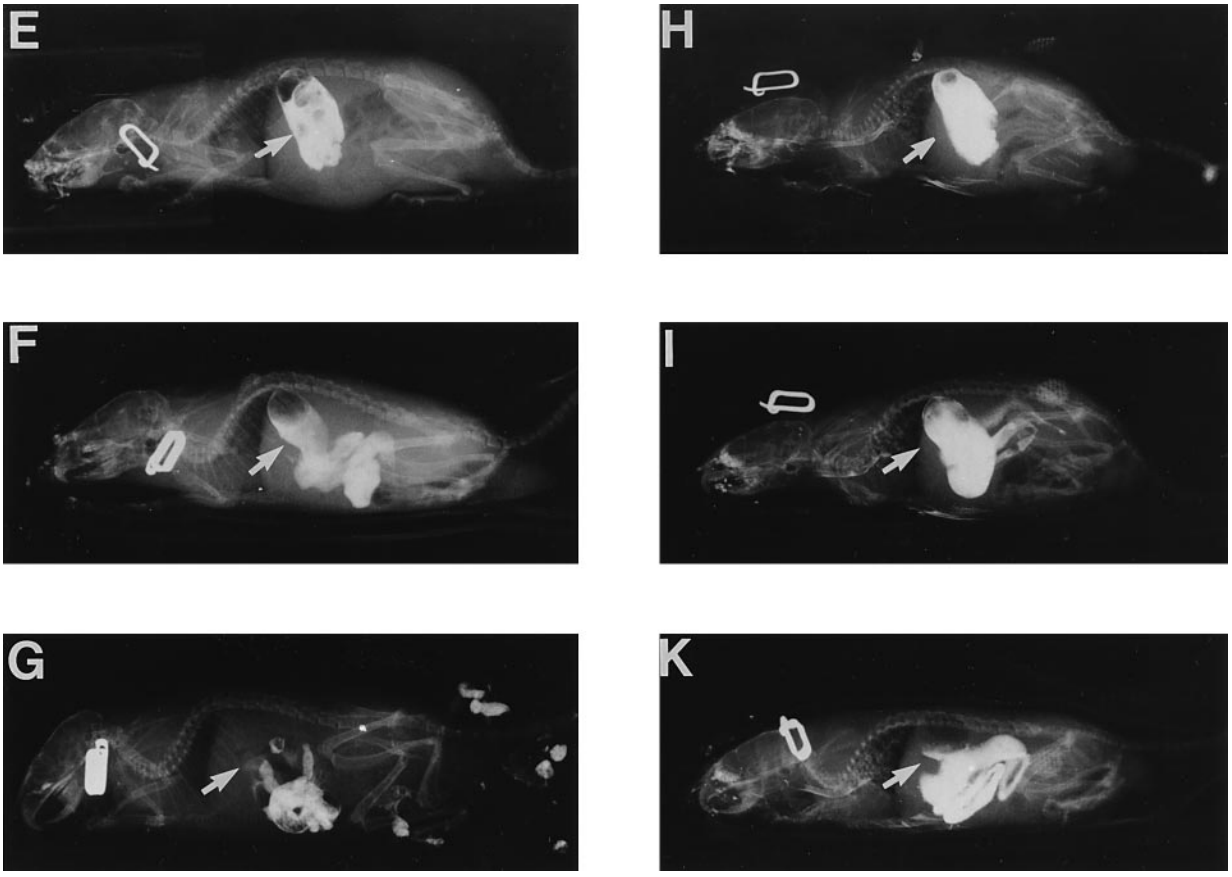


Fig. 4. Functional analysis of isolated intestinal muscle strips. (**A**) Representative tension recordings of gastric fundus strips isolated from a cGKI+/+ (upper trace) and a cGKI–/– (lower trace) mouse. Muscle strips were pre-contracted with 10 nM endothelin-1 (ET), followed by transmural electrical stimulation (EFS) (1–16 Hz). After washout for 20 min (W), the muscles were incubated with 300 μM L-NNA (NNA) for the indicated time (horizontal line). For a second time, 10 nM ET was added to the tissue bath followed by EFS (2 Hz). Finally, the cAMP analogue cBIMPS (50 μM) was added. (**B**) Original traces of EFS-induced relaxation in cGKI+/+ and cGKI–/– gastric fundus. In the wild-type fundus (upper trace), two relaxant phases (arrow heads) can be distinguished: a first (I) and a second (II) phase. The cGKI-deficient muscle (lower trace) has mainly a slow, second phase (II) preceded by a paradox contraction (asterisk) at 16 Hz stimulation frequency. (**C**) Effect of electrical stimulation (2 or 8 Hz) in the absence (Co) or presence of 300 μM L-NNA (NNA) on the first relaxant phase of gastric fundus and ileocaecal junctions (8 Hz ICJ) of cGKI+/+ (white columns) and cGKI–/– (black columns) mice. The asterisks indicate significant differences. (**D**) Relaxant effects of VIP (1 μM) and cBIMPS (50 μM) on the gastric fundus of cGKI+/+ (white columns) and cGKI–/– (black columns) mice. All values are the means ± SEM of at least six muscle strips from 3–6 animals of each genotype. (E–K) Analysis of intestinal motility. X-ray analysis of litter-mached (7-week-old) wild-type (**E**–**G**) and cGKI–/– (**H**–**K**) mice. The pictures were taken immediately (E and H), 30 min (F and I) and 180 min (G and K) after feeding the mice with barium sulfate. The arrows indicate the position of the stomach. The arrowhead in (G) marks contrast-stained faeces. This experiment was repeated in two other litter-matched pairs with identical results.

that cGKI is the physiological and specific mediator of the cGMP signal in smooth muscle *in vivo*. The cAMP pathway is unaffected by the loss of cGKI in smooth muscle, demonstrating independent regulation of smooth muscle relaxation by cGMP and cAMP. Given the outstanding protective effects of NO/cGMP in the vascular system and the high incidence and mortality of cardiovascular diseases in western societies, the cGKI-deficient animal model is also of clinical relevance.

Materials and methods

Production of cGKI–/– ES cells and mutant mice

cGKI DNA was isolated from a genomic library made from 129/sv mouse tissue (Genome Systems, St Louis, MO). A 7 kb *Sph*I–*Sph*I fragment containing the target exon was subcloned into pGEM7 (Promega, Madison, WI). From this vector, a 6 kb *Xho*I–*Bam*HI fragment, containing intronic sequences 5′ to exon IX, and a primer (forward: GCCGCTCGAGTAAGGGAAACTAATGAGAAACTGCT; reverse: GCCGATCGATCAACCTCCAACTCCAAGGGTGTCAAT)-generated 0.5 kb *Bam*HI–*Cla*I fragment, containing the 80 nucleotides of the target exon and adjacent 5′ intronic sequences, were subcloned into the *Xho*I–*Cla*I cloning site of pNTK 5′ to PGK-*neo*. Finally, a 5.5 kb *Spe*I–*Sph*I fragment 3′ to the target exon was cloned 3′ of PGK-*neo*. Culture, selection of ES cells and screening for homologous targeted clones were carried out as described (Fässler and Meyer, 1995). Germline chimeras were obtained by injection of two ES clones into C57Bl/6 blastocysts.

Mice

Mice were bred and maintained in the animal facility of the Institut für Pharmakologie und Toxikologie, TU München, and in the tightly controlled, specific pathogen-free facility of the Max Planck Institute for Biochemistry, Martinsried. No differences in the phenotypes of the animals were noted. Animals from both facilities were assigned randomly to the different experiments. The overall constitution of the homozygous mice deteriorates between 5 and 6 weeks of age. At that age, the weight of mutant mice was 70 ± 5.2% (n = 9) of the litter-matched controls, and already 49% of the cGKI–/– mice had died. A detailed analysis of the life span of 27 randomly chosen cGKI–/– mice revealed that 79% of these mice died before day 57. This phenotype was observed in mice that were kept either in a conventional or specific pathogen-free animal facility.

Northern and immunoblot analyses

Northern and immunoblot analyses were carried out as described (Pfeifer *et al*., 1996). Northern blots were hybridized with [^{32}P]dCTP-labelled mouse cGKI DNA probe. Immunoblot analysis was performed as described (Pfeifer *et al*., 1996). VIP phosphorylation was analysed by use of a monoclonal antibody (Dianova, Hamburg, Germany).

Functional intestinal studies

The gastrointestinal tract of 5- to 6-week-old mice was removed *en bloc* and dissected under a stereomicroscope. Strips of gastric fundus (without mucosa), pylorus and ICJ were transferred to 5 ml organ baths containing Krebs solution [(in mM) NaCl 119, KCl 4.6, $NaHCO_3$ 15, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 5.5, pH 7.4] gassed with 5% CO_2 and 95% O_2, and mounted on force transducers (Grass FT03) (Ny *et al.*, 1995). After equilibration for 30 min, field stimulation (1–16 Hz) of intramural nerves (5 s trains of square wave pulses of 0.5 ms duration and supramaximal voltage) was applied to the preparation via platinum wire electrodes. The recordings were made on a Grass polygraph, 7D. All experiments were carried out in the presence of indomethacin (1 μM), phentolamine (1 μM), propranolol (1 μM) and scopolamine (1 μM).

Contrast X-ray examination

Wild-type mice and their litter-matched cGKI–/– siblings were fed with 0.8 ml of barium sulfate suspension (Micropaque, Guerbet, Sulzbach/Ts, Germany) via stainless feeding needles (Ejay International, Glendora, CA). The awake animals were put into a restrainer bag and X-rays were taken at 0, 4, 10, 30, 40, 60, 90, 130 and 180 min after feeding for 10 s (Siemens Polymat 70, Siemens, Germany; 48 kV, 0.2mA).

Contractility of isolated aortic rings

Thoracic aortas were placed in Krebs–Henseleit solution [(mM): NaCl 115, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, CaCl 2.5, glucose 10, pH 7.4, 35°C], segmented into 3.5 mm long rings and mounted horizontally onto two L-shaped hooks in a tissue bath (volume: 8 ml) containing Krebs–Henseleit solution which was gassed with 5% CO_2 in O_2. The bath solution contained 1 μM diclofenac, 70 μM ascorbic acid and 10 nM tetrodotoxin. The rings were equilibrated at 600 mg resting tension for 1.5 h, with an exchange of the bath solution every 20 min. Aortic rings were pre-contracted with 100 nM NE (EC_{50} 25 nM) and, after reaching a steady state, increasing concentrations of ACh were added. The effect of 8-BrcGMP and cBIMPS was studied in stripped aortic rings without functional endothelium in the presence of 100 μM L-NNA, to block residual NOS activity. Isometric tension was recorded using an inductive force transducer (Q11, 10p; Hottinger Baldwin Meβtechnik, Darmstadt, Germany) connected to an oscilloscope and a pen recorder (Linear Corder mark VII Watanabe). Relaxation was calculated as the percentage of the maximal increase in tension caused by NE.

Measurement of $[Ca^{2+}]_i$ in isolated aortic smooth muscle cells

Vascular smooth muscle cells were prepared as previously described (Kleppisch and Nelson, 1995). In brief, the aortas were incised longitudinally, washed with physiological saline solution (PPS) [containing (in mM) NaCl 130, KCl 5.6, $CaCl_2$ 2, MgCl 1, HEPES 10, glucose 11, pH 7.4]. The aorta was first incubated in Ca^{2+}-free PPS supplemented with 0.4 mg/ml papain, 1 mg/ml dithiothreitol and 1 mg/ml bovine serum albumin (BSA) at 37°C for 18–20 min. Thereafter, the buffer was changed to PPS with 0.1 mM Ca^{2+} containing: 0.8 mg/ml collagenase (type H), 0.8 mg/ml hyaluronidase and 1 mg/ml BSA. After 10–15 min digestion at 37°C, the tissue was placed in PPS with 0.4 mM Ca^{2+} and was triturated gently. Subsequently, the cells were loaded with Fura-2 AM, washed with PPS, and $[Ca^{2+}]_i$ was measured using the dual-wavelength microfluorescence technique (Ruth *et al*., 1993).

Measurement of blood pressure

Mice of 4 weeks of age were anaesthetized using inhalational anaesthesia (1.75% isoflurane, 34–35% O_2 and 64–65% N_2O). Catheters were implanted into the left carotid artery and the left jugular vein. The arterial catheter was connected to a pressure transducer (Statham Transducer P23 ID, Statham Instruments, Oxnard, CA) attached to an amplifier (Siemens Pressure Evaluator 864, Siemens, Germany). The blood pressure was measured under anaesthesia for 12 min. Thereafter, anaesthesia was discontinued and mice recovered for 60 min. Blood pressure was measured in conscious, unrestrained mice. Drugs were delivered via the venous catheter or via intraperitoneal injection in physiological salt solution.

Calculations and statistics

All data are expressed as mean ± SEM. Statistical differences between two means were determined by the Student′s t-test, whereas for multiple comparisons a one-way analysis of variance (ANOVA) was applied. $P < 0.05$ was regarded as significant, n indicates number of experiments.

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