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Aging-Induced Modulation of Pituitary Adenylate Cyclase-Activating Peptide- and Vasoactive Intestinal Peptide-Induced Vasomotor Responses in the Arteries of Mice

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PACAP-KO mice · Neuropeptides · Carotid artery · Relaxation

Abstract
Pituitary adenylate cyclase-activating peptide (PACAP; 1-38 and 1-27) and vasoactive intestinal peptide (VIP) are related neuropeptides of the secretin/glucagon family. Overlapping signaling through G-protein-coupled receptors mediates their vasomotor activity. We previously showed that PACAP deficiency (PACAP-KO) shifts the mechanisms of vascular response and maintains arterial relaxation through the VIP backup mechanism and (mainly) its VPAC1R, but their age-dependent modulation is still unknown. We hypothesized that backup mechanisms exist, which maintain the vasomotor activity of these peptides also in older age. Thus, we investigated the effects of exogenous VIP and PACAP peptides in isolated carotid arteries of 2- and 15-month-old wild-type (WT) and PACAP-KO mice. All peptides induced relaxation in the arteries of young WT mice, whereas in young PACAP-KO mice PACAP1-27 and VIP, but not PACAP1-38, induced relaxation. Unlike VIP, PACAP-induced vasomotor responses were reduced in aging WT mice. However, in the arteries of aging PACAP-KO mice, PACAP1-27- and VIP-induced responses were reduced, but PACAP1-38 showed a greater vasomotor response compared to that of young PACAP-KO animals. There were no significant differences between the vasomotor responses of aging WT and PACAP-KO mice. Our data suggest that, in the absence of PACAP both in young and old ages, the vascular response is mediated through backup mechanisms, most likely VIP, maintaining proper vascular relaxation in aging-induced PACAP insufficiency.

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Introduction

Pituitary adenylate cyclase-activating peptide (PACAP) is a neuropeptide with a diverse array of biological functions. Among others, it affects hormone release, influences motility and growth, and it exerts protective effects in various cell types in the nervous system and peripheral organs [1]. PACAP occurs in 2 biologically active forms, PACAP1-38 (dominant) and PACAP1-27. Due to the high structural similarity between PACAP and vasoactive intestinal peptide (VIP), PACAP is classified as a member of the VIP-secretin-glucagon superfamily. Structurally, PACAP1-27 emerges from PACAP1-38 by cleavage (which has the same amino acid order), and PACAP1-27 shares 68% sequence similarity with VIP [1, 2]. PACAP exerts its effects through G-protein-coupled receptors, namely through the PACAP-specific PAC1 receptor and through the VPAC1/VPAC2 receptors, which transmit the effects of both PACAP and VIP [1, 2]. In the vasculature, these receptors are located mainly in the smooth muscle cells of arteries and arterioles [1, 3].

PACAP, like VIP, is a potent vasorelaxant peptide [4, 5], and it causes a decrease in local and systemic blood pressure when given systemically [4]. For example, infusion of PACAP1-38, PACAP1-27, or VIP (0.25–25 pmol/kg) in dog femoral arteries induces a concentration-dependent increase in blood flow [6]. The vasodilator activity of PACAP has also been recorded in vitro in vessels of various organs of mice [7, 8], rats [9–11], rabbits [12], pigs [5], and humans [13].

The endogenous role of PACAP has been studied using PACAP-deficient (PACAP-KO) mice. PACAP-KO mice display several abnormalities, including alterations in locomotion, behavior, and changes in biochemical and metabolic parameters, resulting in several pathological features, in addition to higher mortality [1, 14, 15]. The cardiovascular system is also affected by PACAP deficiency, indicated by the decreased dilatator ability of arteries [8], reduced cardiac function, increased fibrosis, and myocardial degenerative changes [14]. Moreover, we have recently observed increased arterial relaxations to PACAP1-27 and VIP in PACAP-KO mice, and reduced vasomotor response to PACAP1-38 [7].

It has been shown that aging greatly affects the vasomotor function of vessels [7]. In our recent study on rats, we have observed that relaxations induced by PACAP1-38 were reduced in the arteries of older rats [11]. Studies have also shown an age-related reduction of PACAP levels in cerebral microvessels [16, 17]. In addition, Banki et al. [17] reported that reduced levels of PACAP lead to impaired angiogenic capacity of the cerebral microvasculature in older age. The link between age-related neurodegenerative diseases and PACAP has also been addressed [1, 18]. Thus, it would be important to elucidate whether aging modulates PACAP- and VIP-mediated vasomotor responses of peripheral arteries.

We hypothesized that the vasomotor responses to PACAP isoforms and VIP are substantially modulated by aging, both in PACAP-wild-type (WT) and PACAP-KO mice. Thus, in the present study, we aimed to investigate the relaxations of carotid arteries of young and aging PACAP-WT and PACAP-KO mice in response to exogenous PACAP1-38, PACAP1-27, and VIP.

Methods

Animals

Two- and 15-month-old male PACAP-KO mice on a CD-1 background and their WT littermates were used (total $n = 28$; 17 aged 2 months, 11 aged 15 months) [15]. The mice weighed WT: 2 months 42.73 ± 1.77 g, 15 months 41.5 ± 1.03 g; KO: 2 months 36.8 ± 0.89 g, 15 months: 35.3 ± 0.49 g at the time of experiment. Animal breeding, housing, and experimental procedures were performed according to ethical guidelines (University of Pecs, ethical permission No.: BA02/2000-15024/2011).

Surgery

Common carotid arteries were isolated using a surgical microscope (Olympus SZX7; Olympus Inc., Tokyo, Japan) under ketamine/xylazine anesthesia induced by intraperitoneal administration (ketamine: Gedeon Richter PLC., Budapest, Hungary; xylazine: Eurovet Animal Health B.V., Bladel, The Netherlands). The anesthetics were given in 81.7 and 9.3 mg/kg doses, respectively. The proximal and distal ends of the isolated vessel segments were ligated; the vessel was excised between the ligations, and then transferred to refrigerated Krebs solution. Carotid arteries from both left and right sides were isolated, then the animals were killed with an intraperitoneal injection of pentobarbital (100 mg/kg; Ceva Sante Animale, Libourna, France).

Measurement of Isometric Force of Isolated Carotid Arteries

Preparation and measurement of the isometric force of isolated carotid arteries were performed according to Mulvany and Halpern [19] and our previous experiments [11]. After their removal, the vessel segments were quickly transferred into cold oxygenated physiological Krebs solution (mixture of 95% O_2/5% CO_2; Linde, Repcelak, Hungary; NaCl: 119 mM, KCl: 4.7 mM, KH_2PO_4: 1.2 mM, $NaHCO_3$: 25 mM, Mg_2SO_4: 1.2 mM, $CaCl_2 \times 2H_2O$: 1.6 mM, EDTA: 0.026 mM, glucose: 11.1 mM). NaCl and KCl were obtained from VWR International (Radnor, PA, USA). All other chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. Two-millimeter-long rings were dissected from the arteries. Each ring was positioned between 2 tungsten wires in the chamber (the diameter of the wire was 0.04 mm) in a 5-mL Krebs bath solution. The bath solution was continu-

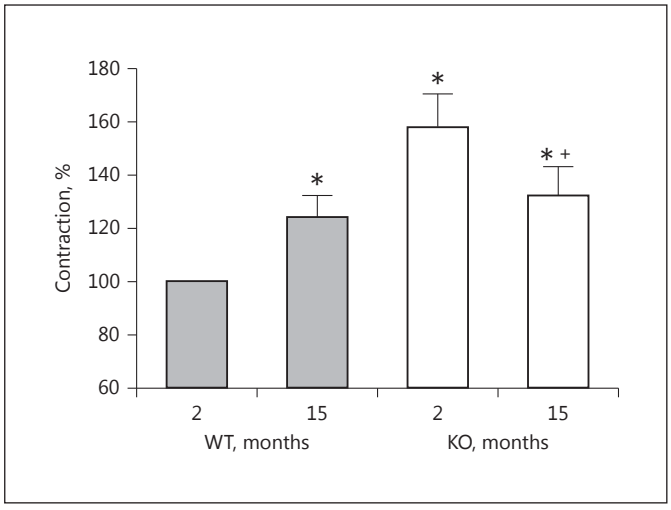

Fig. 1. KCl (60 mM)-induced contractions of isolated carotid arteries of young (2 months old) and aging (15 months old) WT and PACAP-KO mice. Arterial contractions are expressed as the percent increase in tone. Data are expressed as means ± SEM (n = 3–6/group). * $p < 0.05$ versus 2-month-old WT mice, $^{+}$ $p < 0.05$ versus 2-month-old KO mice.

ously oxygenated with a gas mixture of 95% O_2 and 5% CO_2 and kept at 36.9 ± 0.1°C.

Isometric contraction forces were measured with a 4-chamber system, a DMT 610M Wire Myograph (Danish Myo Technology, Aarhus, Denmark). LabChart 8 (ADInstruments, Dunedin, New Zealand) and Myodaq 2.01 (Danish Myo Technology) software were used for data acquisition and display, according to previous descriptions [11]. Vessels were allowed to stabilize for 60 min after normalization, then 60 mM KCl was administered for establishing a tone [11, 20]. Once the vessel reached the plateau phase, the chosen drug was tested.

Pharmacological Agents Used in the Study

The vasomotor function of the vessels was studied in response to cumulative doses (consecutive administration of an increasing dose of substance every 5 min without washing out the previous one) of PACAP1-38 and PACAP1-27 (from 10^{-9} to 10^{-6} M), which were synthesized as previously described [21], and of VIP (from 10^{-9} to 10^{-6} M; Bachem, Bubendorf, Switzerland). Peptides are added to the chamber containing 5 mL of Krebs solution, resulting in a 10^{-9} to 10^{-6} M final concentration. All drugs were dissolved in distilled water. When only the solvent (distilled water) was applied, there was no change in isometric force.

Calculations

Maximal contractions to 60 mM KCl were calculated as percentage changes of the average contraction force of the 2-month-old WT group in response to 60 mM KCl (Fig. 1). Relaxation responses to peptides were calculated for each vessel individually as percentage changes of their own force in response to the maximal contraction to 60 mM KCl, which represented a baseline (Fig. 2).

Statistical Analysis

All data collected as time series were compared across genotypes, age, and dose points by 3-way ANOVA (post hoc, Holm-Sidak). Analyses were performed using Sigma Plot 12.5 (Systat, Chicago, IL, USA). Differences were considered significant with p values <0.05. The data are given as the mean ± standard error of the mean (SEM).

Results

KCl-Induced Constrictions of Carotid Arteries

In the arteries of young mice, contraction elicited by 60 mM KCl was significantly greater in PACAP-KO than in WT mice, whereas this difference diminished between the arteries of aging WT and PACAP-KO mice. While the KCl-induced vasomotor response was greater in the arteries of aging WT mice than in young mice, the vasomotor response was reduced in aging PACAP-KO mice compared to that of young ones (Fig. 1).

Effect of Cumulative Doses of PACAP1-38 on the Relaxation of Carotid Arteries Isolated from Young and Aging Mice

First, we obtained the responses of arteries to PACAP1-38 in young (2 months old) and aging (15 months old) WT and PACAP-KO mice (Fig. 2a). In the arteries of young WT mice, PACAP1-38 elicited a significant dose-dependent relaxation (10^{-8} to 10^{-6} M), whereas in the arteries of aging WT mice, relaxation to PACAP1-38 was observed only at the highest dose (10^{-6} M) and it was significantly reduced compared to young WT mice at 10^{-8} to 10^{-6} M (Fig. 2a, left panel). In the arteries of young PACAP-KO mice, PACAP1-38 induced relaxation only at the highest dose (10^{-6} M). In contrast, in the arteries of aging PACAP-KO mice, PACAP1-38-induced relaxation (10^{-7} to 10^{-6} M) was significantly improved as compared to the arteries of young PACAP-KO mice at 10^{-7} to 10^{-6} M (Fig. 2a, right panel). While arterial relaxation to PACAP1-38 was reduced in young PACAP-KO mice compared to WT mice (at 10^{-8} to 10^{-6} M), aging led to radical changes in arterial response by diminishing these differences.

Effect of Cumulative Doses of PACAP1-27 on the Relaxation of Carotid Arteries Isolated from Young and Aging Mice

The administration of PACAP1-27 resulted in significant dose-dependent relaxation (10^{-7} to 10^{-6} M vs. baseline) of arteries isolated from both young and aging WT mice. However, relaxations of arteries from aging WT

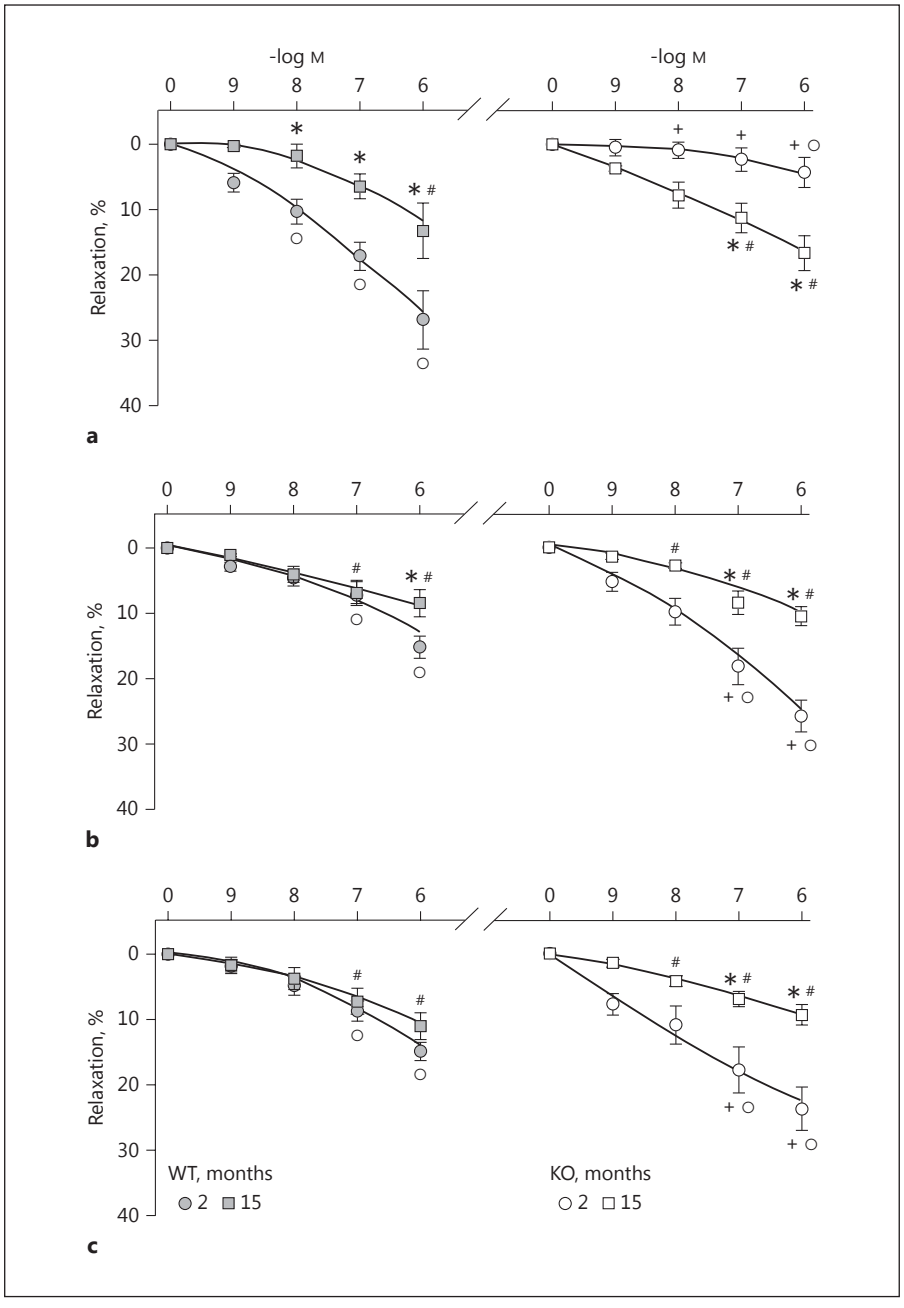

Fig. 2. Effect of cumulative dose-dependent administration of PACAP1-38 (**a**), PACAP1-27 (**b**), and VIP (**c**) on vasomotor response in 2-month- and 15-month-old carotid arteries of WT (left panel) and PACAP-KO (right panel) mice. Arterial relaxations are expressed as the percent decrease in tone (as compared to maximal tone induced by 60 mM KCl). Data are expressed as means ± SEM ($n = 3$–6/group). * $p < 0.05$ in 15-month-old versus 2-month-old mice, ° $p < 0.05$ versus baseline in 2-month-old mice, # $p < 0.05$ versus baseline in 15-month-old mice, + $p < 0.05$ in 2-month-old KO mice versus 2-month-old WT mice.

mice were significantly reduced compared to those from young WT mice (only at 10^{-6} M; Fig. 2b, left panel). In the arteries of both young and aging PACAP-KO mice, PACAP1-27 elicited relaxation as compared to baseline (young: 10^{-7} to 10^{-6} M; aging: 10^{-8} to 10^{-6} M). Relaxations of arteries to PACAP1-27 were significantly reduced in aging PACAP-KO mice compared to those of young PACAP-KO mice (at 10^{-7} to 10^{-6} M; Fig. 2b, right panel). However, while relaxation to PACAP1-27 was augmented in arteries from young PACAP-KO mice compared to young WT mice (at 10^{-7} to 10^{-6} M), there was no significant difference in the arterial relaxations between aging WT and PACAP-KO mice.

Effect of Cumulative Doses of VIP on the Relaxation of Carotid Arteries Isolated from Young and Aging Mice

Administration of VIP resulted in a relaxation of the arteries of both young and aging WT mice. The observed

relaxation was significant at 10^{-7} to 10^{-6} M (for both young and aging mice), but there was no significance between them (Fig. 2c, left panel). In the PACAP-KO mice, VIP elicited a significant relaxation in the arteries of young (at 10^{-7} to 10^{-6} M) and aging (at 10^{-8} to 10^{-6} M) mice, yet relaxation in aging PACAP-KO mice was significantly reduced compared to young PACAP-KO at 10^{-7} to 10^{-6} M (Fig. 2c, right panel). However, while relaxation to VIP was augmented in arteries from young PACAP-KO mice compared to young WT mice (at 10^{-7} to 10^{-6} M), there was no significant difference in the arterial relaxations between aging WT and PACAP-KO mice.

In young WT mice, the greatest relaxation was observed in the presence of PACAP1-38. However, in young PACAP-KO mice, PACAP1-27 and VIP responses were augmented whereas PACAP1-38 virtually did not induce arterial relaxation.

Discussion

In this study we showed that PACAP1-38 elicited significantly greater relaxations in the arteries of young WT mice than PACAP1-27 and VIP. In contrast, PACAP1-38 did not induce relaxation in the arteries of young PACAP-KO mice (except at the highest dose), whereas PACAP1-27- and VIP-induced relaxations were significantly augmented compared to the vasomotor responses of young WT mice. These results are in accordance with our earlier findings [7].

The novel findings are: (1) KCl-induced arterial constriction is augmented in aging WT and reduced in PACAP-KO mice compared to young mice, but there was no difference between the constrictions of arteries from aging WT and PACAP-KO mice; (2) PACAP peptides elicited reduced relaxation in aging WT compared to young WT mice, whereas relaxation to VIP was unaltered, and (3) PACAP1-27 and VIP elicited reduced relaxation of arteries from aging PACAP-KO mice, whereas PACAP1-38 induced significantly greater relaxation compared to young PACAP-KO mice.

KCl-Induced Tone of Arteries of Young and Aging Mice

KCl is used to test the contractile abilities of arteries without receptor mediation, which can be influenced by aging as we have previously shown [20]. In aging WT mice, we observed increased contractile force development, which is in accordance with previous findings [11, 20]. Increased arterial contractility in young PACAP-KO mice corresponds to our previous findings [7]. On the contrary, in aging PACAP-KO mice, there is a decrease, or rather restoration (normalization) of vasomotor relaxation to the level which corresponds to carotid arteries of aging WT mice. One explanation for this phenomenon could be the fact that VIP-induced relaxation is maintained during aging. VIP is similar to PACAP, especially PACAP1-27, in structure and function [1, 2]. Therefore, VIP may stimulate the same second level signaling related to PACAP, thus it can counterbalance the increased contractility. These findings could have physiological importance as VIP can provide a counterbalancing mechanism preserving vasodilation in PACAP-deficiency or PACAP-insufficiency, which can be present in pathological conditions [14] and aging [16].

PACAP- and VIP-Induced Vasomotor Responses of Carotid Arteries with Aging

PACAP has multifunctional regulatory roles both locally in various organs and systemically, by exerting general effects on tissues, such the vasculature, where both PACAP and VIP are potent vasodilators [1, 4, 5, 22]. In the present study, we found that PACAP and VIP elicited arterial relaxations in young male WT and PACAP-KO mice, similarly to our earlier report [7]. In young WT mice, relaxation induced by PACAP1-38 was greater than that by PACAP1-27 and VIP. These observations are in accordance with some reports [23, 24], whereas other studies indicate higher efficacy of either PACAP1-27 or VIP [6, 23]. The vasculature of humans and rats contains PACAP, VIP, and their receptors, and their distribution can differ from organ to organ [1, 10, 13, 25]. However, it seems that there is no general rule concerning the potency of PACAP peptides and VIP in the vasculature. Previous studies showed vasodilatory effects of PACAP and VIP in different vessels of different regions, such as the brain [7–10, 23, 25], abdominal cavity [23], lung [23, 24], and limbs [7] of various species.

In young PACAP-KO mice, relaxation of the carotid artery to PACAP1-38 was detectable only at the highest concentration (10^{-6} M). In contrast, substantially and significantly increased relaxations were observed after administrations of PACAP1-27 and VIP. These findings are in accordance with our earlier report [7]. As we reported, the reason for such vascular behavior could be a "biological switch" from the PACAP to VIP system, due to the overlapping receptorial mechanism and structural similarity between the PACAP1-27 and VIP [1]. Other reports show that the lack of PACAP results in cardiovascular defects, such as reduced cardiac function and dilata-

tor ability, increased fibrosis, and myocardial degenerative changes [8, 14].

PACAP and VIP are highly conserved through evolution [1], indicating the importance of PACAP in a large array of physiological and pathophysiological processes [1, 26]. During prenatal development and shortly after birth, there is a rapid increase in the mRNA and protein levels of PACAP and its receptors [1, 26]. In contrast, these levels decrease with aging. Tripathy et al. [16] reported that the cerebrovascular level of PACAP is higher in young rats and lower in old rats. An interesting observation has been made regarding PACAP and VIP receptors in the brain of old rats showing increased PAC1R, decreased VPAC1R, and unchanged VPAC2R levels [26, 27]. Several research groups have already attempted to identify possible compensatory mechanisms in PACAP deficiency. Ogawa et al. [28] reported that serotonin or tyrosine hydroxylase expression did not alter in PACAP-KO mice. As the most logical possible compensation is VIP, given the structural and receptorial overlapping with PACAP, an earlier study by Girard et al. [29] investigated the postnatal expression of PACAP and VIP in knockout animals. Surprisingly, they found no postnatal developmental compensation by VIP in PACAP-KO animals, or vice versa, of PACAP in VIP-KO mice. In PACAP-KO mice, no increase of VIP expression in the early postnatal brain could be observed. Although these results still leave the question open regarding what compensatory mechanisms exist in the lack of PACAP, our results show that VIP is involved in the compensation of the vasomotor functions, even if expressional alterations cannot be found in the postnatal brain. Differences in age, strain, and organ-specific mechanisms can also explain the discrepancy between our findings and those of Girard et al. [29]. Another study suggested that the compensatory mechanisms may be very complex, and not due to one molecule, as rearing PACAP-KO mice in an enriched environment could increase the protein levels of the NMDA receptor NR2B subunit, phospho-ERK, phospho-CaMKII, and brain-derived neurotrophic factor, resulting in the amelioration of memory impairments shown in the PACAP-KO mice [30]. The vascular relaxation to PACAP1-38 is reduced with aging, which is similar to our previous observations in old rats [11]. An age-related decrease in PACAP mRNA and protein levels [16, 31] can alter PACAP-induced relaxation. Accordingly, in the old rat brain, Lee et al. [26] reported increased PAC1R and unchanged VPAC2R levels with aging, which implies the adaptation of the PACAP system for either compensation (in the form of receptor overexpression) or switch to "alternative" PACAP-mediated responses (most likely VIP). The responses of arteries of aging PACAP-KO mice correspond with those of aging WT mice, suggesting that the signal transduction pathway is able to adapt as age progresses. These observations clearly demonstrate that the VIP-mediated vasomotor effects provide an "alternative mechanism" when PACAP is absent (in young PACAP-KO mice; Fig. 3) or the PACAP level is insufficient (in aging WT mice; Fig. 4), allowing the proper vascular response to be maintained. Normal vascular responses are most likely achieved through receptor adaptation, as demonstrated already in young PACAP-KO mice [7]. It is unclear why carotid arteries of aging PACAP-KO mice improve PACAP1-38-induced relaxation, but we propose that increased receptor binding serves as a compensatory mechanism, especially if there is an increase in levels of PAC1R, as previously observed by Lee et al. [26]. In addition, age-related vascular impairments and dysfunction [32] could also influence the magnitude of the vasomotor response to PACAP and VIP. The translational value of our stud-

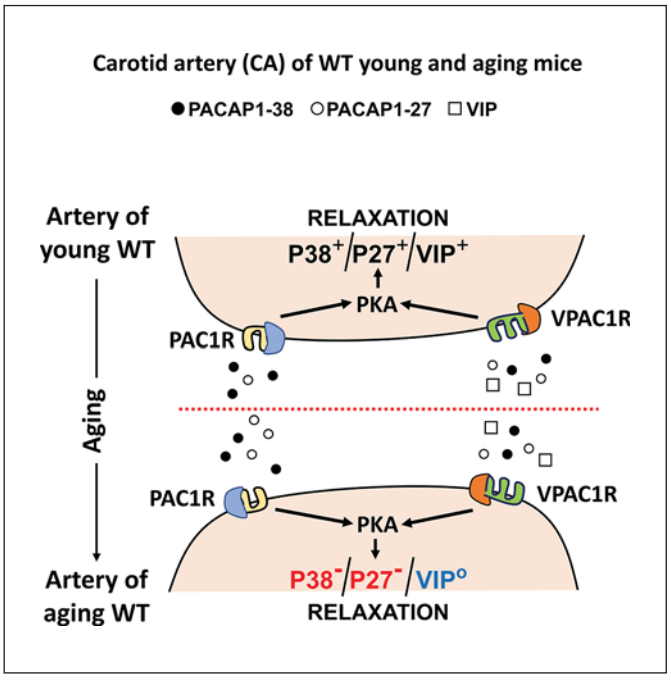

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Fig. 3. Schematic illustration of vasomotor signaling of PACAP and VIP of carotid arteries of young (upper section) and aging (lower section) WT mice. Based on our previous work [7], we observed relaxation to all 3 peptides in young WT mice. In aging WT mice, relaxation was reduced to PACAP1-38 and PACAP1-27, but not to VIP, which does not change relaxation. +/–/o, relaxation present/reduced/unchanged; P38, PACAP1-38; P27, PACAP1-27.

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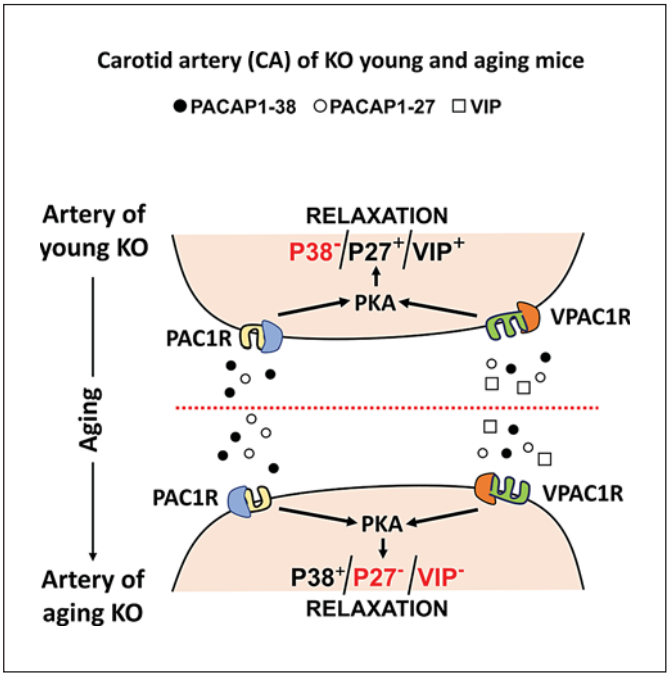

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Fig. 4. Schematic illustration of vasomotor signaling of PACAP and VIP of carotid arteries of young (upper section) and aging (lower section) KO mice. On the basis of our previous work [7], in young KO mice we observed a loss of relaxation to PACAP1-38 peptide, but improved relaxation to PACAP1-27 and VIP as compared to young WT mice. In aging KO mice, increased relaxation was observed to PACAP1-38, but relaxation was reduced to PACAP1-27 and VIP compared to young KO mice (upper section). Magnitude of achieved relaxations to all peptides in aging KO mice correspond to aging WT mice (Fig. 3, lower section). +/–, increased/reduced relaxation; P38, PACAP1-38; P27, PACAP1-27.

ies awaits further investigation, as presently available data show that the vasodilator effects of PACAP depend on the pathological conditions and are different in different organs. For example, PACAP seems to play a major role in the vasodilation of meningeal arteries in migraine patients [33] and general blood flow changes upon intravenous administration [34], while its physiological role in cavernous blood vessels is questioned due to the markedly stronger effect of VIP [35].

The physiological role of PACAP in aging is emphasized, among others, by links between PACAP and neurodegenerative diseases. Age-related neurodegenerative diseases are associated with reduced levels of PACAP, and symptoms of Parkinson's disease can be attenuated by the administration of exogenous PACAP [18]. PACAP-KO mice exhibit early aging symptoms, including early retinal aging signs [36], impairment of memory, and learning ability [17], which has been shown to be ameliorated by enriched environment [30] or reversed with PACAP treatment [31].

Conclusions

This is the first study aiming to characterize the effects of aging on the relaxant function of isolated carotid arteries in normal conditions and in PACAP deficiency by utilizing exogenous PACAP1-38, PACAP1-27, and VIP neuropeptides. We found that both PACAP deficiency and aging shift the response from PACAP-38 to the VIP system, which seems to be preserved in aging. These ideas are depicted in Figures 3 and 4 showing that the VIP system may be an alternative/backup pathway that maintains the vasomotor function of arteries in aging-induced PACAP deficiency/insufficiency, underlying the physiological importance of both the PACAP and VIP pathways in the regulation of the vasomotor function of arteries and perhaps coupling it to neural functions.

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Disclosure Statement

The authors declare no conflicts of interest.

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