Responses to human CGRP, ADM, and PAMP in human thymic arteries

HUNTER C. CHAMPION, TRINITY J. BIVALACQUA, ROBERT L. PIERCE, WILLIAM A. MURPHY, DAVID H. COY, ALBERT L. HYMAN, AND PHILIP J. KADOWITZ
Department of Pharmacology, Tulane University Health Sciences Center, New Orleans, Louisiana 70112
Submitted 7 June 2002; accepted in final form 13 September 2002

Champion, Hunter C., Trinity J. Bivalacqua, Robert L. Pierce, William A. Murphy, David H. Coy, Albert L. Hyman, and Philip J. Kadowitz. Responses to human CGRP, ADM, and PAMP in human thymic arteries. Am J Physiol Regul Integr Comp Physiol 284: R531–R537, 2003; 10.1152/ajpregu.00337.2002.—Responses to human CGRP, adrenomedullin (ADM), and proadrenomedullin NH2-terminal 20 peptide (PAMP) were studied in small human thymic arteries. CGRP, ADM, and PAMP produced concentration-dependent vasodilator responses in arteries preconstricted with the thromboxane mimic U-46619. Responses to ADM and PAMP were attenuated, whereas responses to CGRP were not altered by endothelial denudation. Inhibitors of nitric oxide synthase and guanylyl cyclase attenuated responses to ADM and PAMP but not to CGRP. The CGRP1 receptor antagonist CGRP(8–37) attenuated responses to CGRP and ADM but not to PAMP. Responses to CGRP were reduced by SQ-22536 and Rp-cAMPS, inhibitors of adenyl cyclase and PKA. These data suggest that responses to CGRP and ADM are mediated by CGRP(8–37)-sensitive receptors and that the endothelial ADM receptor induces vasodilation by a nitric oxide-guanylyl cyclase mechanism, whereas a smooth muscle CGRP receptor signals by a cAMP-dependent mechanism. A different endothelial receptor recognizes PAMP and signals by a nitric oxide-dependent mechanism. CGRP1 receptor; vascular endothelium; vascular tone; human thymus

ADRENOMEDULLIN (ADM) and proadrenomedullin NH2-terminal 20 peptide (PAMP) are distinct products encoded by the ADM gene (20–23). The human form of ADM consists of 52 amino acids and a six-membered ring structure that shares homology with CGRP and amylin (20–23). PAMP consists of 20 amino acids and does not share the six-membered ring structure (20–22). ADM was discovered in human pheochromocytoma cells and has been localized in many tissues, including vascular smooth muscle cells and the endothelium (11, 13, 15, 16, 20). ADM plasma levels are increased in disorders, such as hypertension, heart and renal failure, and hypoxia, and ADM induces a different spectrum of hemodynamic effects than do nitrovasodilators (8, 12–14, 18, 23, 26, 35). PAMP is found in the NH2-terminal portion of the precursor for ADM and has a similar distribution, including vascular smooth muscle and the endothelium (17, 20, 24, 36). ADM is a spliced variant of the calcitonin gene and this neuropeptide is present in perivascular sensory nerves (29). ADM, PAMP, and CGRP have vasodilator activity with different mechanisms of action (4, 6, 7, 16, 17, 27–29, 35). ADM has been reported to relax vascular muscle in an endothelium-dependent manner in some vascular beds studied, and those responses are species or vascular bed dependent, whereas PAMP has modest activity and has been reported to inhibit norepinephrine release or decrease vascular resistance by a nitric oxide-independent mechanism (4–6, 17, 27, 28, 33–35). CGRP is a potent vasodilator that has been reported to act as an endothelium-dependent vasodilator agent in most arteries but has been reported to act by releasing nitric oxide in some arteries studied (7, 29, 30, 32).

The functional expression of receptors for CGRP and other vasoactive peptides has been described in the human thymus gland, and it has been postulated that these receptors may play a functional role in pathophysiological processes in this endocrine organ (25). Although CGRP receptors are present in the human thymus, little if anything is known about the actions of CGRP and related peptides on vascular function in small arteries from the thymus (25). Moreover, there is a paucity of information about the actions and mechanisms of action of ADM, PAMP, and CGRP in small human arteries (9, 27, 29, 30, 32, 36). The present study was, therefore, undertaken to investigate responses and the role of endothelium-derived nitric oxide in mediating responses to CGRP, ADM, and PAMP in small arteries from the human thymus.

METHODS

Whole thymus tissue was obtained from pediatric patients undergoing cardiothoracic surgery at Tulane University Medical Center under a protocol for discarded tissue approved by the Institutional Committee on Human Tissue Research and follows the “Guiding Principles for Research

Address for reprint requests and other correspondence: P. J. Kadowitz, Dept. of Pharmacology SL83, Tulane Univ. Health Sciences Center, 1430 Tulane Ave., New Orleans, LA 70112 (E-mail: pkadowi@tulane.edu).

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Involving Animals and Human Beings” by the American Physiological Society (1). The tissue was immediately immersed into cold Krebs bicarbonate buffer (119 mM NaCl, 24 mM NaHCO3, 4.7 mM KCl, 1.18 mM KH2PO4, 1.17 mM MgSO4, 1.6 mM CaCl2, and 5.5 mM glucose) aerated with 95% O2-5% CO2. Branched artery segments 1.5 to 2 mm in length were gently dissected from the thymus and cleared of adhering connective tissue. The arterial segments were transferred to the myograph chamber (Living Systems Instrumentation) containing submaximally precontracted buffer aerated with 95% O2-5% CO2, mounted on a glass micropipette of known internal diameter, and secured with strands of 10-0 nylon suture. Luminal cellular debris was gently flushed with buffer solution, and the distal lumen of the vessel was tied with suture to create a blind pouch configuration. The vessels were allowed to equilibrate for 45 min as temperature was slowly increased to 37°C and intraluminal pressure was gradually increased to 50 to 70 mmHg. Transmural pressure was maintained constant (±1 mmHg) with a microliter peristaltic pump with an automatic pressure servo-control.

The myograph chamber was placed on the mechanical stage of a TMS-F inverted stage microscope (Nikon Instruments), and video images were captured with a high-resolution CCD camera (Burle) and monitor (Panasonic TR-930B, Matsushita Electronic Industrial). Lumen diameter and wall thickness were monitored by a Video Dimension Analyzer (Living Systems Instrumentation) calibrated against a stage micrometer and recorded on a chart recorder (Western Graphtec).

All agonists and antagonists were added to the buffer superfusion reservoir and were allowed to recirculate through the myograph chamber (total volume 80 ml). The myograph chamber was continuously perfused at 37°C with Krebs bicarbonate buffer equilibrated with 21% O2-5% CO2, balance N2 at pH 7.4. Both superfusion reservoir and were allowed to recirculate through the myograph chamber (total volume 80 ml). As an index of the maximal vasoconstrictor response, the arteries were initially challenged with 60 mM KCl solution. Arteries that demonstrated <50% decrease in resting diameter were not used. After washout of the KCl solution, the arteries were submaximally precontracted with the thromboxane mimic U-46619 at 30–100 nM or with endothelin-1 (ET-1) (30–100 nM).

Preparation of drugs. Human PAMP, PAMP(12–20), human synthetic ADM, CGRP(8–37) (Peptide Research Labs, Tulane Medical School), and human CGRP (Peptides International) were dissolved in 0.9% NaCl. Acetylcholine bromide, sodium nitroprusside, and ET-1 (Sigma) were dissolved in 0.9% NaCl. Nω-nitro-l-arginine (L-NA) (Sigma) was dissolved in acidified saline. 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Sigma) was dissolved in 95% ethanol and diluted with 0.9% NaCl. U-37883A (Upjohn), Rp-cyclic 3’5’-hydrogen phosphorothiate adenosine (Rp-cAMPS), and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ-22536; Sigma) were dissolved in 0.9% NaCl with sonication. U-46619 (Upjohn) was dissolved in 100% ethanol and diluted with 0.9% NaCl. L-NA. Leucromakalim (SmithKline Beecham) was dissolved in a 20% ethanol-saline solution at a concentration of 1 mg/ml and was then diluted with 0.9% NaCl. The vehicles for these agents had no significant effect on resting vessel diameter or on responses to the vasoactive agents. The drug solutions were stored in a freezer in amber bottles, and working solutions were prepared on a frequent basis and kept on crushed ice.

Statistics. Data are expressed as percent relaxation of vessels preconstricted with U-46619 as means ± SE. Data were analyzed using a one-way analysis of variance with repeated measures and Scheffe’s F-test or a paired t-test. A P value of <0.05 was used as the criterion for statistical significance.

RESULTS

Responses to the peptides. The effects of human CGRP, ADM, PAMP, and PAMP(12–20) on human thymic arteries 90 to 249 μm in diameter with intact endothelium preconstricted to 60 to 75% of the response to 60 mM KCl with U-46619 are shown in Fig. 1. Superfusion with CGRP, ADM, and PAMP at bath concentrations of 10–18 to 10–5 M produced concentration-dependent increases in arterial diameter with a similar maximal response (Fig. 1). In terms of relative vasodilator activity, CGRP and ADM had similar activity with an EC50 of ~10–8 M, whereas the dose-response curve for PAMP was 1 log unit to the right of the curves for CGRP and ADM with an approximate EC50 of 10–7 M (Fig. 1). PAMP(12–20) was a partial agonist, and the maximal response to PAMP(12–20) was significantly smaller than the maximal vasodilator response observed with CGRP, ADM, or PAMP (Fig. 1). The dose-response curve for the truncated PAMP analog had a shallower slope (Fig. 1).

Role of vascular endothelium. The role of the vascular endothelium in mediating vasodilator responses to CGRP, ADM, and PAMP was investigated in small thymic arteries, and these data are shown in Fig. 2A. After removal of the endothelium with 1 ml of air perfused over a 20- to 25-min period, vasodilator responses to ADM and PAMP were significantly reduced when the arteries were preconstricted with U-46619 (Fig. 2A). Vasodilator responses to ACh were abolished after endothelial denudation, and a small but statisti-
cally significant decrease in arterial diameter (vaso-
constrictor response) was observed (Fig. 2A). Vasodila-
tor responses to sodium nitroprusside (10^-7 M) and
levromakalim (10^-6 M) were not altered, whereas the
response to PAMP(12–20) (10^-6 M) was reduced signi-
ficantly after endothelial denudation (data not shown).

Role of nitric oxide formation. The role of nitric oxide
release in mediating vasodilator responses to CGRP,
ADM, and PAMP was investigated, and these data are
summarized in Fig. 2B. L-NA decreased baseline diam-
eter by 18 ± 2% from an average resting diameter of
175 ± 14 μM, and the vessels were then preconstricted
with U-46619 to a similar level of tone as observed in
endothelium-intact arteries with U-46619. After super-
fusion with the nitric oxide synthase inhibitor L-NA
(10^-3 M), vasodilator responses to ADM, PAMP, and
ACh were significantly reduced compared with control
responses when the thymic arteries were precon-
stricted with U-46619 (Fig. 2B). In these experiments,
vasodilator responses to CGRP were not changed by
the administration of L-NA (Fig. 2B). Vasodilator re-
sponses to PAMP(12–20) (10^-6 M) were reduced sig-
nificantly, whereas the response to levromakalim (10^-7 M)
was enhanced after administration of L-NA (10^-3 M) (data not shown).

Role of cGMP. The role of the activation of nitric
oxide-sensitive guanylyl cyclase and cGMP in mediat-
ing vasodilator responses to ADM, PAMP, and CGRP
was investigated in experiments with the soluble gua-
nylyl cyclase inhibitor ODQ. ODQ decreased baseline
diameter by an average of 15% from a resting diameter of
170 ± 12 μM, and after superfusion with ODQ (3 μM), vasodilator responses to ADM, PAMP, sodium
nitroprusside, and ACh were reduced significantly when baseline diameter was decreased to similar val-
ues with U-46619 (Fig. 3A). Vasodilator responses to
CGRP were not altered by the administration of ODQ
(Fig. 3A). The administration of ODQ significantly de-
creased the vasodilator response to PAMP(12–20)
(10^-6 M) but did not change the vasodilator response to
levromakalim (10^-7 M) (data not shown).

Role of the CGRP1 receptor. The role of the CGRP1
receptor in mediating vasodilator responses to the pep-
tides was investigated in experiments with the CGRP1
receptor antagonist CGRP(8–37), and these data are
shown in Fig. 3B. Vasodilator responses to CGRP and
ADM were significantly reduced after superfusion with
CGRP(8–37) (10^-6 M) when the thymic arteries were
preconstricted with U-46619 (Fig. 3B). Vasodilator re-
sponses to PAMP and ACh were not altered by the
administration of CGRP(8–37) (10^-6 M) (Fig. 3B). This
concentration of CGRP(8–37) did not alter resting ves-
sel diameter. Vasodilator responses to PAMP(12–20)
(10^-6 M) and levromakalim (10^-7 M) were not altered
by CGRP(8–37) (data not shown).

Role of the K^+ ATP channel. The role of K^+ ATP channel
activation in mediating responses to the vasodilator
peptides was investigated, and these data are summa-
rized in Fig. 4A. Superfusion with the K^+ ATP channel
antagonist U-37883A in a concentration of 10^-6 M did
not alter vasodilator responses to CGRP, ADM, and
PAMP, whereas the vasodilator response to the K^+ ATP
channel opener levromakalim was reduced signifi-
cantly in preconstricted thymic arteries (Fig. 4A).

Effect of SQ-22536 and Rp-cAMPS. The role of ad-
ennyyl cyclase activation and PKA in mediating the
vasodilator response to CGRP was investigated, and
these data are summarized in Fig. 4B. Superfusion
with the adenyl cyclase inhibitor SQ-22536 in a con-
centration of 3 × 10^-4 M significantly decreased vaso-
dilator responses to CGRP and to isoproterenol without
altering the response to ACh (Fig. 4B). Superfusion

AJP-Regul Integr Comp Physiol • VOL 284 • FEBRUARY 2003 • www.ajpregu.org
with the PKA inhibitor Rp-cAMPS in a concentration of $3 \times 10^{-5}$ M significantly attenuated vasodilator responses to CGRP and to isoproterenol without changing the response to ACh (Fig. 4B).

The effects of the nonselective cyclooxygenase inhibitor sodium meclofenamate on responses to the peptides were investigated, and superfusion with the cyclooxygenase inhibitor ($10^{-6}$ M) did not alter vasodilator responses to CGRP, ADM, PAMP, or ACh but decreased the vasodilator response to superfusion with the prostaglandin precursor arachidonic acid in preconstricted thymic arteries (data not shown).

**DISCUSSION**

The results of the present investigation show that human CGRP, ADM, and PAMP have significant vasodilator activity in small arteries from the human thymus. Vasodilator responses were concentration dependent, and the three peptides elicited similar maxi-
nal vasodilator responses when the vessels were preconstricted with U-46619, with similar vasodilator responses in vessels preconstricted with ET-1. In terms of relative potency, CGRP and ADM were similar with an approximate ED_{50} of 10^{-8} M and were 10-fold more potent than PAMP. The truncated PAMP analog PAMP(12–20) was a partial agonist that elicited ~50% of the maximal vasodilator response to the full-sequence peptide, suggesting that activity was retained when the first 11 amino acids were deleted, but that these residues may be necessary for the full expression of vasodilator activity. Although PAMP(12–20) is a partial agonist in human thymic arteries, this truncated peptide does not inhibit Ca^{2+}-dependent agonist-stimulated aldosterone secretion (2). The reason for the difference in activity is uncertain but may suggest differences in activity of the truncated peptide on receptors in small thymic arteries and in the human adrenal cortex. After endothelial removal, responses to ADM and PAMP were reduced, whereas the response to CGRP was not altered, and the vasodilator response to ACh was blocked, indicating that responses to ADM and PAMP are, in part, endothelium dependent in small human thymic arteries. Vasodilator responses to ADM, PAMP, and ACh were reduced by L-NA, whereas the nitric oxide synthase inhibitor did not reduce the response to CGRP, suggesting that responses to ADM and PAMP and to ACh are dependent, in part, on the release of nitric oxide from the endothelium. Nitric oxide activates soluble guanylyl cyclase and, following administration of ODQ, vasodilator responses to ADM and PAMP were significantly reduced, whereas the response to CGRP was not altered and responses to sodium nitroprusside and ACh were significantly decreased (3). These results suggest that endothelium-dependent vasodilator responses to ADM and PAMP, as well as to ACh, and the endothelium-independent response to nitroprusside are mediated by the activation of soluble guanylyl cyclase in small thymic arteries by nitric oxide released from the endothelium or from the nitric oxide donor sodium nitroprusside (3). The observation that the response to sodium nitroprusside was attenuated by ODQ is consistent with the observation that responses to this nitric oxide donor are inhibited by methylene blue and methemoglobin and provides support for the hypothesis that the response is mediated by activation of nitric oxide-sensitive guanylyl cyclase and increased cGMP formation in small thymic arteries (10).

The role of the CGRP1 receptor was investigated using the CGRP1 receptor antagonist CGRP(8–37) and shows that responses to CGRP and ADM are attenuated, whereas the response to PAMP was not altered. These data suggest that responses to CGRP and ADM are mediated by the activation of a CGRP(8–37)-sensitive receptor. However, the location of the CGRP(8–37)-sensitive receptor and signal transduction mechanism appears to be different for CGRP and ADM. Experiments with endothelial removal suggest that the ADM-sensitive CGRP(8–37) receptor is located on the vascular endothelium, whereas the CGRP-sensitive CGRP(8–37) receptor is not on the endothelium and is probably located on smooth muscle. The results with L-NA and ODQ indicate that the response to ADM stimulation of the endothelial CGRP(8–37)-sensitive receptor involves the release of nitric oxide and the activation of soluble guanylyl cyclase, whereas the response to CGRP activation of the CGRP(8–37)-sensitive receptor is independent of the vascular endothelium, nitric oxide release, or the activation of soluble guanylyl cyclase. The effects of selective ADM receptor antagonists on responses to ADM and CGRP would be of interest in future studies.

CGRP has been reported to act as a hyperpolarizing vasodilator by opening K_{ATP} channels in small arteries from the rabbit mesentery (30). However, the present results show that responses to CGRP, as well as to ADM and PAMP, are, in part, endothelium dependent in small human thymic arteries. Vasodilator responses to ADM, PAMP, and ACh were reduced by L-NA, whereas the nitric oxide synthase inhibitor did not reduce the response to CGRP, suggesting that responses to ADM and PAMP and to ACh are dependent, in part, on the release of nitric oxide from the endothelium. Nitric oxide activates soluble guanylyl cyclase and, following administration of ODQ, vasodilator responses to ADM and PAMP were significantly reduced, whereas the response to CGRP was not altered and responses to sodium nitroprusside and ACh were significantly decreased (3). These results suggest that endothelium-dependent vasodilator responses to ADM and PAMP, as well as to ACh, and the endothelium-independent response to nitroprusside are mediated by the activation of soluble guanylyl cyclase in small thymic arteries by nitric oxide released from the endothelium or from the nitric oxide donor sodium nitroprusside (3). The observation that the response to sodium nitroprusside was attenuated by ODQ is consistent with the observation that responses to this nitric oxide donor are inhibited by methylene blue and methemoglobin and provides support for the hypothesis that the response is mediated by activation of nitric oxide-sensitive guanylyl cyclase and increased cGMP formation in small thymic arteries (10).

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In regard to a physiological role for CGRP and other vasoactive peptides in small thymic arteries, the molecular and functional expression of CGRP receptors...
has been characterized in the human thymus (25). CGRP receptor transcripts were identified, and CGRP increased cAMP levels in human thymic epithelial cell cultures (25). It has been suggested that CGRP may exert a functional role in pathophysiological processes, such as cancer and during production of antinicotinic receptor antibodies in the human thymus (25). The present data show that CGRP has significant vasodilator activity in small arteries from the human thymus, suggesting that CGRP could play a role in the regulation of vascular tone in this endocrine organ.

In summary, the present results show that CGRP, ADM, and PAMP dilate small arteries from the human thymus. Vasodilator responses to ADM and PAMP are endothelium dependent and involve the release of nitric oxide and the activation of soluble guanylyl cyclase. In contrast, vasodilator responses to CGRP are endothelium independent and are mediated by a cAMP-dependent mechanism. Vasodilator responses to ADM and CGRP are attenuated by CGRP(8–37), suggesting that distinct CGRP(8–37)-sensitive receptors recognizing ADM are located on the endothelium and that CGRP(8–37)-sensitive receptors recognizing CGRP are located on smooth muscle cells of small thymic arteries. The present data, along with results showing the presence of CGRP receptors in the human thymus, are consistent with the hypothesis that CGRP may have a role in regulating vascular function in the thymus gland in physiological and pathophysiological conditions. The results of the present study also indicate that ADM and PAMP could have a significant regulatory effect on vascular tone in the human thymus.

This study was supported by National Institutes of Health Grant HL-62000 and a grant from the American Heart Association.

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