cGMP signals mainly through cAMP kinase in permeabilized murine aorta

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Wörner R, Lukowski R, Hofmann F, Wegener JW. cGMP signals mainly through cAMP kinase in permeabilized murine aorta. Am J Physiol Heart Circ Physiol 292: H237–H244, 2007. First published August 18, 2006; doi:10.1152/ajpheart.00079.2006.—GMP affects vascular tone by multiple mechanisms, including inhibition of the Rho/Rho kinase-mediated Ca2⁺ sensitization, a process identified as Ca2⁺ desensitization. Ca2⁺ desensitization is mediated probably by both cGMP- and cAMP-dependent protein kinases (cGKI and PKA). We investigate to which extent Ca2⁺ desensitization is initiated by cGKI and PKA. cGMP/cAMP-induced relaxation was studied at constant [Ca2⁺] in permeabilized aortas from wild-type and cGKI-deficient mice. [Ca2⁺] increased aortic tone in the absence and presence of 50 μM GTPγS with EC50 values of 160 and 30 nM, respectively. In the absence of GTPγS, the EC50 for [Ca2⁺] was shifted rightward from 0.16 μM to 0.43 and 0.82 μM by 1 and 300 μM 8-bromo-cGMP (8-Br-cGMP), and to 8 μM by 10 μM Y-27632. Contractions induced by 300 nM [Ca2⁺] were relaxed by 8-Br-cGMP with an EC50 of 2.6 μM. Surprisingly, [Ca2⁺]-induced contractions were also relaxed by 8-Br-cGMP in aortas from cGKI−/− mice (EC50 of 19 μM). Western blot analysis of the vasodilator-stimulated phosphoprotein indicated “cross”-activation of PKA by 1 μM 8-Br-cGMP in aortic smooth muscle cells from cGKI−/− mice. Indeed, the PKA inhibitor peptide (PKI 5–24) completely abolished the relaxant effect of 8-Br-cGMP in muscles from cGKI−/− mice and to 65% in wild-type aortas. The thromboxane analogue U-46619 induced contraction at constant [Ca2⁺], which was only partially relaxed by 8-Br-cGMP but completely relaxed by Y-27632. The effect of 8-Br-cGMP on U-46619-induced contraction was attenuated by PKI 5–24. These results show that cGKI has only a small inhibitory effect on Ca2⁺ sensitization in murine aortas.

calcium sensitization; Rho kinase; U-46619

PHOSPHORYLATION of the smooth muscle myosin regulatory light chain (RLC-20) correlates with smooth muscle tone and is determined by the balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activity. To initiate contraction, this balance is moved toward phosphorylation via activation of MLCK by an increased intracellular Ca2⁺ concentration ([Ca2⁺]) that is triggered by an action potential or contractile agonists (14, 23, 24). Contraction can also be initiated by certain agonists at constant [Ca2⁺], a phenomenon called Ca2⁺ sensitization, which increases phosphorylation by inhibition of MLCP activity mostly due to phosphorylation of the myosin phosphatase targeting subunit 1 (MYPT1) and/or the protein kinase C-potentiated inhibitor CPI-17 by Rho kinase (ROCK) and protein kinase C (PKC), respectively (9, 42).

Relaxation is achieved either passively by removal of the respective stimulus or actively by agonists that move the balance toward dephosphorylation via reducing [Ca2⁺] and increasing MLCP activity. The most prominent representative of those agonists in vascular smooth muscle is nitric oxide (NO), which relaxes mainly via production of the cyclic nucleotide cGMP and subsequent activation of cGMP-dependent protein kinase I (cGKI) (29). The involvement of cGKI in this cascade was verified by deleting the cGKI gene in mice (36). At least four different downstream pathways exist by which cGKI can promote relaxation (21). Intracellular [Ca2⁺] is reduced by 1) inhibiting Ca2⁺ release from intracellular stores via phosphorylation of the inositol receptor-associated G kinase substrate (IRAG) (16); 2) attenuating inositol (1,4,5)-trisphosphate [Ins(1,4,5)P3]-mediated Ca2⁺ release by calming the synthesis of Ins(1,4,5)P3 via modulation of the regulator of G protein signaling 2 (RGS-2), which regulates GTPase activity of Gq (44); and 3) hyperpolarization of the membrane potential and closure of L-type calcium channels via cGKI-induced increase in the open probability of Ca2⁺-activated K⁺ channels (BKCa) (1, 13, 38, 50). An alternative mechanism to induce relaxation at constant [Ca2⁺] is to increase MLCP activity, a process that has been described as Ca2⁺ desensitization (35, 42). cGMP/cGKI has been reported to increase the activity of myosin phosphatase leading to dephosphorylation of regulatory light chain (RLC) (9, 28, 49), probably via phosphorylation of MYPT1 (43, 49), albeit at another phosphorylation site than ROCK (49).

However, in recent times it became obvious that cGMP relaxed smooth muscle preparations in the absence of cGKI (3, 38). It was possible that some of the cGKI targets previously identified by NO-cGMP-dependent phosphorylation (6, 38, 41, 49) were phosphorylated by cGMP-activated, cAMP-dependent protein kinase (PKA). Consequently, we hypothesized that part of cGMP-mediated Ca2⁺ desensitization may be performed by activation of PKA rather than of cGKI. For this purpose, the contribution of cGKI and PKA to the regulation of vascular tone by cGMP was investigated at constant [Ca2⁺] in permeabilized murine aortic strips from wild-type and cGKI-deficient mice. The results show that cGKI only slightly affects Ca2⁺ desensitization in permeabilized aortic smooth muscle.

MATERIALS AND METHODS

All experiments complied with the animal protection laws of Germany. Wild-type mice and mice lacking cGMP-dependent protein kinase I (cGKI−/− mice) were bred as described (47). Mice were killed according German legislation, under a license granted from the German government (Regierung von Oberbayern, 2531-5/05) to conduct animal research as described in our study. The thoracic aorta was isolated and cleaned from connective tissue. Aortic rings of 5 mm in width were mounted to organ baths (Myograph 601, www.dmt.de) according to Wegener et al. (48). Resting tension was set to 4–5 mN. Tension was recorded isometrically at room temperature to minimize

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rundown of Ca\(^{2+}\)-induced contraction (19). Endothelium was removed by mechanical treatment. The absence of endothelium was verified by the missing relaxant effect of carbocarb (10 \(\mu\)M).

Permeabilization was performed as described (26). Briefly, rings were incubated in Ca\(^{2+}\)-free solution (in mM: 97 NaCl, 5.4 KCl, 6.47 MgCl\(_2\), 5 EGTA, 0.1 DTT, 5.5 Na\(_2\)ATP, 9.5 Na\(_2\)-creatine phosphate, 5 glutathione, 5.6 glucose, and 10 HEPEP; pH 7.4) with 520 U/ml of \(\alpha\)-toxin from *Staphylococcus aureus*, which produced nearly maximal contraction in rings incubated at 1 \(\mu\)M [Ca\(^{2+}\)]. The concentrations of free Ca\(^{2+}\) were reached by the addition of a specified amount of CaCl\(_2\) to the Ca\(^{2+}\)-free solution, the amount being calculated according to Brooks and Storey (4). In the experiments with the PKA inhibitor peptide (PKI 5–24), \(\beta\)-escin was used for permeabilization to allow free diffusion for molecules up to 17,000 Da as previously described (27, 45); 0.04\% bovine serum albumin was added to saturate unspecific protein binding sites. Contraction to 300 nM Ca\(^{2+}\) was 0.37 ± 0.04 (n = 36) and 0.23 ± 0.01 (n = 98) N/m in muscles permeabilized with \(\alpha\)-toxin and \(\beta\)-escin, respectively. The effect of 300 \(\mu\)M 8-bromo-cGMP (8-Br-cGMP; www.biolog.de) was not significantly different in \(\beta\)-escin- versus \(\alpha\)-toxin-permeabilized muscles (22 ± 2.6\%; n = 6 vs. 21 ± 4.4\% of control; n = 8).

Smooth muscle cells were isolated from the aortas of wild-type and cGKI\(^{-/-}\) mice by enzymatic digestion and grown in DMEM (Life Technologies) as previously described (10). Cells were kept in serum-free medium 2 days before the start of the experiments and used without any passage. Drugs or vehicle were applied for 30 min in serum-free medium.

Western blot analysis was performed by using polyclonal rabbit antisera detecting cGKI (15), Akt (cell signaling), and vasodilator-stimulated phosphoprotein (phospho-Ser\(^{117}\)/VASP; Alexis). All salts and substances were used as pure as commercially available and purchased from Sigma (www.sigma-aldrich.com) unless otherwise indicated. Drugs were applied as single quantity or cumulatively to achieve the concentrations as indicated.

Results are presented as original recordings, blots, or expressed as means ± SE. Tension generated by the muscle is expressed in Newtons per meter with meter corresponding to the length of the aortic ring or in percentage of Ca\(^{2+}\)-induced contraction. Effects of drugs were analyzed in steady-state conditions. Changes in tension were determined with respect to the resting tension in Ca\(^{2+}\)-free conditions. Statistical comparisons of data sets were performed by a repeated measurement design using Prism 4 software (www.graphpad.com). Differences were considered significant at \(P < 0.05\).

**RESULTS**

Ca\(^{2+}\)-induced contraction and cGMP-dependent relaxation in permeabilized aorta. Raising the extracellular [Ca\(^{2+}\)] to 1 \(\mu\)M had no effect on the tone of an intact aortic strip, whereas it induced a strong contraction in a permeabilized preparation. After permeabilization, aortic tone increased concentration dependently in response to a rise in [Ca\(^{2+}\)] with an EC\(_{50}\) of 0.16 \(\mu\)M free [Ca\(^{2+}\)] (Fig. 1). The concentration-response curve to Ca\(^{2+}\) was not different between aortas from control and cGKI\(^{-/-}\) mice (\(P > 0.05\); Fig. 1), confirming the results reported for intestinal smooth muscle (3). Addition of GTPyS (50 \(\mu\)M) shifted the dose-response curve to the left, resulting in an EC\(_{50}\) value of 30 nM free [Ca\(^{2+}\)] (Fig. 1). These experiments confirmed the presence of a Ca\(^{2+}\)-sensitizing mechanism in murine aorta.

The phosphodiesterase-resistant cGMP analogue 8-Br-cGMP (51) relaxed concentration dependently permeabilized aortic rings contracted by 300 nM [Ca\(^{2+}\)] (Fig. 2A) with an apparent EC\(_{50}\) of 2.6 \(\mu\)M (Fig. 2B). Contraction induced by low [Ca\(^{2+}\)] (200 nM) was more sensitive to 1 \(\mu\)M 8-Br-cGMP than contraction induced by high [Ca\(^{2+}\)] (10 \(\mu\)M) (Fig. 2C), indicating that 8-Br-cGMP is able to relax only those contractions mediated by moderate [Ca\(^{2+}\)] via desensitization. The [Ca\(^{2+}\)] needed to half maximally contract the aortic rings were shifted to higher concentrations when 8-Br-cGMP was added, demonstrating that cGMP induced Ca\(^{2+}\) desensitization of the aortic tone (Fig. 2C). The EC\(_{50}\) value was shifted from 160 to 430 nM and 820 nM in the presence of 1 and 300 \(\mu\)M 8-Br-cGMP, respectively. The Ca\(^{2+}\)-desensitizing effect of 8-Br-cGMP was weaker than that of the Rho kinase inhibitor Y-27632 (10 \(\mu\)M), which shifted the EC\(_{50}\) to 8 \(\mu\)M (Fig. 2C).

**Dependence of cGMP-induced relaxation on PKA.** 8-Br-cGMP (300 \(\mu\)M) relaxed contractions induced by 300 nM [Ca\(^{2+}\)] in permeabilized aorta not only from wild-type mice but also from cGKI\(^{-/-}\) mice that lack a functional cGKI (Fig. 3, A and B). Preincubation with PKI 5–24 (200 \(\mu\)M) attenuated the relaxant effect of 8-Br-cGMP in both preparations (Fig. 3, C and D). Maximal inhibition by PKI 5–24 was observed at 3 \(\mu\)M. At this concentration, 300 \(\mu\)M 8-Br-cGMP did not relax the tonus of aortic rings from cGKI\(^{-/-}\) mice [96 ± 2\% (n = 11) of control, \(P > 0.05\)] and only by 28 ± 3\% (n = 10) of those from wild-type mice (Fig. 3E). These results supported the hypothesis that the relaxant effects of 8-Br-cGMP at constant [Ca\(^{2+}\)] are mediated mostly by activation of PKA and only to a minor extent by cGKI. This interpretation was further strengthened by the cumulative concentration-response curves for 8-Br-cGMP shown in Fig. 3F. The fit of the data revealed that 8-Br-cGMP relaxed precontracted, permeabilized wild-type and cGKI\(^{-/-}\) aortas with EC\(_{50}\) values of 2.6 and 19 \(\mu\)M to values of 34 and 52\% of control, respectively. In the presence of 3 \(\mu\)M PKI 5–24, the fit showed that 8-Br-cGMP relaxed precontracted aorta with an EC\(_{50}\) of 0.7 \(\mu\)M to 57\% of control (Fig. 3F). This result demonstrates that cGKI affected relaxation at constant [Ca\(^{2+}\)] in murine wild-type mice aorta, although only to a small extent. In the next series of experiments, the effect of PKI 5–24 on Ca\(^{2+}\) desensitization induced by the phosphodiesterase-resistant cAMP analogue dibutyryl-cAMP (db-cAMP) and 8-Br-cGMP was studied. The concentration-response curve for Ca\(^{2+}\) in \(\beta\)-escin-permeabilized aorta was shifted to the right by db-cAMP (300 \(\mu\)M) as well as by 8-Br-cGMP (300 \(\mu\)M) but not in the presence of PKI 5–24 (3 \(\mu\)M) (Fig. 4). A similar shift was observed in the \(\beta\)-escin-permeabilized aorta of cGKI\(^{-/-}\) mice in the presence of 8-Br-cGMP that was abolished in the presence of PKI 5–24 (3
8-Br-cAMP (1 mM) induced phosphorylation of VASP in the absence of cGKI in vascular tissue, most probably via “cross”-activation of PKA. Stimulation of β-escin-permeabilized tissue with 1 mM 8-Br-cGMP likewise induced phosphorylation of VASP, which was attenuated in the presence of 3 μM PKI 5–24 (not shown).

**cGMP-induced relaxation after G-protein activation at constant [Ca\(^{2+}\)].** As noted previously (3, 26, 42) and shown in Fig. 1C, activation of G proteins increased contractions at constant [Ca\(^{2+}\)]. Stimulation of the permeabilized aorta with the stable thromboxane analogue U-46619 (30 μM) and GTPγS (50 μM) at 300 nM [Ca\(^{2+}\)] increased the aortic tone by 49 ± 6% (n = 15) and 111 ± 18% (n = 6), respectively (Fig. 6, A, B, and D). The α\(_1\)-adrenergic agonist phenylephrine (3 μM) was almost without effect [12 ± 2% (n = 18); Fig. 6, C and D]. In the absence of an agonist that activated G proteins, 8-Br-cGMP (300 μM) relaxed the tone at 300 nM Ca\(^{2+}\) by 79 ± 4% (n = 8) and 28 ± 3% (n = 10) in the absence and presence of 3 μM PKA inhibitor peptide, respectively (Fig. 6E). U-46619-induced contraction to a similar level in permeabilized wild-type and cGKI\(^{-/-}\) aortas (P > 0.05), which was relaxed to a similar level by 8-Br-cGMP in both preparations [from 149 ± 6% (n = 15) and 132 ± 6% (n = 3) to 98 ± 7% (n = 8) and 97 ± 6% (n = 3), respectively; Fig. 6E]. 8-Br-cGMP-induced relaxation was attenuated by the PKA inhibitor peptide (P < 0.05; Fig. 6E). In contrast, 8-Br-cGMP was unable to affect the tension elicited by 50 μM GTPγS (Fig. 6F), whereas the Rho kinase inhibitor Y-73623 relaxed contractions induced by GTPγS at 300 nM [Ca\(^{2+}\)] to 38 ± 3% (n = 6) of control, demonstrating that the Ca\(^{2+}\)-desensitization process was still intact in these experimental conditions (Fig. 6G). These results show that activation of the thromboxane receptor is much more efficient to build up contraction via Ca\(^{2+}\) sensitization than that of the α\(_1\)-adrenergic receptor. In agreement with the above results, cGMP-dependent Ca\(^{2+}\) sensitization via cGKI had only a marginal effect on the tone elicited by G protein-dependent Ca\(^{2+}\) sensitization.

**DISCUSSION**

The present study shows that 8-Br-cGMP-induced Ca\(^{2+}\) sensitization is mediated by PKA rather than by cGKI in permeabilized murine aorta. This view is strongly supported by the findings that 8-Br-cGMP relaxed permeabilized muscles from cGKI\(^{-/-}\)-deficient mice in the absence, but not in the presence, of the PKA inhibitor peptide. Several former studies (11, 31) have described a cross-activation between cyclic nucleotides and their respective cyclic nucleotide-dependent protein kinases with respect to relaxation in smooth muscle. For example, cAMP-elevating agents relaxed pig and sheep arteries via cGKI activation (8, 22). In skinned rat mesenteric artery, cAMP and cGMP induced Ca\(^{2+}\) sensitization that was abolished by Rp-8-Br-cGMPs but was not inhibited by Rp-cAMPS (25). Evidence for a cross-talk between cGMP and PKA has been reported using smooth muscle from cGKI\(^{-/-}\) mice (38). In intact aorta, cGKI-independent relaxation induced by NO was attenuated by inhibition of soluble guanylyl cyclase with 1H-1,2,4oxadiazolo[4,3-α]quinoxalin-1-one or...
of PKA with an inhibitory cAMP analogue (38). cGKI-independent relaxation was also observed in permeabilized ileal muscle strips from wild-type and cGKI−/− mice. A–D: original recording of tension in β-escin-permeabilized aortic rings from wild-type (A and C) and cGKI−/− mice (B and D). Lines indicate presence of 300 nM [Ca^{2+}] (A–D), 300 μM 8-Br-cGMP (A–D), and 200 nM PKI 5–24 (C and D). E: concentration-response curve of PKI 5–24 on relaxation induced by 8-Br-cGMP (300 μM). Data points represent means ± SE; n = 4–11. F: concentration-response curve of 8-Br-cGMP on permeabilized aorta at 300 nM [Ca^{2+}] in muscles from wild-type and from cGKI−/− mice. The experiments on muscles from wild-type mice were performed in the absence and presence of 3 μM PKA inhibitor peptide. Data points represent means ± SE; n = 4–6.

According to Francis et al. (12), the association constant (K_a) values for activation of cGK and PKA by 8-Br-cGMP in pig coronary artery are 25 nM and 2.8 μM, respectively. Slightly higher values have been reported using purified cGK and PKA.
conditions and in the presence of db-cAMP or 8-Br-cGMP, respectively. The highest nominal concentration of the PKI peptide from porcine aorta was 71 nM and 6.3 μM, respectively. Further experiments with the PKA-specific peptide interacted mostly caused by activation of PKA. The PKI peptide interacts with the PKA and cGKI with IC50 values of 127 nM and 37.8 μM, respectively. Further experiments with the PKA-specific peptide. The IC50 for cGKI. These considerations strongly support the notion that 8-Br-cGMP relaxed to 65% of the skinned wild-type aorta with EC50 values of 2.6 and 19 μM, respectively (25).

This study did not directly address the question of whether or not activation of PKA by cGMP could be relevant in an intact vessel. Previous reports suggested that NO can induce extremely high concentrations of cGMP in intact tissues. cGMP concentrations reached 50 μM in the murine aorta (38), 50 pmol/10⁶ cell (equal to 50 μM assuming a cell volume of 1 μl) in endothelial cells (32), 30 μM in cerebellar cells (2), 0.4 μM in platelets (33), and 10.5 μM in the rat aorta (34). These in vivo values raise the possibility that NO can increase cGMP in vascular smooth muscle to levels that desensitize contraction by activating not only cGKI but also PKA. It should be noted that cGMP-dependent relaxation involves not only modulation of the contractile machinery but also modulation of [Ca²⁺].

Another important finding of the study is that 8-Br-cGMP-induced desensitization of the contractile machinery via cGKI is rather ineffective after G-protein activation in the murine aorta. As reviewed recently (42), G-protein activation by G-protein-coupled receptors (GPCR) induce contraction, at least partially, by activation and membrane translocation of the small GTPase RhoA. Activated RhoA, in turn, stimulates Rho kinase to subsequently phosphorylate MYPT1, leading to inhibition of MLCP activity and, thus, Ca²⁺ sensitization. GPCR activating G-proteins of the Gα12/13 type, like the prostaglandin C and RhoA (18, 46). In line with this view, the prostaglandin receptor agonist U-46619 and GTPγS induced additional contraction at constant [Ca²⁺] in permeabilized aortic rings, demonstrating Ca²⁺ sensitization in this particular vascular smooth muscle. In contrast, the α-adrenergic receptor agonist phenylephrine barely affected contraction at constant [Ca²⁺], indicating that activation of this GPCR did not induce

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**Fig. 4.** Ca²⁺ concentration-response curves of β-escin-permeabilized aorta. A: data obtained from CTR mice under control conditions and in the presence of 300 μM dibutyryl-cAMP with and without PKI 5–24. B: data obtained from CTR mice under control conditions and in the presence of 300 μM 8-Br-cGMP with and without PKI 5–24 and data from cGKI−/− mice in the presence of 300 μM 8-Br-cGMP with and without PKI 5–24. Data points represent means ± SE; n = 4–14. Curves represent the fit of data obtained under control conditions and in the presence of db-cAMP or 8-Br-cGMP, respectively.

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**Fig. 5.** Western blot analysis of aortic smooth muscle cells from wild-type (top) and cGKI−/− mice (bottom) with antibodies detecting cGKI (top), vasodilator-stimulated phosphoprotein (VASP), and phospho-Ser157-VASP (p-VASP; bottom). Cells were kept 48 h in serum-free conditions and incubated with vehicle, 0.1 and 1 mM 8-Br-cGMP, or 0.1 and 1 mM 8-Br-cAMP for 30 min before lysis. Antibody detecting Akt was used as a loading control. Similar results were observed in two other experiments.
additional contraction via Ca\(^{2+}\) sensitization in the murine aorta, in contrast to rabbit vascular smooth muscle (20, 37). Whereas relaxation of Ca\(^{2+}\)-induced contraction by cGMP was similar in the absence and presence of phenylephrine, cGMP poorly relaxed aortic rings after Ca\(^{2+}\) sensitization by U-46619 or GTP\(\gamma\)S, indicating an ineffective Ca\(^{2+}\) desensitization in this condition. Ca\(^{2+}\) desensitization by cGKI or PKA are supposed to involve mainly activation of MLCP by phosphorylation of MYPT1. Several phosphorylation sites of MYPT1 have been recently identified, including a phosphorylation site for MYPT1 kinase and likely Rho kinase at threonine-696 and a phosphorylation site for cGKI/PKA at serine-695 (49). Interestingly, cGKI/PKA-dependent phosphorylation of serine-695 prevented phosphorylation at threonine-696 and, thus, the
inhibition of phosphatase activity. As a consequence, contraction via Ca\(^{2+}\) sensitization was attenuated after cGKI activation with cGMP (49). The results obtained in permeabilized murine aorta indicate that this situation exists also vice versa. If contraction was performed via Ca\(^{2+}\) sensitization, which may lead to phosphorylation of threonine-696, the Ca\(^{2+}\)-desensitizing effect of cGKI/PKA activation was greatly attenuated. Thus the results suggest that phosphorylation of MYPT1 at threonine-696 by Rho kinase may likewise prevent the phosphorylation at serine-695 by cGKI/PKA and, consequently, abolish cGKI/PKA mediated relaxation.

In conclusion, 8-Br-cGMP induced Ca\(^{2+}\) desensitization mainly via activation of PKA in murine aorta, particularly at higher concentrations, whereas cGKI played a minor part in this regulatory mechanism. After global G-protein activation, 8-Br-cGMP-induced desensitization of the contractile machinery is rather ineffective. Instead, cGMP/cGKI-mediated relaxation may use other mechanisms in murine aortic smooth muscle, i.e., relaxation of hormone-induced contractions by inhibition of Ca\(^{2+}\) release from intracellular stores via IRAG phosphorylation (16).

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