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ORIGINAL ARTICLE

Protein kinase enzymes in the human vagina—relation to key mediators of the cyclic AMP and cyclic GMP pathways

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Aside from phosphodiesterase (PDE) isoenzymes, protein kinases (cAK = cyclic AMP-binding protein kinase, cGK = cyclic GMP-binding protein kinase) have also been identified as important receptors for cyclic nucleotides. A significance of protein kinases in the control of the function of the male and female reproductive tract has been suggested; however, up until today, only a few approaches have addressed these enzymes in female genital tissues. The present study aimed to investigate by means of biochemical and immunohistochemical methods the expression of cAK and cGK. The distribution of cAK(I) and cGK(I) in relation to the vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP) and PDE type 4 (PDE4) was also evaluated. Cytosolic supernatants prepared from specimens of vaginal wall smooth muscle or epithelium were subjected to anion exchange chromatography and the activities of cAK and cGK(I) measured. To evaluate the distribution of cAK(I) and cGK(I) in relation to VIP, CGRP and PDE4, immunohistochemistry was conducted in sections of the human vaginal wall (full-wall specimens). Activities representing cGK(I) and cAK(I) were resolved from the chromatography column. Staining specific for cAK(Iα) was identified in both vascular and non-vascular vaginal smooth musculature, immunoreactivity for cGK(Iβ) was observed in the smooth muscle and endothelium of small arteries interspersing the sections. cAK(Iα)-positive vessels were found innervated by slender varicose nerve fibers presenting the expression of VIP and CGRP. These arteries also expressed PDE4. Localization of cAK and cGK in close relation to key mediators of the cyclic AMP (PDE4, VIP) and cyclic GMP (CGRP) pathways indicate that both signaling systems may synergistically work together in human vaginal tissue.

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INTRODUCTION

Within the normal female sexual response cycle, the vagina has a significant role in the perception of coital stimulation leading to sexual arousal and, finally, orgasm. With visual and sensory sexual stimulation, relaxation of vascular smooth musculature of the vagina occurs, resulting in an increase in local blood flow and vaginal lubrication. Alterations in vaginal luminal diameter, brought about by a reduction of tension of non-vascular smooth musculature, have also been shown, thus allowing penetration of the male penis during sexual intercourse.[1,2] The signaling mechanisms regulating the function of vaginal vascular and non-vascular smooth muscle are not yet completely understood. Nitric oxide (NO)/cyclic GMP-mediated responses are involved in smooth muscle relaxation in the urogenital region, for example, in the erectile tissues of the penis and clitoris.[3,4] Isoforms of the NO synthase (neuronal NO synthase and endothelial NO synthase) have been identified in the human vagina, including nerve fibers, the vascular endothelium and vaginal wall. An increase in cyclic GMP triggered by NO may be involved in the control of local vaginal blood flow and the relaxation response of non-vascular smooth muscle,[5,6] Aside from phosphodiesterase (PDE) isoenzymes and cyclic nucleotide-gated cationic channels, protein kinase enzymes (cAK, cyclic AMP-binding protein kinase; cGK, cyclic GMP-binding protein kinase) have also been identified major downstream targets for cyclic nucleotide monophosphates.[7] It is currently thought that cAK and cGK has a major role in mediating the cyclic AMP/GMP-dependent reduction of cytosolic Ca^{2+} or the sensitivity of the myosin light chain towards Ca^{2+}, presumably by the cGK-mediated phosphorylation and inhibition of Rho-kinase (ROK), thus resulting in smooth muscle relaxation.[8,9] Pathophysiological pathways contributing to sexual dysfunction in both male and female subjects possibly include Ca^{2+} sensitization mediated by the Rho/Rho-kinase system, a reduction of the bioavailability of NO and cyclic GMP, downregulation of the activity of adenylyl cyclase, and a dysregulation of the cyclic AMP/cyclic GMP/protein kinase signaling.[10] Up until today, studies evaluating the expression and functional significance of protein kinases have been conducted using mainly cardiovascular tissue. Hence, there is limited knowledge on the role of cAK and cGK in human male and female genital tissues, such as the penile erectile tissue (corpus cavernosum), clitoris and vagina. With particular regard to the human vagina, the localization of NO synthase and cyclic AMP- and cyclic GMP-degrading PDE isoenzymes has been comprehensively evaluated while, in contrast, the issue of protein kinases has only seldomly been addressed.[11–13] Therefore, it was the aim to investigate in human vaginal tissue by means of biochemical and immunohistochemical methods the expression (localization/distribution) of cAK(I) and cGK(I) in relation to the vasoactive

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intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP) and PDE type 4 (PDE4, cyclic AMP PDE).

MATERIALS AND METHODS

Tissue source

Human vaginal tissue (full-wall specimens) was obtained from 10 women (four menopausal and six perimenopausal) aged 46–88 years (mean age: 65 years) who had undergone colporrhaphy surgery. Macroscopically normal tissue was taken from the distal portion of the anterior and posterior vaginal wall, immediately placed in a chilled (4 °C) solution of Custodiol (Franz Köhler Chemie GmbH, Alsbach, Germany) and then transported to the laboratory.

Tissue preparation

The vaginal epithelium was carefully dissected from the smooth muscle portion. All subsequent steps were performed at 4 °C. Approximately 5 g of tissue (epithelial layer or smooth musculature) was homogenized in four volumes (v/w) of buffer A (pH 6.8) containing 10 mM KH_2PO_4, 1 mM EDTA, 1 mM DTT and a mixture of the following protease inhibitors: 1 mM benzamidine, 0.2 mM phenylmethanesulfonyl fluoride, 10 mM mercaptoethanol, 0.5 μg ml^{-1} leupeptin. The homogenate was filtered through four layers of gauze and centrifuged at 25.500 x*g* for 30 min. The pH of the resulting supernatant was adjusted to pH 5.4 by adding 0.5 M CH_3COOH, gently stirred for 30 min, and spun again at 25.500 x*g* for 30 min. The pellet (precipitate) was redissolved in 5 ml of buffer, centrifuged again and the resulting supernatant used as a source of cytosolic protein kinase activity.

Anion exchange chromatography

Separation of protein kinase activity was performed on a DEAE-sepharose column (26 × 39 mm, total volume: 20 ml) equilibrated with 10 bed volumes of buffer (20 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 0.5 μg ml^{-1} leupeptin, 10 mM β-mercaptoethanol) containing 60 mM NaCl. One millilitre of the 25.500 x*g* supernatant was applied to the column. The column was then washed with at least five bed volumes of buffer to remove unbound material. Protein kinases were then eluted with five bed volumes of a linear NaCl gradient (0.06–0.3 M) in the buffer. Flow rate was set at 2 ml min^{-1}. Three millilitre fractions were collected over the entire gradient and kept on ice.

Assays for protein kinase activity

The activities of cAK and cGK(I) in the volume eluting from the column were measured using the CycLex Cyclic GMP Protein Kinase Assay Kit (CycLex, Ina, Nagano, Japan) or Protein Kinase A Assay Kit (Merck Biosciences, Beeston, Nottingham, UK), respectively.

Immunohistochemistry

Full-wall preparations of the vaginal wall were taken from the lower mid portion of the vagina. After immersion-fixation for 4 h, tissue preparations were rinsed several times with PBS containing 15% (w/w) sucrose and then embedded in Tissue-Tec (Miles Laboratories, Elkhart, IN, USA). Tissue specimens were sliced to sections of 8–10 μm thickness and thaw-mounted onto glass slides. Sections were pre-incubated for 2 h in PBS with 0.2% Triton X-100 and 0.1% bovine serum albumin, followed by incubation for 24 h with the primary antibodies directed against cAK (4–5 μg ml^{-1}), cGKI(β) (10–12.5 μg ml^{-1}), CGRP (1.5 μg ml^{-1}, 1:500), VIP (0.75 μg ml^{-1}) and PDE4A (4–5 μg ml^{-1}). After rinsing, Alexa Fluor-conjugated secondary antibodies (4.3 μg ml^{-1}) were applied for 90 min; thereafter, sections were mounted using phenylendiamine. For double-labelling, more than one antibody was incubated on a section in a consecutive manner. Visualization was done using an Olympus 3 × 50 System fluorescence microscope (Olympus, Osaka, Japan). Imaging was performed using the Viewfinder program (Version 2.0, Pixera, Egham, UK).

Chemicals and antibodies

All chemicals, unless otherwise indicated in the text, were purchased from Carl Roth GmbH (Karlsruhe, Germany) or Sigma Chemical (St Louis, MO, USA). HiTrap DEAE sepharose was obtained from General Electric (GE) Healthcare Europe GmbH (Munich, Germany). Antibodies directed against cAK were obtained from Calbiochem (La Jolla, CA, USA), the anti-cGKI(β) antibody (1:100) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-CGRP and -VIP antibodies were from Euro Diagnostica (Malmoe, Sweden), the anti-PDE4 antibody was purchased from FabGennix (Frisco, TX, USA). Alexa Fluor-conjugated secondary antibodies were from Molecular Probes Europe BV (Leiden, The Netherlands).

RESULTS

DEAE sepharose anion exchange chromatography

For identification of protein kinase activities, fractions were assayed for the phosphorylation of recombinant cAK and cGK substrates (histone type IIa or serine heptapeptide). Typical elution profiles of protein kinases from the vaginal epithelium and vaginal wall are shown in Figures 1 and 2. cAK from the cytosolic fractions of the epithelial layer (Figure 1a) and vaginal wall (Figure 2a) eluted in single peaks each at a medium ionic strength. While cAK resolved by means of anion exchange chromatography from the vaginal wall was characterized by a pronounced increase in activity from baseline level to peak fraction followed by a respective descend, activity from the epithelial tissue presented a course that more transiently descended towards baseline following the peak fraction. Western blot analysis of volume fractions from the ascending sides of both peaks using specific anti-cAKI(α) ABs revealed a distinct signal with a size of 55 kD in the supernatant prepared from the vaginal wall; while, in contrast, no such signal could be related to the activity isolated from the vaginal epithelium. Typical patterns of cGK activity from tissue homogenates of the human vaginal epithelium and smooth muscle wall are displayed in Figures 1b and 2b, respectively. cGK activity eluted in single, clear shaped peaks, at a low-to-medium sodium chloride concentration. For further characterization of cGK activity, fractions were subjected to western blot analysis using specific anti-cGKI(β) ABs. Exposure to the ABs resulted in clearly visible bands with a size of 78 kD. This observation in conjunction with the shapes of the (activity) profiles eluting from the column through the entire sodium chloride gradient indicated that peaks did not contain a mixture of two or more cGK enzymes. Signals positive for the isoform cGKI(β) were also detected in the cytosolic supernatants (originating from vaginal wall and epithelium) before application to the anion exchange column. No kinetics on the diminishing effects of inhibitors of cyclic nucleotide-dependent protein kinases (for example, derivatives of isoquinoline sulfonamide, such as HA 1077) on the rate of substrate phosphorylation were conducted in the peak fraction volumes.

Immunohistochemistry

Examination of numerous sections (three to five) revealed distinct immunofluorescence signals related to cAK, cGK(Iβ), cyclic GMP, PDE4, CGRP and VIP. Both, the vascular and non-vascular smooth musculature presented abundant staining for cAK (Figure 3a). Sections double-labelled for cAK and the neuropeptide VIP confirmed the presence of numerous VIP-containing varicose nerve fibers in close relation to the blood vessels (Figures 3b and c). Immunoreactions specific for cGK(Iβ) were observed in the vascular wall of small arteries transversing the tissue sections (Figures 3d and e). A homogenous distribution of immunoreactivity specific for cGK was observed in all layers of the arterial wall, including the vascular endothelium. In addition, some of these vessels contained the second messenger cyclic GMP (Figure 3f). Some nerve fibers running underneath the endothelial layer of subepithelial vaginal arterioles were immunopositive for CGRP, known as a marker of sensory nerve terminals (Figure 3g). The non-vascular smooth musculature surrounding the arteries also presented staining for cGK(Iβ) and, to a certain degree, cyclic GMP (see Figures 3e and f). Sections double-labelled for the cyclic AMP-specific PDE4 and CGRP revealed that PDE4 immunoreactive vascular structures are innervated by nerve terminals containing

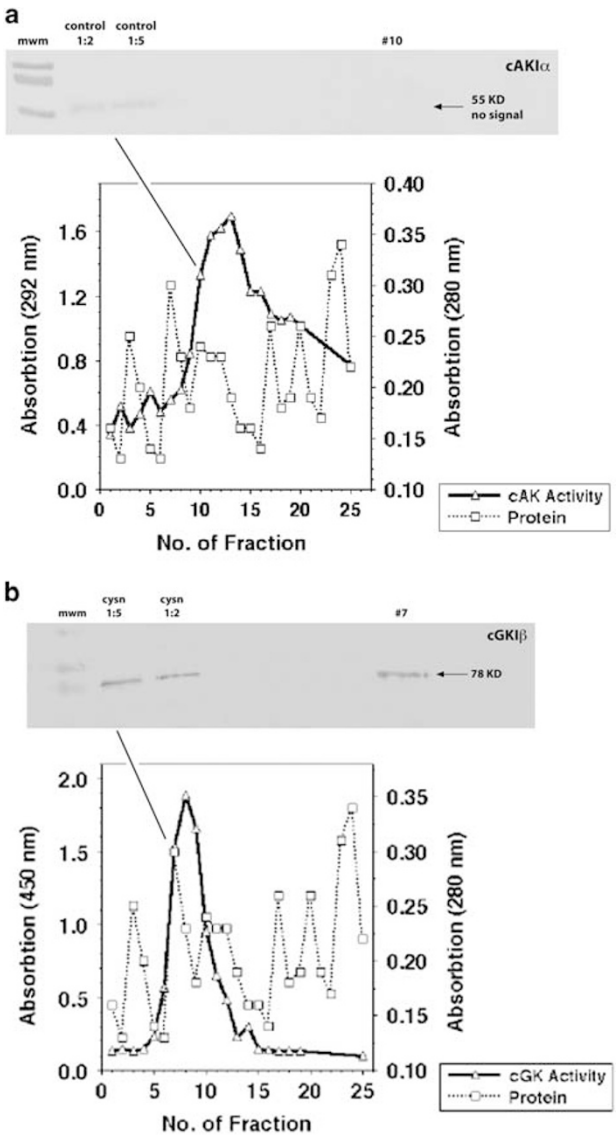

Figure 1. (**a** and **b**) DEAE anion exchange chromatography of cytososolic supernatants prepared from specimens of the human vaginal epithelium: typical profiles of activities of the cAK (**a**) and cGK (**b**) eluting from the column. Activities were eluted using a linear NaCl gradient ranging from 0.06–0.3 M. The presence of protein specific for cAK (isoform Iα) or cGKI (isoforms Iα or Iβ) in peak fractions was verified by means of WB analysis: (**a**, inset) WB analysis revealed no signal specific for cAKIα. (**b**, inset) WB exemplifying the elution of cGKIβ from the column. *mwm* = molecular weight marker, # = number of fraction applied to WB immunodetection. WB, western blot.

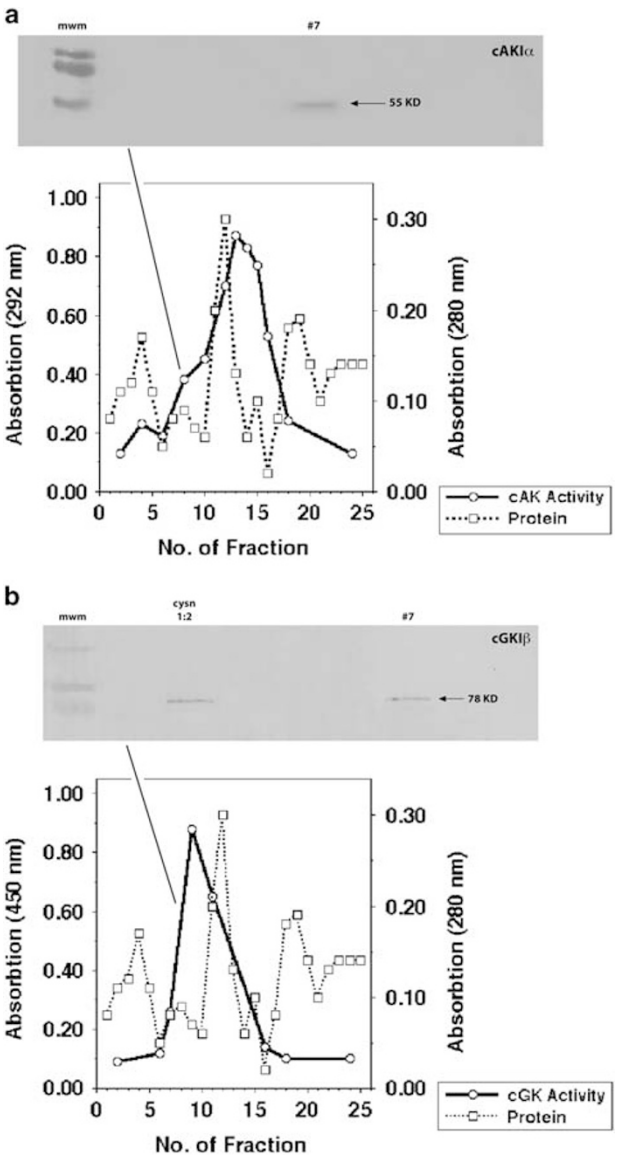

Figure 2. (**a** and **b**) DEAE anion exchange chromatograpy of cytososolic supernatants prepared from specimens of the human wall (denuded of the epithelial layer): typical profiles of activities of the cAK (**a**) and cGKI (**b**) eluting from the column. The presence of protein specific for cAK (isoform Iα) or cGKI (isoforms Iα or Iβ) in peak fractions was verified by means of WB analysis: WB exemplifying the elution of cAKIα (**a**, inset) and cGKIβ (**b**, inset) from the column. *cysn* = cytosolic supernatant, *mwm* = molecular weight marker, # = number of fraction applied to WB immunodetection. WB, western blot.

the neuropeptide (Figures 3h and i). Control stainings omitting the primary antibodies did not indicate fluorescence signals in any of the different histological components (vascular, non-vascular, neuronal) of the vaginal wall.

DISCUSSION

Numerous studies have demonstrated a role of the cyclic nucleotide-mediated signaling in the control of vascular and non-vascular smooth musculature in the human male and female genital tracts.[3,14] In particular, cyclic AMP/GMP-binding PDE isoenzymes and protein kinases have been identified major targets for cyclic nucleotides. Thus, both signaling pathways and related key enzymes, when negatively affected or impaired—for example, due to hypertension, diabetes, hypercholesterolemia or the ageing process—may contribute to the pathophysiology of sexual dysfunction in both males and females.[10,15] To date, only a few studies have investigated the expression and potential significance of cyclic nucleotide-binding protein kinases in human genital tissues.[16–18] Only one of these approaches exclusively utilized human vaginal tissue. In brief, immunostainings for cGKI (β) were reported in vaginal vascular and non-vascular smooth muscle. cAK-positive subepithelial arterioles were also seen, these vessels were densely innervated by varicose nerve fibers, some of

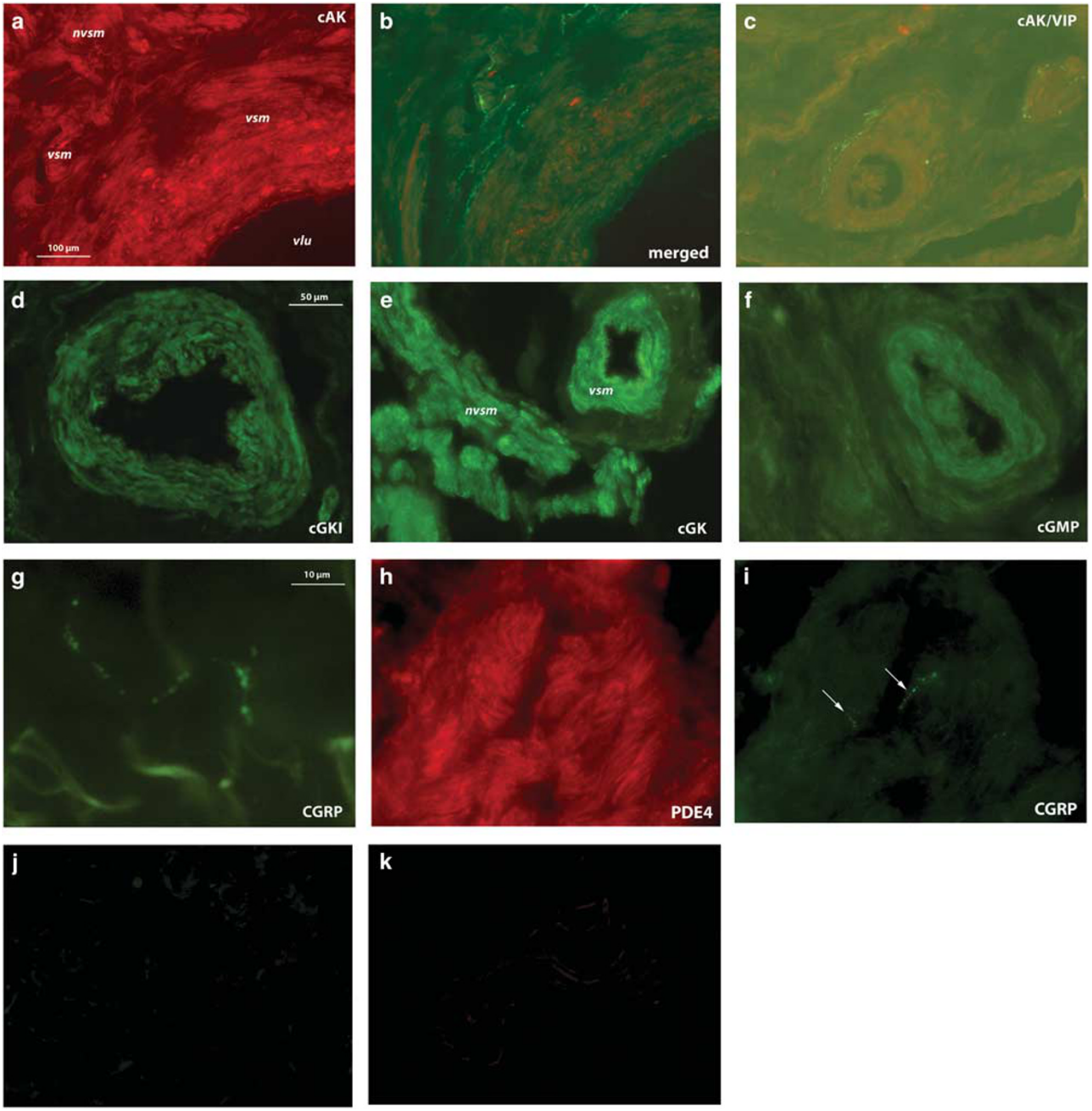

Figure 3. (**a**–**k**): Distribution of cyclic AMP- and cGMP-binding protein kinases (cAK, cGK) in the human vagina. (**a**) Localization of cAK in vaginal vascular and non-vascular smooth muscle (magnification ×40). (**b** and **c**) As shown by double-labeling technique, blood vessels are innervated by slender varicose nerve fibers characterized by the expression of the neuropeptide VIP (magnification ×40). (**d** and **e**) Localization of cGK (isoform I) in the entire vascular wall of a small arterial vessels transversing the tissue section, the non-vascular smooth muscle surrounding the arteries also stained for cGKI (**e**). (**f**) Vessels also present staining for the second messenger cyclic GMP (magnification ×20). (**g**) CGRP-positive varicose nerve fiber running underneath the endothelium of a small vaginal artery (magnification ×40). (**h** and **i**) Cross-section of a vaginal blood vessel presenting abundant immunoreactivity for the cyclic AMP PDE4 (**h**), the vascular smooth muscle is innervated by a slender nerve fiber characterized by the expression of CGRP (**i**, arrow) (magnification ×40). (**j**) control, Alexa Fluor green, (**k**) control, Alexa Fluor red. *vlu* = vascular lumen, *vsm* = vascular smooth muscle, *nvsm* = non-vascular smooth muscle. VIP, vasoactive intestinal polypeptide.

which presented the localization of VIP. No signals specific for the cGKIα were registered.[13] Said findings are confirmed by the present study, as assessed by immunofluorescence. In addition, we also demonstrated that cGKI(β)-positive small arteries contained the second messenger cyclic GMP. Slender nerve fibers staining for the neuropeptide CGRP were seen in close relation to these arteries. Some blood vessels presented relevant immunoreactivity for the cyclic AMP-specific PDE4 and were innervated by nerve terminals containing CGRP. The presence of cAK(I) and cGK(I) in vaginal wall specimens have been proved true as seen in the results from the biochemical experiments (anion exchange chromatograpy) and western blot analysis. According to these observations in conjunction with the results from the immunohistchemistry, it seems unlikely that human vaginal wall

smooth musculature contains more than one cAK or cGK isoenzyme. It has also become apparent that the cAK activity isolated from the vaginal epithelium is not represented by the isoform I alpha. Although it has been suggested that the NO/cyclic GMP-dependent relaxation of smooth muscle is mediated via the activation of cGKI, the precise role of protein kinases in the control of epithelial function remains to be elucidated.[19] The localization of cAK/cGK in epithelial cells might be related to influencing the permeability of the epithelial layer. The localization of cGK, cAK, PDE4 and cyclic GMP in the smooth musculature of vaginal arteries innervated by CGRP- and VIP-positive slender nerve fibers indicates that the cyclic AMP and cyclic GMP signaling systems may work together in a synergistic manner in the control of local vaginal blood flow. This is supported by the following observations: (i) PDE4 is a cyclic AMP-specific PDE isoenzyme that does not bind cyclic GMP. It was demonstrated that the PDE4 inhibitor rolipram, applied at doses of 0.3 mg kg^{-1} body weight, caused a threefold increase in rate of vaginal blood flow in anesthetized female rats, as assessed by means of Laser Doppler flowmetry recording;[20] (ii) VIP is known to produce effects by receptor-mediated increases in intracellular levels of cyclic AMP;[21] (iii) the relaxation of isolated smooth muscle induced by CGRP is abolished in the presence of inhibitors of the cyclic GMP-producing enzyme soluble guanylyl cyclase and NO synthase; and (iv) an enhancement in cyclic AMP production in response to exposure of isolated smooth muscle cells to CGRP has been shown.[22] Such a synergism between the two signal transduction pathways has already been shown in human corpus cavernosum, including the penile vascular smooth muscle, and been assumed to also occur in the prostate.[18,23,24] In general, when looking at the results, it should be taken into consideration that vaginal tissue was obtained mainly from women of an older age who were in the menopausal (or perimenopausal) stage. Since the physiology of the vaginal tract is under the control of reproductive hormones, systematic cellular and molecular changes might occur with menopause. For example, it has been shown (in the rodent model) that sex hormone levels can influence the expression and actions of VIP.[25] The findings from the present study further support the hypothesis of a pivotal role of both the cyclic AMP- and cyclic GMP-pathway in the control of human vaginal vascular and non-vascular smooth muscle.

CONCLUSION

The cyclic AMP and cyclic GMP-binding protein kinases cAK(Iα) and cGK(Iβ) are present in the vascular and non-vascular smooth musculature of the human vagina. The localization of cAK(Iα) and cGK(Iβ) in vaginal vessels in conjunction to cyclic GMP and the cyclic AMP-specific PDE4, and the close relation of such vessels to CGRP- and VIP-positive varicose nerve fibers indicates that the cyclic AMP and cyclic GMP signaling work synergistically together in temporary events controlling vaginal blood flow and smooth muscle tone, including vasodilation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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