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Cyclic cytidine 3′,5′-monophosphate (cCMP) signals via cGMP kinase I

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ABSTRACT

We analysed the function and intracellular signalling of the cyclic pyrimidinic nucleotide cCMP. The membrane-permeable cCMP analogue dibutyryl-cCMP mediated mouse aorta relaxation. cCMP activated purified cGMP-dependent protein kinase (cGK) Iα and Iβ and stimulated cGK in aorta lysates. cCMP-induced relaxation was abolished in cGKI-knockout tissue. Additionally, deletion of inositol–trisphosphate receptor associated cGKI substrate (IRAG) suppressed cCMP-mediated relaxation. Signalling of cCMP via cGKI/IRAG appears to be of broader physiological importance because cCMP-mediated inhibition of platelet aggregation was absent in cGKI- and IRAG-deficient platelets. These results demonstrate that cCMP acts as intracellular messenger molecule, most unexpectedly utilizing the cGMP signal transduction pathway.

1. Introduction

The cyclic nucleotides cAMP and cGMP are established second messengers which are essential for signalling in various mammalian tissues. However, the occurrence of further cyclic nucleotides acting as second messenger has not yet been established. Analysis of purine cyclic nucleotides has resulted in several innovative pharmacological strategies [1]. Formation of the cyclic pyrimidine nucleotide cyclic cytidine 3′,5′-monophosphate (cCMP) by cytidylyl cyclases was previously postulated in mammalian tissues including smooth muscles, blood cells, heart and brain [2,3] but was debated [4]. Functions regulated by cCMP are largely unknown. However, cCMP has been implicated in the control of cell growth and blood cell function [5,6]. Furthermore, intracellular signal transduction pathways mediated by cCMP have not yet been identified. In this report we investigated the effect of a potential third second messenger, cCMP, on intact tissues and cells, namely vascular smooth muscle and platelets, and on purified cyclic nucleotide-dependent protein kinases. Furthermore, for elucidation of cCMP signalling pathways murine mutants of the cGK cascade were analysed.

2. Materials and methods

2.1. Materials

Cyclic nucleotides were purchased from BIOLOG, Bremen, Germany. Dibutyryl-cCMP (Biolog No. D-075) had a purity of >99.7%. Other reagents were purchased as indicated in the different sections.

2.2. Animal strains and maintenance

Wild-type, IRAG-deficient ($IRAG^{-/-}$) or SMIα-rescue mice were bred and analysed as described before [7,8]. Experimental protocols were approved by local authorities for animal research and were conducted according to German law for animal care and European Guidelines for Laboratory Animal Care.

2.3. Myography

Tension of thoracic aorta segments were analysed isometrically on a Myograph (Myograph 601, Danish Myo Technology, Aarhus, Denmark) as described previously [7]. Resting tension was set to 2 mN. After equilibration period, aortic rings were precontracted with 3 μM phenylephrine in the presence of 100 μM L-NAME (endothelial NO-synthase inhibitor, Sigma). Effects of submaximal

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relaxation-doses of db-cCMP, db-cAMP and 8-Br-cGMP on tension were determined.

2.4. Measurement of kinase activity

Aortic lysates were prepared and kinase activity was measured as described previously [8]. In brief, reaction was started by adding 20 μl of protein extract to reaction mix (50 mM MES, pH 6.9, 0.4 mM EGTA, 1 mM Mg-acetate, 10 mM NaCl, 0.1% (w/v) BSA, 10 mM DTT, 40 μM substrate peptide VASPtide (Sequence: RRKVSKQE), 2 μM cAK-inhibitor peptide, 0.1 mM [γ-^{32}P]ATP (100 cpm/pmol) ± cyclic nucleotide at different concentrations. Reaction mixtures were incubated for 15 min at 30 °C and then transferred on Whatman P-81 papers (1.5 × 3 cm). Reactions were stopped by washing in 75 mM H_3PO_4. The dried papers were counted in Rotiszint scintillation liquid. Purified cGKI-isozymes were measured with 20 ng of purified enzyme and 5 min incubation time.

2.5. Determination of platelet aggregation

Blood from wild-type, IRAG$^{-/-}$ or SMIα-rescue mice (mice with cGKI$^{-/-}$-background and smooth muscle specific overexpression of cGKIα which have cGKI-deficient platelets) anesthetized by ether inhalation was collected by cardiac puncture into Alsever's buffer (Sigma). Platelets were isolated and analyzed as described [9] with the following modifications: preincubation of platelets (1.5 × 10^5 platelets/μl) with 200 μM db-cCMP for 10 min at 37 °C and aggregation initiation with thrombin (0.03 U/ml). Aggregation was measured by an optical aggregometer (Chronolog, Havertown, PA, USA) using Aggro/Link Software 5.1 (aggregation: maximal slope).

2.6. Statistical analysis

All data are expressed as mean ± S.E.M. For calculation of statistical differences between two means the unpaired *t*-test was used, for three means, the ANOVA was used. Significance of *P*-value was indicated by asterisks $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; n.s.: not statistically significant). N in or above bars indicates number of independent experiments.

3. Results

3.1. db-cCMP induces smooth muscle relaxation

The effect of cCMP on smooth muscle precontracted with phenylephrine (3 μM) was studied in isolated murine thoracic aorta. Wild-type tissue showed a strong relaxing response of 44 ± 3% to exogenously applied dibutyryl-cCMP (db-cCMP) (300 μM) a membrane-permeable and esterase-cleavable prodrug of cCMP (Fig. 1A and C). db-cCMP relaxed aorta with an EC_{50} of 479 ± 54 μM. As db-cCMP is a prodrug of cCMP, the effective EC_{50} concentration of cCMP inside smooth muscle cells is lower. All further aorta experiments were performed with 300 μM db-cCMP which is in the range of concentration previously used for physiological analysis of smooth muscle with db-cAMP or db-cGMP [10,11]. Addition of 300 μM 8-Br-cGMP, an established membrane-permeable and directly cGKI-activating cGMP analogue, resulted in smooth muscle relaxation of 61 ± 2% (Fig. 1B and C). The EC_{50} of 8-Br-cGMP was

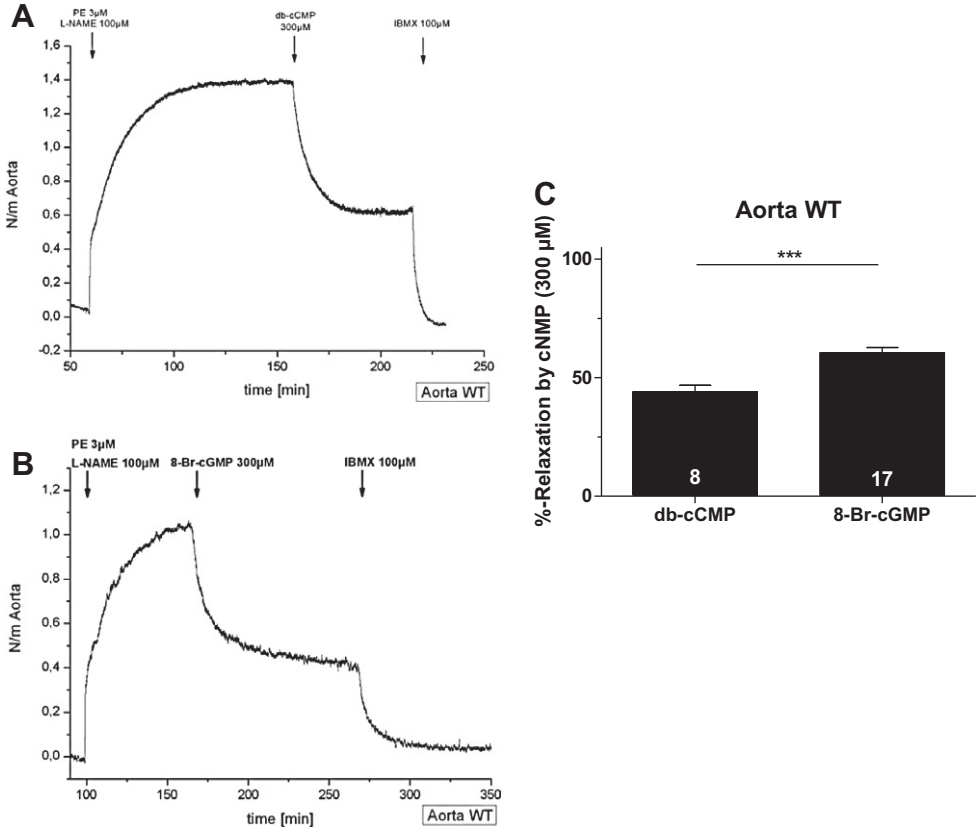

Fig. 1. Relaxation of murine wild-type vascular smooth muscle by cCMP. Phenylephrine-induced contraction of denuded wild-type (WT) aortic tissue with (A) db-cCMP (300 μM) and (B) 8-Br-cGMP (300 μM). (C) Summary of relaxation potency of db-cCMP and 8-Br-cGMP on precontracted aortic tissue ($^{***}P < 0.001$). Numbers in bars indicate number of independent experiments. Error bars denote S.E.M. PE: phenylephrine, IBMX: 3-isobutyl-1-methylxanthine (unspecific inhibitor of PDEs). L-NAME: N_{ω}-nitro-L-arginine methyl ester (endothelial NO-synthase inhibitor).

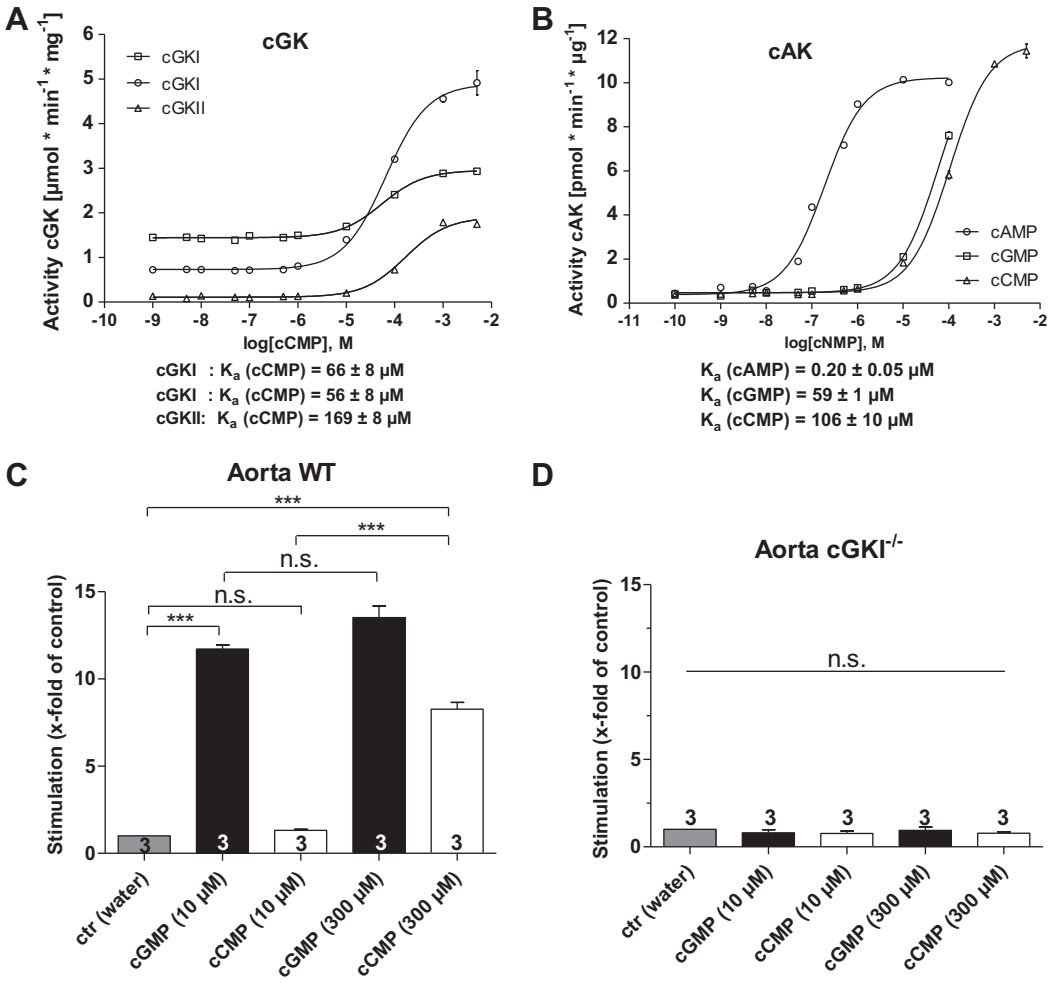

Fig. 2. Activation of cGKI and cAK by cCMP (A) in vitro concentration-response curves of purified bovine cGKI isozymes using cCMP as activator. (B) In vitro concentration–response curve of cAK (from bovine heart, Sigma) using cAMP, cGMP and cCMP. (C) Stimulation of endogenous cGKI in aortic tissue of wild-type animals after activation with water (ctr), cGMP (10, 300 µM) and cCMP (10, 300 µM). (D) Same panel as (C) using cGKI-deficient tissue. All dose–response curves of (A) and (B) were measured in three independent experiments. Values in bars indicate number of independent experiments. (***$P < 0.001$, n.s. $P > 0.05$), error bars denote S.E.M.

27 ± 9 µM. To exclude effects of the short chain fatty acid butyrate on second messenger signalling or vasodilatation [12] we applied butyrate or tributyrin on preconstricted aortic smooth muscle tissue. Butyrate and tributyrin did not influence contractility in µM range which corresponded to the concentrations taken for the experiments using dibutyryl-cCMP. But butyrate slightly enhanced contraction in mM range (Supplementary Fig. S1).

3.2. cCMP activates purified cGKs and cAK

We examined whether cCMP activates cAMP-dependent or cGMP-dependent protein kinases in vitro. Purified cGKIα and cGKIβ isozymes [13–15] or commercially available cAMP-dependent kinase (cAK) were used for peptide-specific radioactive phosphotransferase assays. The K_a (cCMP) for cGKIα and for cGKIβ were 66 ± 8 µM and 56 ± 8 µM, respectively (Fig. 2A). db-cCMP did not activate cGKI isozymes (Supplementary Fig. S2). The K_a (cGMP) and K_a (cCMP) for cAK were 59 ± 1 µM and 106 ± 10 µM, respectively (Fig. 2B). cGMP concentration–response curves for activation of cGKI isozymes revealed no leftward shift by cCMP (10 µM) (Supplementary Fig. S3), indicating that sensitivity of cGKI-stimulation by cGMP was not changed by cCMP. However, enhanced activity of cGKI at submaximally effective concentrations of cGMP in presence of cCMP (10 µM) indicated that cGMP-dependent activity of purified cGKI isozymes was enhanced by addition of low cCMP concentrations.

3.3. cCMP activates cGKI in aortic tissue lysates

We analysed whether cCMP activates endogenously expressed cGKI, determining phosphotransferase activity of wild-type aortic lysates (Fig. 2C). cCMP at concentrations also used for myograph experiments (10 and 300 µM) stimulated substrate peptide (VASP-tide) phosphorylation by cGKI. Phosphorylation by cAK during these experiments was excluded by addition of PKA_{5-24}-inhibitor peptide. cGMP (10 µM) induced 12-fold stimulation, cGMP (300 µM) a 13.5-fold stimulation. cCMP (10 µM) resulted in 1.5-fold stimulation, and cCMP (300 µM) exhibited more than 8-fold stimulation. In contrast, cGKI-deficient aortic lysates exhibited no stimulated phosphorylation of substrate peptide either with cGMP (10 and 300 µM) or with cCMP (10 and 300 µM) (Fig. 2D), suggesting that cGKI achieves cGMP- and cCMP-induced substrate phosphorylation.

3.4. db-cCMP induces smooth muscle relaxation via cGKI/IRAG signalling

In the next set of experiments we examined whether cGKI is involved in cCMP-mediated vascular smooth muscle relaxation using

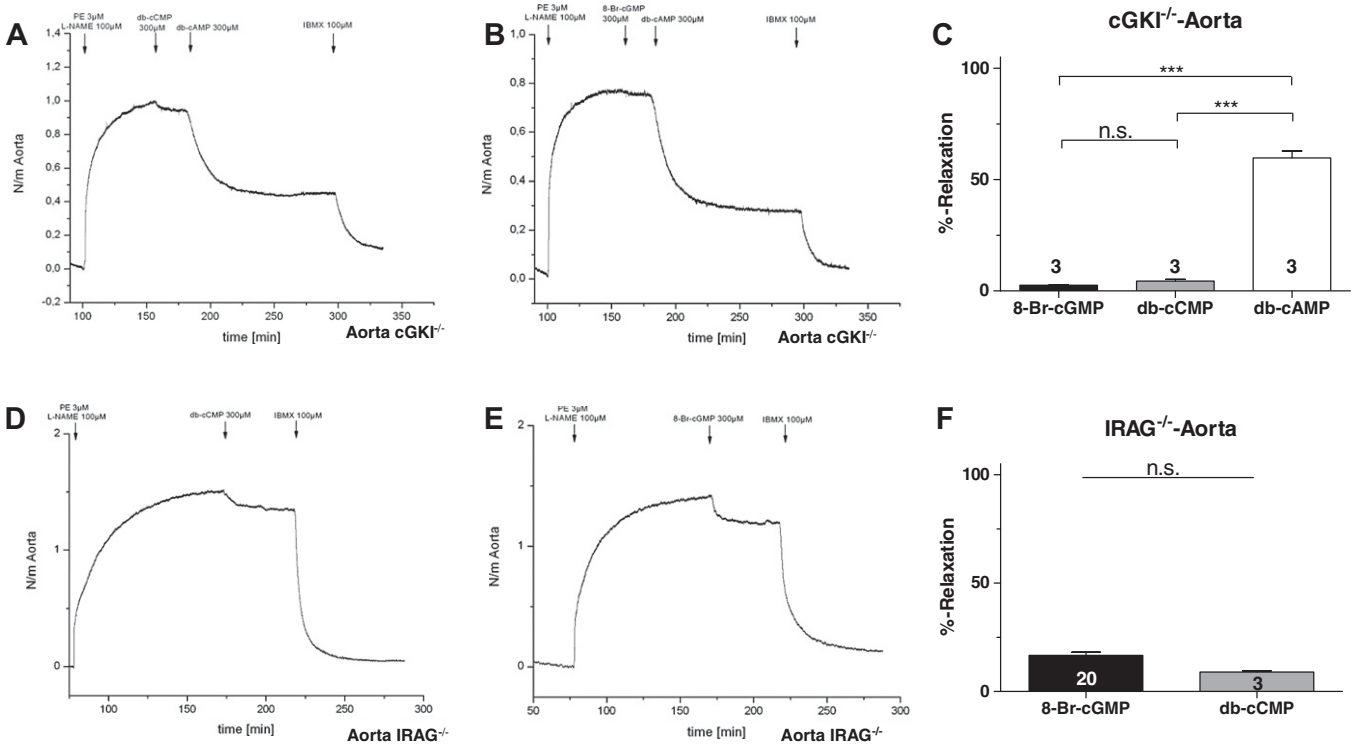

Fig. 3. Strongly reduced cCMP-induced relaxation of cGKI- or IRAG-deficient vascular smooth muscle. Relaxation of phenylephrine (PE)-precontracted cGKI$^{-/-}$ aortic tissue with (A) db-cCMP (300 μM) and (B) 8-Br-cGMP (300 μM). Relaxation was initiated by adding db-cAMP (300 μM), revealing that the cAMP-cascade was not affected. (C) Statistical analysis of experiments shown in (A) and (B). (D and E) Same experiments as in (A) and (B) with IRAG$^{-/-}$ tissue. Total relaxation was induced by application of IBMX (100 μM). (F) Statistical analysis of myograph experiments with IRAG$^{-/-}$ aortic tissue. Values in bars indicate number of independent experiments (***$P < 0.001$, n.s. $P > 0.05$), error bars denote S.E.M.

cGKI$^{-/-}$- aortic tissue (Fig. 3). As described [16], 8-Br-cGMP had almost no effect on precontracted cGKI-deficient tissue (2.5 ± 0.2 %). Interestingly, this tissue showed a much smaller response to db-cCMP than wild-type tissue (cGKI$^{-/-}$: 4.3 ± 0.8 %). To prove that cAMP signalling was still intact in cGKI$^{-/-}$-tissue, we added db-cAMP (300 μM) which relaxed nearly to basal tone. This observation also showed that 8-Br-cGMP and db-cCMP (300 μM each) had only a small cross-activating effect on cAMP. To further analyse the role of db-cCMP in cGKI signalling we tested its effect on IRAG-deficient tissue. IRAG is a substrate of the cGKIβ-isozyme which is essential for NO- and atrial natriuretic peptide-mediated smooth muscle relaxation [7,17]. IRAG-deficient smooth muscle tissue exhibited strongly impaired cGMP-mediated relaxation [7] which was also shown using 8-Br-cGMP (efficacy reduced to 16.7 ± 1.5%) (Fig. 3E). The relaxing effect of db-cCMP was substantially reduced (8.9 ± 0.5%) in IRAG-deficient tissue (Fig. 3D and F). Accordingly, SMIα-rescue tissue (cGKI$^{-/-}$-background with smooth muscle specific overexpression of cGKIα, i.e. cGKIβ-KO) [8] also had a defect in smooth muscle relaxation after db-cCMP addition in comparison to wild-type tissue (data not shown). Additionally, we analysed whether a rise in cGMP levels is provoked by cCMP via inhibition of phosphodiesterase (PDE) 5A, which could lead to smooth muscle relaxation (Supplementary Fig. S4). Precontracted aortic wild-type tissue was preincubated for 30 minutes with 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylyl cyclase inhibitor. This should lead to reduction of intracellular cGMP due to degradation by PDEs. Subsequent PDE5 inhibition with zaprinast mediated reduced relaxation in comparison to zaprinast relaxation without ODQ preincubation. ODQ preincubation with subsequent db-cCMP administration had no effect on relaxation in comparison to db-cCMP addition alone. These results imply that a reduced cGMP level does not alter cCMP-mediated smooth muscle relaxation. Therefore, cGMP does not determine relaxation by cCMP.

Another hypothesis was that cCMP triggers cGKI activation via cGMP. This could be caused either by direct cCMP-binding to one of two cyclic nucleotide binding sites of cGKI or indirectly by inhibition of a cGMP–PDE. Therefore, we examined whether db-cCMP at a submaximally effective concentration of 10 μM enhances relaxation by 8-Br-cGMP (Supplementary Fig. S5). Phenylephrine-precontracted wild-type tissue was preincubated with db-cCMP for 15 minutes, followed by addition of 8-Br-cGMP. However, db-cCMP had no effect on intensity of relaxation suggesting that cCMP does not modulate cGMP-activation of cGKI.

3.5. db-cCMP inhibits platelet aggregation via cGKI/IRAG signalling

db-cCMP strongly inhibited platelet aggregation (aggregation: 36.6 ± 6.5% of control $N = 5$). cGKI/IRAG signalling is mainly involved in cGMP-mediated inhibition of platelet aggregation [9]. Hence, we studied whether cCMP signals via cGKI in isolated platelets. The inhibitory effect of db-cCMP was suppressed in cGKI- or in IRAG-deficient platelets (aggregation: 88.3 ± 5.7% of control $N = 7$; 103.5 ± 9.3% of control $N = 6$, respectively) (Fig. 4).

4. Discussion

Using purified enzymes and isolated vascular smooth muscle and platelets we revealed that cCMP acts as signalling molecule which utilizes the cGKI signalling pathway. Other groups reported potential cross-activation of cGMP- and cAMP-signalling pathways via cAMP or cGMP, respectively, [18,19]. However, substantial activation of cAK by cCMP could be excluded because of strongly

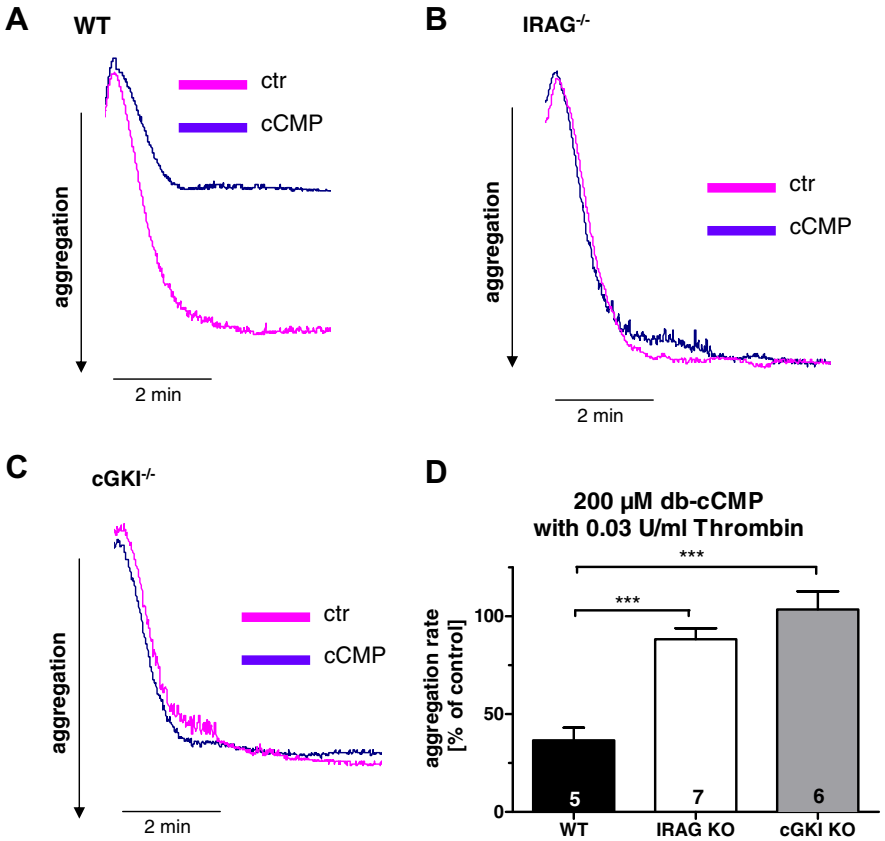

Fig. 4. cCMP inhibits platelet aggregation via cGKI/IRAG. Representative aggregation experiments (A–C) and statistics of aggregation rate (percentage of control) (D) of wild-type, IRAG- and cGKI-deficient platelets (WT, IRAG$^{-/-}$, cGKI$^{-/-}$) precincubated with 200 µM db-cCMP before aggregation initiation by thrombin (0.03 U/ml). Asterisks indicate statistical significant differences $^{***}P < 0.001$. Error bars denote S.E.M.

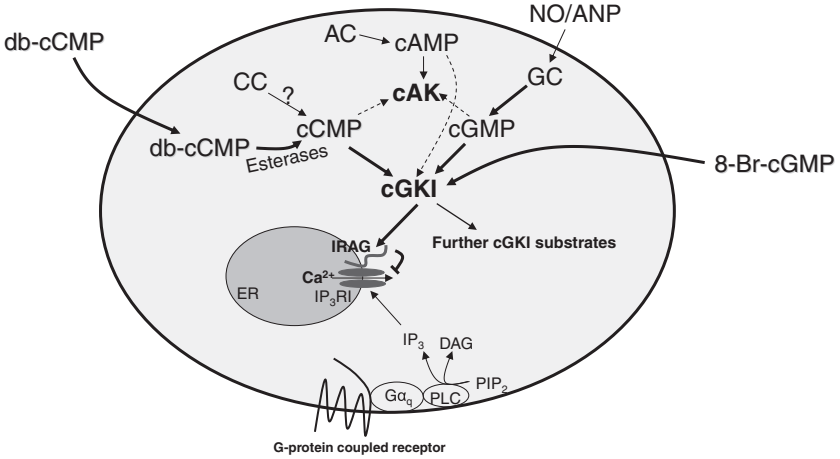

Fig. 5. CMP signalling cascade for smooth muscle relaxation. cCMP mediates smooth muscle relaxation via the cGKI signalling cascade. Murine mutant analysis revealed that the IP_3 receptor associated cGKI substrate (IRAG) inhibiting IP_3 receptor-mediated Ca^{2+} release mainly determines the cCMP/cGKI-induced smooth muscle relaxation. A tentative cytidylyl cyclase (CC) has not yet been identified. cGMP is synthesized by NO (nitric oxide)-activated soluble or ANP (atrial natriuretic peptide)-stimulated membrane bound guanylyl cyclases (GC), respectively. Cross-activation of cGKI by cAMP and of cAK by cGMP is known from other studies. However, activation of cAK by cCMP does not effectively compensate for defects in cGKI signalling. DAG, diacylglycerol; ER, endoplasmic reticulum; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate.

reduced relaxation of cGKI$^{-/-}$-aortae by db-cCMP. Nevertheless, the remaining about 4% relaxation induced by db-cCMP in cGKI$^{-/-}$ aortae could result from cAK.

We have no indication that inhibition of cGMP-stimulated phosphodiesterases including PDE5A alter relaxation by cCMP suggesting that PDE5A is not involved in hydrolysis of cCMP. Therefore, cCMP might accumulate in tissues and cells and, thereby mediate its pharmacological effects. Furthermore, there is no indication that cGMP concentration is effectively altered by db-cCMP application as pre-treatment by ODQ did not change the db-cCMP effect on smooth muscle. Previous work reported the presence of cCMP-phosphodiesterases in mammalian tissues [20]. Comparison

of the long-term relaxing effect of 8-Br-cGMP being completely PDE-resistant and db-cCMP revealed a slight time-dependent reduction of db-cCMP-induced relaxation (Supplementary Fig. S6). Therefore, it cannot be excluded that an as yet un-identified cCMP-PDE or cCMP transport proteins are present in smooth muscle. Interestingly, cCMP or cGMP induced a slight relaxation (Supplementary Fig. S7). In fact, cGMP transporters have already been reported [21,22].

The physiological role of the cCMP is still unclear. A tentative cytidylyl cyclase that synthesizes cCMP in tissues has not yet been identified. Occurrence of cytidylyl cyclase was reported previously but was debated [2,4,23]. The presence of cCMP and increase of cCMP concentration by ranitidine, prostacyclin and prostaglandin E-2 was reported in gastric mucosa [24]. Stability of cCMP against PDEs may result in sufficiently high cCMP concentrations that mediate physiological effects. cCMP-dependent phosphorylation was detected in mouse brain [25], and Rab23 was cCMP-dependently phosphorylated [26]. This observation could lead to identification of specific cCMP-dependent protein kinases which may, e.g. activate cGKI and thereby mediate the observed effects.

Here, we show that cCMP stimulates cGKI and thereby induces smooth muscle relaxation and inhibits platelet aggregation. A summary of our results concerning the cCMP signalling cascade in smooth muscle is given in Fig. 5. This discovery demonstrates that cCMP mediates an intracellular messenger function in specific tissues and cells and actually achieves its effects via the cGMP-signalling cascade. This is most unexpected, challenging the current view regarding base-specificity of cyclic nucleotide second messenger systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.07.059.

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