Modulation of Ca\textsuperscript{2+} Sensitivity by Cyclic Nucleotides in Smooth Muscle from Protein Kinase G-deficient Mice*

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The cGMP-dependent protein kinase (PKG) is the main mediator of nitric oxide-induced relaxation of smooth muscle. Although this pathway is well established, the cellular action of PKG, nitric oxide, and cGMP is complex and not fully understood. A cross-talk between the cGMP-PKG and other pathways (e.g. cAMP-protein kinase A) seems to exist. We have explored cGMP- and cAMP-dependent relaxation of smooth muscle using PKG-deficient mice (cGKI−/−). In intact ileum strips of wild type mice (cGKI+/+), 8-Br-cGMP inhibited the sustained phase of carbachol contractions by −80%. The initial peak was less inhibited (−30%). This relaxation was associated with a reduction in intracellular [Ca\textsuperscript{2+}] and decreased Ca\textsuperscript{2+} sensitivity. Contractions of cGKI−/− ileum were not influenced by 8-Br-cGMP. EC\textsubscript{50}\textsuperscript{1} for 8-Br-cGMP for PKG was estimated to be 10 nM. PKG-independent relaxation by 8-Br-cGMP had an EC\textsubscript{50} of 10 μM. Relaxation by cAMP (−50% at 100 μM), Ca\textsuperscript{2+} sensitivity of force, and force potentiation by GTPγS was similar in cGKI+/+ and cGKI−/− tissues. The results show that PKG is the main target for cGMP-induced relaxation in intestinal smooth muscle. cGMP desensitizes the contractile system to Ca\textsuperscript{2+} via PKG. PKG-independent pathways are activated at 1000-fold higher cGMP concentrations. Relaxation by cAMP can occur independently of PKG. Long term deficiency of PKG does not lead to an apparent up-regulation of the cAMP-dependent pathways or changes in Ca\textsuperscript{2+} sensitivity.

The cGMP-dependent protein kinase (PKG)\textsuperscript{1} is the main mediator of nitric oxide (NO) induced relaxation of smooth muscle (1, 2). Although this pathway is well established, the cellular actions of PKG, NO, and cGMP are complex and not fully understood. In addition to the effects via PKG, both NO and cGMP can have direct effects on other systems in the cell, e.g. NO on cADP-ribose signaling or on Ca\textsuperscript{2+} handling (3, 4) or cGMP on ion channels. The effects of cGMP and PKG on intracellular Ca\textsuperscript{2+} have been shown in different smooth muscle tissues (5–7) and have been suggested to involve several mechanisms including effects on large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK\textsubscript{Ca}) channels (8–10), voltage-gated 1-type Ca\textsuperscript{2+} channels (11), and effects on Ca\textsuperscript{2+} handling by the sarcoplasmic reticulum (cf. Refs. 1 and 2). In addition to direct effects on intracellular [Ca\textsuperscript{2+}], cGMP and PKG also modulate the sensitivity of the smooth muscle contractile system to Ca\textsuperscript{2+}. Nitroglycerine and nitroprusside, which increase cellular cGMP levels, and the cell-permeable cGMP analogue 8-Br-cGMP lower force at constant [Ca\textsuperscript{2+}] in high K\textsuperscript{+}–activated vascular and tracheal tissue (12, 13). Experiments on chemically skinned preparations where effects on Ca\textsuperscript{2+} sensitivity can be directly examined have revealed that cGMP lowers force at constant [Ca\textsuperscript{2+}] (14–16). The mechanisms involved in this Ca\textsuperscript{2+} desensitization are not fully resolved but are considered to be the result of an activation of the myosin light chain phosphatase (16, 17), possibly via effects on RhoA (18), Telokin (19), and/or CPI-17 (20).

A cross-talk between the cGMP and cAMP pathways has been proposed to exist. This can involve cross-activation of PKG by cAMP and of cAMP-dependent protein kinase (PKA) by cGMP as well as actions of the nucleotides or the kinases on phosphodiesterases (21–24).

Recently, a mouse strain lacking cGKI, which is the PKG isoform normally present in smooth muscle, was generated (7). Intact smooth muscles of these animals are not relaxed by NO or cGMP, but their responses to cAMP are essentially unaltered (7, 8, 25). However, a detailed characterization of the cGMP and cAMP dependencies is difficult in intact tissue in which diffusion and metabolism of nucleotide analogues might influence the responses, and the effects on Ca\textsuperscript{2+} sensitivity cannot be directly assessed and distinguished from effects on intracellular Ca\textsuperscript{2+} levels.

We have explored cGMP- and cAMP-dependent relaxation mechanisms of smooth muscle using PKG-deficient (cGKI−/−) and wild type (cGKI+/+) mice. The effects of cGMP on intracellular [Ca\textsuperscript{2+}] and force were examined in intact preparations of small intestine. Using α-toxin-permeabilized preparations in which the concentration of free Ca\textsuperscript{2+} can be held constant, the effects of cGMP and cAMP on Ca\textsuperscript{2+} sensitivity were examined. The following questions were addressed. 1) Does cGMP-mediated relaxation of agonist-induced contractions of intact intestinal smooth muscle involve a reduction of intracellular [Ca\textsuperscript{2+}], a decreased Ca\textsuperscript{2+} sensitivity, or both? 2) Is cGMP-induced relaxation of intestinal smooth muscle dependent on PKG? 3) Are cGMP and cAMP able to desensitize the contractile machinery to Ca\textsuperscript{2+} in the absence of PKG? 4) In what concentration range can cGMP desensitize the contractile system to Ca\textsuperscript{2+} via PKG-independent (e.g. PKA) pathways? (5) To what extent...
can the smooth muscle cells compensate via up-regulation of the related PKA pathway or via resetting of the light chain phosphorylation/dephosphorylation systems for a chronic loss of PKG-activity? A preliminary report of some of these results has been presented previously (26).

**EXPERIMENTAL PROCEDURES**

**Animals and Smooth Muscle Preparations—** PKG knock-out mice (cGKI−/−) and their wild type controls (cGKI+/+) were obtained as described previously (7). The animals were killed by cervical dislocation. The distal part of the small intestine was cut in centimeter-long pieces. The longitudinal (external) muscle layer was peeled off using microscope and forceps and mounted for experiments, intact or chemically skinned.

**Experiments on Intact Preparations—** Muscle strips measuring 4 × 1 mm with a thickness of ~0.05 mm were cut using microscissors and stainless steel surgical blades. The strips were mounted in open organ baths at 22 °C using thin silk thread between a stainless steel pin on an adjustable stand and a Grass FT03 transducer (Grass Instruments, Freeport, IL) for recording of isometric force. The preparations were equilibrated in physiological saline solution (PSS) (solution composition see below) with 1.6 mM Ca2+ for 1 h at a passive tension corresponding to optimal length for active force. In each muscle preparation, a reference contraction was recorded after adding 10 μM carbachol to the PSS. We measured the initial peak (after ~50 s) and the sustained force (after 20 min) of the contractions. The initial peak force was used as reference for all of the latter force responses. The strips were then relaxed and treated with 100 μM 8Br-cGMP (a cell-permeable cGMP analogue). Preliminary experiments showed that the effect of 8-Br-cGMP was obtained within 5 min. Therefore, we treated the preparations with 8-Br-cGMP for 20 min to ensure full intracellular effect of 8-Br-cGMP. Preparations were then activated with 10 μM carbachol (100 μM 8-Br-cGMP still present). Peak and sustained forces were measured and expressed relative to the reference force. These experiments were carried out on wild type (cGKI+/+) and knock-out mice (cGKI−/−) examined in parallel.

**Measurements of Intracellular [Ca2+].—** Fura-2 technique was used to measure intracellular Ca2+ changes as described previously (27, 28). Muscle strips were incubated in 0.5 ml of PSS with 10 μM acetoxy methyl ester of fura-2 (Fura-2/AM) dissolved in Me2SO premixed with Pluronic F127 (final concentrations, 1 and 0.02%, respectively). Prior to loading, the preparations were kept in PSS at 4 °C for 30 min to minimize non-cytotoxic compartmentalization of Fura-2/AM (29). Loading was performed under dark conditions at 22 °C for 4 h with gentle shaking. After loading, the muscle strips were thoroughly washed in PSS and mounted with aluminum foil clips on a myograph for measuring isometric tension (DSC-60E-4-110-force transducer, Eurosonor, London, United Kingdom) in a 1-ml cuvette (22 °C). The set-up was maintained at the temperature of an inverted Nikon (TMD) microscope. The preparation was illuminated with alternating wavelengths (340 and 380 nm), and the emitted fura-2 fluorescence was measured through a 500-nm interference filter (bandwidth, 40 nm) and recorded via a ×10 objective using a photomultiplier (R 374, Hamamatsu Photonics). The fluorescence and force signals were recorded with an Analog Devices RTI board and a personal computer. The fluorescence intensities were integrated, and a 340/380 intensity-ratio value was obtained approximately every 50 ms. After mounting, the strips were allowed to equilibrate for ~30 min. They were then stimulated with carbachol (10 μM). Fluorescence signals and force were recorded during the initial 4 min of the contraction and again after 25 min of stimulation. The strips were then relaxed in PSS, and fluorescence and force were recorded for 1 min. The strips were thereafter treated with 8-Br-cGMP (100 μM) for 20 min followed by a second stimulation with carbachol (10 μM) and recording of fluorescence and force.

**Experiments on α-Toxin-permeabilized Preparations—** The small strips were wrapped at both ends with aluminum foil and mounted horizontally on the extended arm of a force transducer (AE 801, SensoNor a.s., Horten, Norway) and a steel pin enabling length adjustment using small holes in the aluminum. The experiments were performed at room temperature (22 °C) in the surface tension bubble of small plastic cups containing 140 μl of solution. The solution was continuously stirred throughout the experiments using small stirring magnets. The PSS and solutions were equilibrated for approximately 45 min before the HEPES-buffered PSS (see below) before a test contraction using 10 μM carbachol was made. The preparations were permeabilized using α-toxin essentially as described previously (30). The strips were thoroughly relaxed in Ca2+-free HEPES-buffered PSS for 10 min. The preparations were transferred to relaxing solution for 5 min before being treated with relaxing solution with Staphylococcus aureus α-toxin (5000–10,000 units/ml) for 45 min. Finally, the preparations were kept 25 min in α-toxin-free relaxing solution containing 10 μM of the calcium ionophore A23187.

After the α-toxin-skinnning procedure, a reference contraction was recorded in contracting (pCa 4.5) solution. Each strip was held in relaxing solution for 10 min before being exposed to increasing [Ca2+] (pCa 7.0, 6.8, 6.6, 6.4, 6.2, 6.0, and 4.5) for 6 min at each concentration or until a steady force level had been reached. Force was expressed relative to the maximal contraction at pCa 4.5. In separate experiments, permeabilized strips were activated with intermediate [Ca2+] (pCa 6.2) resulting in a submaximal sustained contraction. At the plateau of the contraction, increasing concentrations of 8-Br-cGMP or the cAMP analogue cBIMPS were added to the solution. The drop in force induced by 8-Br-cGMP and cBIMPS was expressed relative to force of the submaximal contraction. In another set of experiments, permeabilized submaximally activated (pCa 6.2) preparations were treated with 10 μM GTP·S. The rise in force induced by GTP·S was expressed relative to force of the submaximal contraction.

**Solutions—** The HEPES-buffered PSS contained (in mM) NaCl 118, KCl 5, Na2HPO4 1.2, MgCl2 1.2, CaCl2 1.6, HEPES 24, and glucose 10. The pH was adjusted to 7.4. The Ca2+-free PSS was made by omitting CaCl2. Prior to use, the PSS was gassed with 100% O2 for at least 15 min. The solutions used for permeabilized preparations were stored at −20 °C until usage. The relaxing solution (pCa 9) contained (in mM) TES 30, phosphocreatine 10, Na2ATP 5.14, magnesium acetate 7.92, K*methanesulfonate 46.6, and EGTA 10. The maximal contracting solution was made by replacing EGTA with CaEGTA. Ionic strength and free [Mg2+] were held constant by varying [K∗-methanesulfonate] and [magnesium acetate]. The pH was adjusted to 7.1 at room temperature. [Ca2+] was varied by mixing relaxing and contracting solutions and thereby obtaining the desired CaEGTA/EGTA ratio (30, 31).

**Statistics—** All of the values are given as mean ± S.E. with the number of observation within parentheses. Curve fitting was performed using routines in SigmaPlot 2000 (SPSS Inc.). Analysis of variance was performed using Statistica for Windows (StatSoft Inc.).

**RESULTS**

Force recordings from intact longitudinal muscle preparations from the small intestine of wild type (cGKI+/+) and PKG-deficient (cGKI−/−) mice are shown in Fig. 1, panel A. Carbachol (10−5 M) evoked a sustained contraction in both cGKI+/+ and cGKI−/− preparations. In the cGKI+/+ mice, a second activation in the presence of 100 μM 8-Br-cGMP resulting in a transient contraction with an initial peak response followed by a sustained force component was markedly inhibited (to ~20% of the maximal force) and replaced by irregular phasic contractions. The peak force was slightly decreased (to ~70% of the reference force). In cGKI−/− mice, no effect of 8-Br-cGMP was observed. Fig. 1, panel B, summarizes these results.
To explore the mechanisms involved in the cGMP inhibition of force, we measured changes in intracellular \([Ca^{2+}]\) and force in preparations from cGKI\(^{+/+}\) mice before and after treatment with 100 \(\mu M\) 8-Br-cGMP. Fig. 2 shows the transients in force and the 340/380-nm light intensity ratio of fura-2 as a measure of changes in intracellular \([Ca^{2+}]\). Force after the initial peak was inhibited by 8-Br-cGMP (Fig. 2, panel A). These changes were associated with lowered intracellular \([Ca^{2+}]\) (Fig. 2, panel B). To obtain a measure of the changes in \([Ca^{2+}]\) sensitivity of force, we related the force values to the activation-induced increase in 340/380-intensity ratio. The activation-induced increase was calculated by subtracting the fura-2 ratio determined in the relaxed state between contractions from all of the values recorded after activation. As seen in Fig. 2, panel C, the ratio of active force to the fura-2 signal decreased after 8-Br-cGMP treatment, suggesting that the lower sustained force after 8-Br-cGMP treatment involved a decreased sensitivity to \([Ca^{2+}]\) as well as a decrease in total intracellular \([Ca^{2+}]\).

As seen in Fig. 2, panel A, activation with carbachol in the absence of 8-Br-cGMP was associated with a second peak of contraction after \(\sim 50\) s where the increase in force occurred without an increase in intracellular \([Ca^{2+}]\) (Fig. 2, cf. panels B and C). In the presence of 8-Br-cGMP, the second peak was still present and at approximately the same point in time (note that error bars in the time-direction are within the size of the symbols).

The calcium dependence of force was identical in cGKI\(^{+/+}\) and cGKI\(^{-/-}\) preparations permeabilized with Staphylococcus aureus alpha-toxin (Fig. 3A). Force at saturating \([Ca^{2+}]\) was \(\sim 50\%\) of equal magnitude to high K\(^+\) (80 mM KCl) induced responses in the intact muscle for both the cGKI\(^{+/+}\) and cGKI\(^{-/-}\) groups.

At submaximal \([Ca^{2+}]\) (pCa 6.2), the addition of 10 \(\mu M\) GTP\(_{\gamma}\)S was associated with a 2-fold increase in force, indicating increased sensitivity to \([Ca^{2+}]\). The responsiveness to GTP\(_{\gamma}\)S was similar in cGKI\(^{+/+}\) and cGKI\(^{-/-}\) preparations (Fig. 3B).

The relaxant effects of 8-Br-cGMP and the cAMP analogue cBIMPS were examined at a submaximal \([Ca^{2+}]\) concentration (pCa 6.2), giving \(-50\%\) of the force at saturating \([Ca^{2+}]\). As seen in the Fig. 4, upper panel, the cGKI\(^{+/+}\) preparations relaxed in a dose-dependent manner in response to increasing concentrations of the cGMP analogue. If cGKI\(^{+/+}\) or cGKI\(^{-/-}\) preparations were exposed to 100 \(\mu M\) 8-Br-cGMP at maximal \([Ca^{2+}]\) (pCa 4.5), no effect on force was noted, suggesting that the inhibition of force observed at submaximal \([Ca^{2+}]\) was because of a change in \([Ca^{2+}]\) sensitivity rather than a general effect on force generation. Preparations from cGKI\(^{-/-}\) mice relaxed to a small extent in response to maximal [8-Br-cGMP] (100 \(\mu M\)). Similar experiments were performed using the cAMP analogue cBIMPS.

Fig. 5 summarizes the effects of 8-Br-cGMP and cBIMPS on force at submaximal \([Ca^{2+}]\). The cGKI\(^{+/+}\) preparations relaxed...
in response to 8-Br-cGMP with an apparent half-maximal response (EC\textsubscript{50}) of \(50 \text{ nm} \). The cGKI\textasciitilde preparations relaxed slightly at the highest concentrations of 8-Br-cGMP. The estimated EC\textsubscript{50} for 8-Br-cGMP (Fig. 5, panel C) was \(>250 \) times higher (EC\textsubscript{50} \(10 \text{ \mu M} \)), and the maximal extent of relaxation was \(50\%\) in the cGKI\textasciitilde compared with the cGKI\textasciitilde preparations. The relaxant responses to 8-Br-cGMP in the cGKI\textasciitilde preparations (Fig. 5, open circles, panel A) deviated from the fitted dose-response relationship. No saturation was reached at the highest concentrations. If we assume that the additional relaxation at higher 8-Br-cGMP concentrations is the result of a cGKI-independent mechanism similar to that responsible for the small relaxation in the cGKI\textasciitilde preparations, we can obtain an estimate of the 8-Br-cGMP dependence of the cGKI by correcting for this component. Therefore, cGMP responses from cGKI\textasciitilde were subtracted from the cGKI\textasciitilde responses, rendering a modified relationship (Fig. 5, panel A, open squares), which was adequately described by a hyperbolic relationship with EC\textsubscript{50} of \(10 \text{ nm} \). The CAMP analogue cBIMPS relaxed both cGKI\textasciitilde and cGKI\textasciitilde preparations with an EC\textsubscript{50} of \(1 \text{ \mu M} \) to a similar maximal extent in both the cGKI\textasciitilde and cGKI\textasciitilde preparations. In cBIMPS experiments, concentrations of 1, 10, and 100 \(\mu M\) were examined. The lowest concentration gave \(50\%\) of the relaxant response, and no differences were observed between the cGKI\textasciitilde and the cGKI\textasciitilde preparations. The maximal extent of relaxation at saturating [cBIMPS] was identical in the two groups.

**DISCUSSION**

The relaxant effect of cGMP in intact smooth muscle of the small intestine involves a decrease in intracellular [Ca\textsuperscript{2+}] as well as a reduced Ca\textsuperscript{2+} sensitivity of the contractile activation. We show using \(\alpha\)-toxin-permeabilized preparations of cGKI\textasciitilde mice that the Ca\textsuperscript{2+}-desensitizing effect of 8-Br-cGMP in small intestinal smooth muscle is dependent on activation of PKG. In the lower cGMP concentration range (<1 \(\mu M\)), the tissues lacking PKG did not relax. The experiments on cGKI\textasciitilde tissues enabled us to correct for PKG-independent effects without using pharmacological tools, and we determined the EC\textsubscript{50} value for 8-Br-cGMP to \(10 \text{ nm} \) in the intestinal smooth muscle. This EC\textsubscript{50} value corresponds well to the apparent activation constants (\(K_a\)) estimated for the purified cGMP kinase (18 and 71 nm, for types I and II (32) and with the EC\textsubscript{50} value in permeabilized rabbit femoral artery preparations (8 nm) (17). At high concentrations (10 and 100 \(\mu M\)), a small relaxant effect of 8-Br-cGMP was also observed in cGKI\textasciitilde animals. One likely mechanism is that 8-Br-cGMP activates CAMP-dependent kinase at these higher concentrations. Using the cGKI\textasciitilde tissues where PKG is completely absent, we estimate the EC\textsubscript{50} value for this mechanism to be \(10 \text{ \mu M} \), which is consistent with the \(K_a\) values of 8-Br-cGMP for purified CAMP-dependent kinase (6.3 \(\mu M\)) (32). It has been suggested that the action of CAMP is partially mediated by the activation of PKA (23). Our data from intestinal muscle show that a maximal Ca\textsuperscript{2+} desensitization via PKG can be achieved at concentrations not activating PKG-independent pathways. In view of the almost 1000-fold difference in EC\textsubscript{50} values, it seems unlikely that a substantial cross-activation of PKA by CAMP contributes to Ca\textsuperscript{2+} desensitization. However, in extreme situations, e.g. in response to large doses of NO donors, CAMP appears to be able to reach levels high enough to activate PKA and contribute to relaxation by desensitization (8). Because CAMP, even at 100 \(\mu M\), failed to relax intact carbachol activated cGKI\textasciitilde tissues, we conclude that PKA and other PKG-independent relaxation mechanisms are not involved in the 8-Br-cGMP-induced relaxation in this case.

It has also been suggested that CAMP can cross-activate PKG and that PKG activation might contribute to the responses at
Figure 5. Dose-dependence of 8-Br-cGMP (panel A) and cBIMPS (panel B) effects on force at intermediate [Ca\(^{2+}\)] (pCa 6.2) in small intestinal preparations from cGKI\(^{+/+}\) (open symbols) and cGKI\(^{-/-}\) (filled symbols) animals is shown. The force values are expressed relative to the force level before the addition of 8-Br-cGMP and cBIMPS. A hyperbolic equation, \( F = 1 - A \times (E_{50} + c) \), was fitted to the force (F) and concentration (c) data of the individual experiments to obtain estimates of the concentration giving half-maximal relaxation (E_{50}) and the extent of the relaxation (A). The mean E_{50} values are shown in panel C. The extent of relaxation (parameter A) for 8-Br-cGMP was 0.41 and 0.25 for cGKI\(^{+/+}\) and cGKI\(^{-/-}\), respectively. The A value for cBIMPS was ~0.5 in both groups. The curves inserted in panels A and B show fits of the equation above to the mean values. The open squares show data obtained by subtracting the relaxant responses in the cGKI\(^{-/-}\) group from the cGKI\(^{+/+}\) data at respective concentrations. A fit of the equation to these data gave an E_{50} value of 11 nM and an A value of 0.32. * and ** indicate significant differences (Student’s t test) compared with cGKI\(^{+/+}\) group; n = 4–7.

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