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Adrenomedullin, a Multifunctional Regulatory Peptide*

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ABSTRACT

Since the discovery of adrenomedullin in 1993 several hundred papers have been published regarding the regulation of its secretion and the multiplicity of its actions. It has been shown to be an almost ubiquitous peptide, with the number of tissues and cell types synthesizing adrenomedullin far exceeding those that do not. In *Section II* of this paper we give a comprehensive review both of tissues and cell lines secreting adrenomedullin and of the mechanisms regulating gene expression. The data on circulating adrenomedullin, obtained with the various assays available, are also reviewed, and the disease states in which plasma adrenomedullin is elevated are listed. In *Section III* the pharmacology and biochemistry of adrenomedullin binding sites, both specific sites and calcitonin gene-related peptide (CGRP) receptors, are discussed. In particular, the putative adrenomedullin receptor clones and signal transduction pathways are described. In *Section IV* the various actions of adrenomedullin are discussed: its actions on cellular growth, the cardiovascular system, the central nervous system, and the endocrine system are all considered. Finally, in *Section V*, we consider some unresolved issues and propose future areas for research. (*Endocrine Reviews* **21:** 138–167, 2000)

I. Introduction

A NEW regulatory peptide was discovered when a group of scientists in Japan were screening a panel of peptides extracted from a pheochromocytoma. They were looking for biological activity by testing whether the peptides could raise platelet cAMP levels. The scientists found a peptide with this activity, purified and sequenced it, and termed it "adrenomedullin" as it was derived from the adrenal medulla. The first paper on adrenomedullin, published in April 1993, described not only the purification of this peptide, but also its action on blood pressure, and the development of a specific RIA to measure circulating adrenomedullin (1). Three months later the gene encoding human adrenomedullin was sequenced (2), followed by the rat gene in September (3). Within 2 yr plasma adrenomedullin had been measured in a wide range of clinical conditions, the first candidate receptor for adrenomedullin had been identified (4), and a new field of endocrine research had begun.

The growth of interest in adrenomedullin has been exponential, with more than 600 papers published in this field to date including a number of reviews (5–10). This review aims to summarize the present state of our knowledge of adrenomedullin biology, and to focus on issues that are currently unresolved, with an indication of likely areas for future research.

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II. Synthesis and Secretion of Adrenomedullin

A. Structure and synthesis of adrenomedullin

Human adrenomedullin is a 52-amino acid peptide with a single disulfide bridge between residues 16 and 21 and with an amidated tyrosine at the carboxy terminus (1). It shows some homology with calcitonin gene-related peptide (CGRP) (1) and has therefore been added to the calcitonin/CGRP/amylin peptide family. Rat adrenomedullin has 50 amino acids, with 2 deletions and 6 substitutions compared with the human peptide (3). Porcine adrenomedullin is nearly identical to the human peptide, with only a single substitution (Gly for Asn) at position 40 (11). The sequences for both canine (12) and bovine (13) adrenomedullin have also been elucidated, and a comparison of the amino acid sequences of adrenomedullin from different species is shown in Fig. 1.

Adrenomedullin is synthesized as part of a larger precursor molecule, termed preproadrenomedullin. In both rat and human this precursor consists of 185 amino acids (2, 3), while the porcine precursor has 188 residues (11). Preproadrenomedullin contains a 21-amino acid N-terminal signal peptide that immediately precedes a 20-amino acid amidated peptide, designated proadrenomedullin N-terminal 20 peptide or PAMP (2). The biological actions of PAMP have been reviewed elsewhere (14) and will not be discussed further here. It has also been suggested that a further biologically active peptide, termed adrenotensin, may be a product of the adrenomedullin gene (15), but this awaits confirmation.

The gene encoding preproadrenomedullin is termed the adrenomedullin gene and has been mapped and localized to a single locus of chromosome 11 (16). The human adrenomedullin gene comprises 4 exons and 3 introns, with TATA, CAAT and GC boxes in the 5′-flanking region (16) (Fig. 2). There are several binding sites for activator protein-2 (AP-2) and a cAMP-regulated enhancer element (16). It has also been found that there are nuclear factor-κB (NF-κB) sites on the promoter of the adrenomedullin gene (16). The organization and chromosomal localization of the murine adrenomedullin gene have also been elucidated (17).

The adrenomedullin gene is expressed in a wide range of tissues. The initial report on the distribution of adrenomedullin mRNA suggested that the highest levels of expression were seen in the adrenal medulla, ventricle, kidney, and lung (2). Since the discovery that the adrenomedullin gene is more highly expressed in endothelial cells than even in the adrenal medulla (18), this peptide has come to be regarded as a secretory product of the vascular endothelium, together with nitric oxide (NO) and endothelin. Clearly, therefore, adrenomedullin expression is seen in all tissues of the body, and comparisons between tissues may simply reflect varying degrees of tissue vascularity. However, evidence from both immunocytochemistry and studies with cultured cell lines reveals that the adrenomedullin gene is expressed by many different cell types, in addition to vascular endothelial cells (Table 1). In addition, many tumor cell lines express the adrenomedullin gene or have been shown to synthesize the immunoreactive peptide (58). Although adrenomedullin is often described as a ubiquitous regulatory peptide, it is worth noting that certain cell types and cell lines do not appear to express the adrenomedullin gene (Table 2). It is widely recognized that adrenomedullin is a product of vascular endothelial cells, but it appears that not all vascular endothelial cells synthesize the peptide. In some tissues, although adrenomedullin immunostaining has been demonstrated in certain cells, the vascular endothelial cells and smooth muscle cells remain unstained. This is particularly noticeable in the rat adrenal gland (25, 29).

The question as to whether adrenomedullin, in common with many other regulatory peptides, is stored by some cell types has been raised. There is evidence that adrenomedullin is stored in secretory granules in the pancreas (54), but to date other endocrine tissues have not been investigated at the electron microscope level. When the adrenomedullin content of cultured cells is compared with the peptide concentration in the culture medium, there is no evidence for intracellular storage of the peptide (27), and it is thought that adrenomedullin is constitutively secreted (59).

B. Circulating adrenomedullin: adrenomedullin assays

After the initial report of picomolar levels of adrenomedullin circulating in plasma (60), several research groups have developed in-house assays to measure plasma adrenomedullin levels. In general these assays appear to have been carefully validated, with evidence presented from HPLC analysis to show that immunoreactive adrenomedullin from human plasma coelutes with authentic human adrenomedullin$_{1-52}$ (60–62). There is a remarkable consistency between these different methods in terms of the absolute concentrations of adrenomedullin reported in the circulation of healthy controls (see Table 3). We can therefore conclude with some certainty that the normal plasma concentration of adrenomedullin is in the range of 1 to 10 pM, with most values between 2 and 3.5 pM. There do not appear to be significant differences between males and females or between different age groups, although to date these questions have not been directly addressed. It has been suggested that the Peninsula assay (Peninsula Laboratories, Inc., Belmont, CA) may over-

Human	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-NH_2
Pig	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDGVAPRSKISPQGY-NH_2
Bovine	YRQSLNNFQGLRSFGCRFGTCTVQKLAHQIYHFTDKDKDGSAPRSKISPQGY-NH_2
Rat	YRQSMN--QGSRSTGCRFGTCTMQKLAHQIYQFTDKDKDGMAPRNKISPQGY-NH_2
Mouse	YRQSMN--QGSRSNGCRFGTCTFQKLAHQIYQLTDKDKDGMAPRNKISPQGY-NH_2
Dog	YRQSMNNFQGPRSFGCRFGTCTVQKLAhqiyqftdkdkdnvaprskispqgy-NH_2

FIG. 1. A comparison of the amino acid sequences of adrenomedullin from different species. Residues are shown as single amino acid codes and compared with the human sequence. −, Deleted residue; X, substituted residue; x, assumed residue in incomplete sequence for dog.

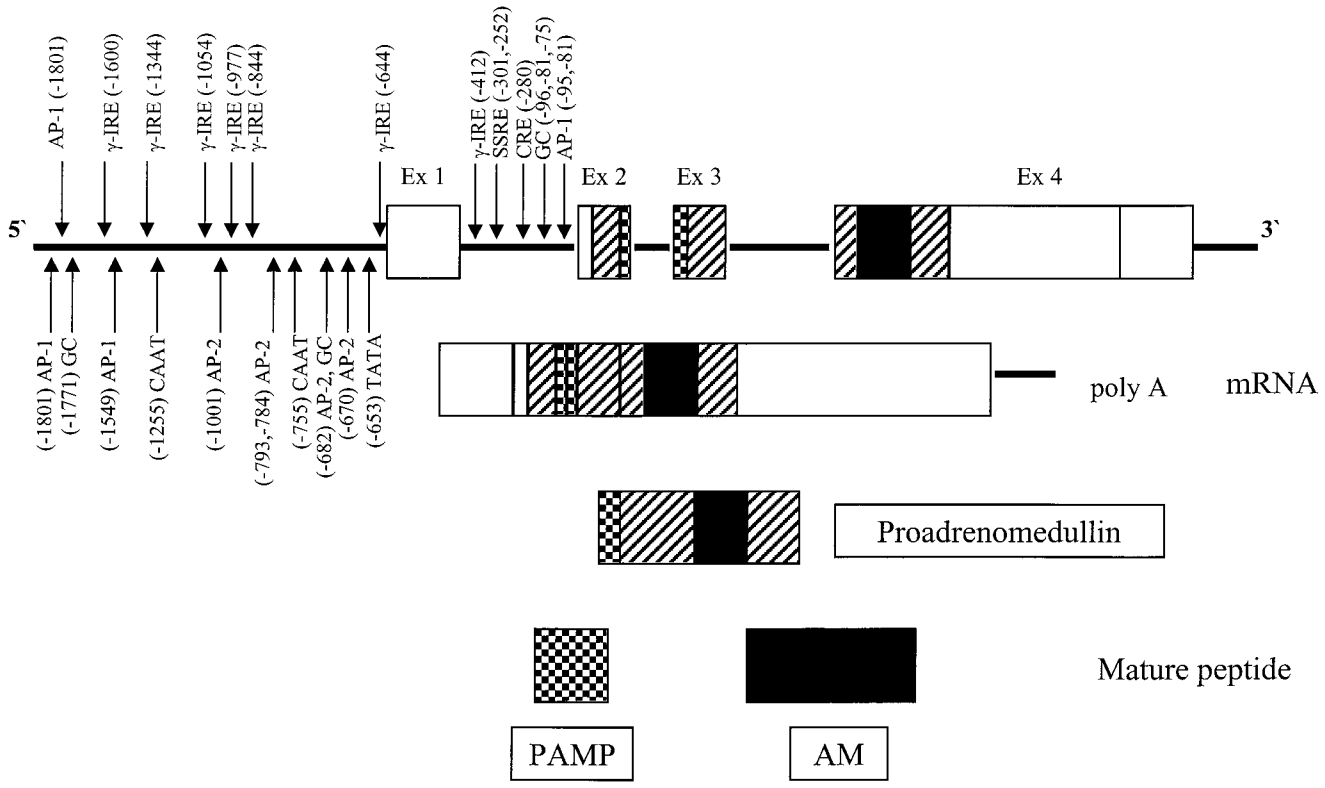

FIG. 2. Schematic presentation of the adrenomedullin gene, pre-proadrenomedullin, and biosynthesis of adrenomedullin. The genomic DNA of human adrenomedullin comprises four exons and three introns, and mature adrenomedullin peptide is coded in the fourth exon. *Arrows* indicate known regulatory sequences in the 5′-noncoding and intron 1 region of the human adrenomedullin gene.

estimate the levels of adrenomedullin (63), and although a comparison of the values obtained with the Peninsula assay in different laboratories shows that these tend to be higher than values obtained with other assays (see Table 3), these values are still within the range of 1 to 10 pM. The sole exception is the value reported by Hata and co-workers (77), which is clearly outside the normal range, although the reason for this is unclear.

There is evidence to suggest that the major circulating form of adrenomedullin is a carboxy-terminal glycine-extended peptide, which is converted to mature adrenomedullin by enzymatic amidation (78). It is thought that this glycine-extended peptide, termed the intermediate form, is the peptide that is processed from preproadrenomedullin. The plasma concentration of glycine-extended adrenomedullin (iAM) has been reported to be 2.7 ± 0.18 pM, with mature adrenomedullin (mAM) present at a concentration of 0.48 ± 0.05 pM (78). It has been observed that in congestive heart failure both iAM and mAM are similarly increased: the ratio of iAM to mAM does not significantly change (79). As far as it may be ascertained from the range of values reported, it appears likely that most assays used for the determination of plasma adrenomedullin are measuring either iAM or total adrenomedullin. Unless future studies demonstrate significant divergence between mAM levels and total adrenomedullin, this situation would appear to be satisfactory. One factor that may challenge this assumption is the recent discovery of adrenomedullin serum-binding protein (AMBP-1), which may affect adrenomedullin bioavailability (80).

Adrenomedullin has also been measured in rat plasma, using an antiserum that recognizes both rat and human forms of adrenomedullin. Adrenomedullin levels in rat plasma were comparable with those measured in man, at 3.6 ± 0.34 pM (81).

C. Circulating adrenomedullin in disease

Adrenomedullin has been measured in a wide range of disease states (see Table 4). In many cardiovascular disorders, plasma adrenomedullin is reported to be elevated, possibly suggesting that increased adrenomedullin is part of the homeostasis of blood pressure, released to compensate for elevated blood pressure. The finding that adrenomedullin is lower in preeclampsia compared with uncomplicated pregnancies may suggest that adrenomedullin is involved in the pathogenesis of this disorder (77). These findings have been questioned, however, on the basis of the exceptionally high adrenomedullin levels measured in the healthy controls in this study (77) (see Table 3). A more recent study has reported no difference in plasma adrenomedullin between preeclamptic and normotensive pregnant women, although adrenomedullin concentrations in amniotic fluid were found to be higher in preeclampsia (89).

In general, it appears that elevated adrenomedullin is a

TABLE 1. Cell types that have been found to express the adrenomedullin gene or to synthesize immunoreactive adrenomedullin

Cell type			Ref.
Cardiovascular system			
Vascular endothelial cells	Rat, pig, cow, human	RIA/mRNA analysis	(18)
Vascular smooth muscle	Rat, cow	RIA/mRNA analysis	(19, 20)
Atrial and ventricular myocardium	Dog	ICC	(21)
Umbilical vein endothelial cells	Human	RIA/mRNA analysis	(22)
Cardiomyocytes	Rat, neonatal	mRNA analysis	(23, 24)
Endocrine cells			
Zona glomerulosa	Rat	ICC/ISH	(25)
Cultured adrenocortical cells	Human	mRNA analysis	(26)
Adrenal cell line SW13	Human	RIA/mRNA analysis	(27)
Chromaffin cells	Rat	ICC	(25, 28)
	Human	ICC	(29)
Pheochromocytoma	Human	mRNA analysis	(26)
Posterior pituitary	Rat	ISH	(30)
Central nervous system			
Paraventricular nucleus	Human	ICC	(29)
	Rat	ICC	(31)
Supraoptic nucleus	Human	ICC	(29)
	Rat	ICC	(31)
Infundibular nucleus	Human	ICC	(29)
Astrocytes	Rat	mRNA analysis	(32)
Glioblastoma cell line T98G and A172	Human	RIA/mRNA analysis	(33)
Blood cells			
Macrophage	Mouse	RIA/mRNA analysis	(34, 35)
	Human	RIA	(36)
Cell line RAW264-7	Mouse	RIA/mRNA analysis	(35)
Cell lines THP-1 and HL60	Human	RIA	(36)
Granulocytes			
Lymphocytes			
Monocytes			
Kidney			
Mesangial cells	Human	RIA/mRNA analysis	(37)
	Rat	RIA	(38)
	Rat	mRNA analysis	(39)
Glomerular epithelial cells	Human	RIA/mRNA analysis	(37)
	Mouse	ISH	(30)
	Rat	mRNA analysis	(39, 40)
	Dog	ICC	(21)
Distal tubules	Dog	ICC	(21)
Collecting duct	Dog	ICC	(21)
	Rat	mRNA analysis	(39)
Renal tubular cell lines MDCK	Dog	RIA	(41)
LLCPK1	Porcine		
MDBK	Bovine		
Respiratory tract			
Bronchial epithelium	Fetal human	ICC	(42)
	Mouse	ISH	(30)
Columnar epithelium	Human	ICC	(43)
Parasympathetic neurons			
Chondrocytes			
Alveolar macrophages			
Smooth muscle cells			
Reproductive system			
Granulosa cell	Rat	mRNA analysis	(44)
Placental trophoblasts	Human	ICC	(45)
Endometrial epithelium	Human	ICC	(46)
	Mouse	ISH	(30)
Endometrial macrophages	Human	ICC	(47)
Mammary ductal cells	Mouse	ICC	(48)
Prostatic epithelial cells	Rat	ISH	(49)
Skin			
Dermal epithelium	Human	ICC/ISH	(50)
Keratinocytes			
Hair follicles			
Skin cell lines: CRL 7922	Human	Western blot/mRNA analysis	(50)
CRL 7729			
CRL 7585			
CRL 7687			
Gastrointestinal tract			
Epithelial cells	Mouse	ISH	(30)
Colorectal carcinoma cell line DLD-1	Human	RIA/mRNA analysis	(51, 52)
Enterochromaffin cells	Rat	ICC	(28)
Pancreas	Rat	ICC	(53, 54)
Gastric mucosa	Rat	ISH	(55)
Fibroblasts			
Rat-2	Rat	RIA	(56)
Swiss 3T3 cells	Mouse	RIA	(57)
Hs68	Human		
NHLF	Human		

ICC, Immunocytochemistry; ISH, *in situ* hybridization.

TABLE 2. Cell lines/cell types/tissues that do not express the adrenomedullin gene or synthesize immunoreactive adrenomedullin

Cell type	Species	Method	Ref.
H-146 (small cell lung carcinoma)	Human	mRNA analysis	(43)
H-187 (small cell lung carcinoma)	Human	mRNA analysis	(58)
H-23 (adenocarcinoma)			
H-460 (large cell carcinoma)			
Thyroid	Human	mRNA analysis	(58)
Thymus	Mouse	ISH	(30)
	Human	mRNA analysis	(58)
Adrenal zona fasciculata	Rat	ICC/ISH	(25)

ICC, Immunocytochemistry; ISH, *in situ* hybridization.

TABLE 3. Comparison of plasma adrenomedullin levels measured in healthy control subjects obtained using different immunoassays

Value/range obtained (pmol/liter)	Subjects (n)	Assay	Ref.
1.56 ± 0.73	24	*Peninsula*	(64)
1.1 to 3.4	11	*Phoenix*	(65)
2.1 ± 0.7	13	Tokyo	(66)
2.19 ± 0.38	11	*Phoenix*	(67)
2.27 ± 1.0	21	*Phoenix*	(68)
2.7 to 10.1	44	New Zealand	(62)
3 ± 0.3	30	Osaka	(61)
3.05 ± 0.6	10	*Phoenix*	(69)
3.3 ± 0.39	8	Mizayaki	(60)
4.53 ± 0.53	15	*Phoenix*	(70)
6.47 ± 1.16	10	*Peninsula*	(71)
7.8 ± 1.4	12	*Peninsula*	(72)
8 (no SD given)	6	*Phoenix*	(73)
8.1 ± 0.7	11	ICSM, London	(74)
12.3 ± 0.68	20	*Phoenix*	(75)
13.7 ± 6.7	41	*Peninsula*	(76)
61.2 ± 7.9 (SD)	10	*Peninsula*	(77)

Assay column refers to site of laboratory where assay was developed or the company marketing the assay (an entry in *italics* indicates a commercially available assay). Only one entry from each laboratory or research group has been included. Where values were given in picograms it has been converted to picomoles using a value of 6028 as the molecular weight of human adrenomedullin.

consequence rather than a cause of the pathology. It is unclear whether systemic increases in adrenomedullin reflect an overflow from local sites of production and action, or whether in certain conditions increased plasma adrenomedullin has a hormonal function causing a general decrease in vascular resistance and a fall in blood pressure. In cirrhosis, a progressive increase in adrenomedullin has been reported with increasing severity of the disease (64). A positive correlation has been demonstrated between PRA, aldosterone concentration, and adrenomedullin (64, 101, 103), with a negative correlation between adrenomedullin and glomerular filtration rate (101), creatinine clearance, and sodium excretion (103), leading to the suggestion that adrenomedullin may have a role in the hemodynamic changes in hepatic cirrhosis that lead to the formation of ascites (103). In congestive heart failure, classified according to the New York Heart Association criteria, a progressive increase in plasma adrenomedullin has been reported from classification I to IV (87), although the association is not strong enough to provide a diagnostic marker for the different stages of heart disease (107). In patients with mitral stenosis, a significant correlation was found between plasma adrenomedullin levels and pulmonary artery pressure (70).

TABLE 4. Disease states associated with elevated plasma adrenomedullin

Disease state	Ref.
Cardiovascular disorders	
Essential hypertension	(61, 72, 82, 83)
Acute myocardial infarction	(84, 85)
Cerebrovascular disease	(86)
Heart failure	(67, 72, 87)
Congestive heart failure	(83, 88)
Preeclampsia	(89)
Hemorrhagic shock	(75)
Pulmonary hypertension (mixed etiology)	(90, 91)
Mitral stenosis	(70)
Subarachnoid hemorrhage	(92)
Respiratory disorders	
Chronic obstructive pulmonary disease	(72)
Asthma, acute only	(93)
Endocrine disorders	
Type I diabetes (with renal complications)	(76)
NIDDM	(94)
Primary adrenal insufficiency	(68)
Thyrotoxicosis (Grave's disease)	(95)
Primary hyperaldosteronism	(96, 97)
Renal disorders	
Chronic renal failure	(72)
Renal failure (mixed etiology)	(71, 74)
End-stage renal failure	(98)
IgA nephropathy	(99)
Glomerulonephritis	(100)
Other conditions	
Hepatic cirrhosis	(64, 65, 72, 101–103)
Cancer	
Lung	(75)
GI tract	(75)
ACTH-secreting adenoma	(104)
Raynauds disease	(105)
Sepsis	(75, 106)
Wegener's granulomatosis	(74)

Of all the conditions investigated, the greatest increment in plasma adrenomedullin has been observed in septic shock (75, 106). It appears that adrenomedullin has a key role in the pathophysiology of septic shock. This is the only pathological condition in which plasma levels of adrenomedullin approach the levels required for receptor activation (see *Section III* below). The plasma levels of adrenomedullin observed in patients with sepsis are likely to be directly responsible for the hypotension characteristic of septic shock, as a correlation has been demonstrated between plasma adrenomedullin concentrations and relaxation of vascular tone in this condition (108). One corollary of this is that the actions of adrenomedullin under normal conditions must therefore be autocrine or paracrine in nature.

D. Origins of circulating adrenomedullin

Adrenomedullin is synthesized in most tissues of the body (see Table 1). Although the gene encoding adrenomedullin is very highly expressed in the adrenal gland, in both zona glomerulosa and the adrenal medulla (25, 109), there is considerable evidence against the adrenal as the major source of circulating peptide. Adrenal venous sampling reveals levels of adrenomedullin that are not significantly different from arterial plasma, in contrast to epinephrine and norepinephrine, which show marked trans-adrenal gradients (110). Insulin-induced hypoglycemia has been used in an attempt to

provoke the release of medullary adrenomedullin, and while plasma epinephrine levels were increased 20-fold, no significant change in circulating adrenomedullin was observed (75). Furthermore, in a patient with a pheochromocytoma, no change in plasma adrenomedullin concentration was seen during a hypotensive attack, although both epinephrine and norepinephrine concentrations increased significantly (111). As it has been shown that adrenomedullin is cosecreted with catecholamines, at least by bovine chromaffin cells in culture (112), these data suggest that the adrenal medulla is unlikely to be a significant source of circulating adrenomedullin.

Selective arterial and venous sampling across various vascular beds (including heart, lungs, kidney, and adrenal) in patients with a variety of cardiovascular pathologies, failed to identify a site of significant adrenomedullin production (111). In congestive heart failure, however, significant cardiac secretion of adrenomedullin has been reported (88). It has been suggested that the increased plasma adrenomedullin seen in pregnancy (see below) may derive from the placenta, but in the study that measured both venous and arterial umbilical cord plasma, no difference in adrenomedullin concentrations was found, suggesting that no net production or clearance of adrenomedullin occurs in the placenta (113). In certain disease states, notably cerebrovascular disease, the reported increase in plasma adrenomedullin concentration is thought to reflect the degree of endothelial cell damage (86).

In measuring plasma adrenomedullin concentrations in disease states, it is unclear whether the generally elevated levels reflect increased production or decreased clearance. This question has not been directly addressed. However, in septic shock there is evidence for increased adrenomedullin production by several different cell types (19, 34–36, 57, 59). In congestive heart failure there is also evidence for increased cardiac production of adrenomedullin (67).

E. Metabolic clearance of adrenomedullin

The plasma half-life of adrenomedullin has been reported to be 22.0 ± 1.6 min with a MCR of 27.4 ± 3.6 ml/kg·min and with an apparent volume of distribution of 880 ± 150 ml/kg (74). The effects of plasma membrane enzymes on adrenomedullin have been investigated. It appears likely that adrenomedullin is degraded initially by metalloproteases to yield adrenomedullins 8–52, 26–52, and 33–52, followed by an aminopeptidase action to yield adrenomedullins 2–52, 27–52, and 28–52 (114). It has been suggested that the lung may be a major site of adrenomedullin clearance in man (111).

F. Adrenomedullin in other biological fluids

In addition to peripheral plasma, significant levels of adrenomedullin have been measured in urine (66, 115), milk (48), cerebrospinal fluid (CSF) (116, 117) saliva (S. Kapas, unpublished data), amniotic fluid (89), sweat (50), and in umbilical vein blood (89). Using healthy subjects, urinary adrenomedullin concentrations have been reported to be approximately 6-fold higher than plasma levels (66). The authors suggest that the lack of correlation between urinary and plasma levels argues against the kidney as a major site of adrenomedullin excretion (66). However, urinary adrenomedullin is reported to be decreased in various renal disorders, such as IgA nephropathy (99), with an increase in plasma peptide levels, possibly suggesting impaired excretion. In healthy subjects, however, the high urinary adrenomedullin concentrations relative to plasma may suggest that the kidney itself is the major source of urinary adrenomedullin (66). The concentration of adrenomedullin in CSF is lower than that in plasma, and while plasma adrenomedullin increases in pregnancy, no change in CSF concentration is seen, suggesting independent regulation of adrenomedullin in the two compartments (116).

Although the presence of adrenomedullin in murine milk has been demonstrated (48), no data are presently available on the concentration, so it is unclear whether it is actively secreted into milk. In urine (66) and sweat (50) the concentration of adrenomedullin appears to be significantly higher than in plasma, and there is evidence for production of adrenomedullin in both skin and kidney (see Table 1).

G. Regulation of adrenomedullin gene expression and peptide synthesis in vivo

The effects of various physiological manipulations on plasma adrenomedullin concentrations have been investigated in both man and other species. Exercise has been reported by some studies (83), but not others (118, 119), to increase plasma adrenomedullin in man, with a correlation between plasma adrenomedullin and blood pressure (83). Moving from low to high altitude was also associated with an increase in plasma adrenomedullin probably related to the degree of hypoxia experienced by the subjects (69). In dogs, as in man, hemorrhagic shock causes an increase in plasma adrenomedullin (120), and endotoxic shock increases adrenomedullin gene expression in blood vessels (12). In rat and mouse a consistent finding is that experimentally induced sepsis increases adrenomedullin gene expression and plasma concentration (121–125). In rats, a period of fasting causes an increase in adrenomedullin concentration in the gastrointestinal tract (126).

Pregnancy is associated with increased circulating adrenomedullin concentrations in both rats (127) and women (77, 89, 116). The plasma concentration of adrenomedullin has been reported to increase progressively from the first to third trimester, with a further increase postpartum, although these data have been questioned due to the excessively high concentrations of adrenomedullin found in this study (see Table 3) (63, 128). A more recent study reported that, while plasma adrenomedullin was increased on average 5-fold in pregnant women compared with nonpregnant women, there was no correlation with gestational age, and within 48 h post partum plasma adrenomedullin concentrations had significantly decreased (89). An interesting observation is that babies delivered by the vaginal route had significantly higher umbilical cord adrenomedullin concentrations than babies delivered by elective cesarean section (129). In rats it is possible to mimic the effects of pregnancy on plasma adrenomedullin concentrations by the administration of a progesterone derivative (127), suggesting that the increased adrenomedullin has a role in the cardiovascular changes of

pregnancy. It has been shown that adrenomedullin mRNA in the rat uterus is significantly increased in pregnancy (130), suggesting that the uterus itself may be the source of plasma adrenomedullin.

The effects of various endocrine manipulations have also been investigated. Hyperthyroid rats were found to have increased plasma adrenomedullin concentrations and also an increased adrenomedullin mRNA level in the lung (131). Glucocorticoids are also implicated in the regulation of adrenomedullin: patients with Addison's disease (primary adrenal insufficiency) had their plasma adrenomedullin levels reduced by glucocorticoid replacement (68). However, insulin-induced hypoglycemia, a potent stimulus to glucocorticoid secretion, had no effect on plasma adrenomedullin concentrations (75). In rats with septic shock, dexamethasone did not alter plasma adrenomedullin levels, but in control adrenalectomized animals dexamethasone significantly increased both lung mRNA levels and plasma adrenomedullin (122). In the rat ventral prostate adrenomedullin expression is highly androgen dependent, with a 25-fold reduction in mRNA after castration, which is fully reversible by androgen administration (132).

Adrenomedullin is implicated in the regulation of fluid and electrolyte status (5), and it has been shown that adrenomedullin concentrations are reduced by hemodialysis in patients with renal disease (71). Altering the renin-angiotensin system by the use of captopril or furosemide was found to have no effect on plasma adrenomedullin in normal subjects (133), and an infusion of ACTH was also without effect (133). Similarly it has been found that feeding rats a diet either high (4%) or low (0.02%) in salt has no effect on renal adrenomedullin gene expression (40). In the Dahl salt-sensitive rat strain, however, those on a high-salt diet had increased plasma and ventricular adrenomedullin by comparison with those on a control diet (134). In human subjects, changes in salt intake, either acute or chronic, had no effect on plasma adrenomedullin in either normotensive or hypertensive subjects (135).

It has been demonstrated that an infusion of atrial natriuretic peptide increases plasma adrenomedullin levels in healthy control subjects (73). In this study blood was taken for adrenomedullin measurement at 30-min intervals for 5 h. During this time there was apparently no change in plasma peptide levels in the control subjects, while the test subjects showed an elevated plasma adrenomedullin concentration for only the 60-min duration of the infusion. A steady-state 4-fold increase was achieved within 20 min of the onset of the infusion, and levels returned to basal within 30 min of the cessation of the infusion (73). Two models of pressure overload have also been used to investigate the regulation of adrenomedullin in the rat: hormonally induced overload, using either arginine vasopressin (AVP) or angiotensin II, resulted in an increase in cardiac adrenomedullin mRNA and peptide (136). No effect on adrenomedullin expression was seen in the surgical model, however, despite a marked increase in atrial natriuretic peptide (137).

From the data outlined above it is difficult to describe the exact mechanisms that regulate adrenomedullin synthesis and secretion *in vivo*. The question is clouded by the fact that these data were obtained from several different species and using different techniques. However, the major consistent findings are of increased adrenomedullin in two conditions: sepsis and pregnancy. It also appears likely that adrenomedullin is not, in general, subject to regulation by electrolyte balance, although in some conditions of altered blood pressure, adrenomedullin levels appear to change in a manner consistent with the possible role of this peptide in a compensatory mechanism. The data concerning hormonal regulation of adrenomedullin *in vivo* are, at present, conflicting. It also appears likely that specific regulatory mechanisms may exist in different tissues for the local control of adrenomedullin production.

H. Experimental regulation of adrenomedullin gene expression and peptide synthesis in vitro

The *in vitro* regulation of adrenomedullin gene transcription and peptide synthesis has been studied in a number of comprehensive papers by Kangawa and co-workers (19, 20, 22), using either rat vascular smooth muscle cells (VSMCs), or rat endothelial cells. Adrenomedullin production by vascular smooth muscle cells is increased by a range of cytokines, growth factors, and hormones, including tumor necrosis factor α and β, interleukin-1α and β, (19, 20), dexamethasone, cortisol, aldosterone, retinoic acid, and thyroid hormone (138, 139). Other hormones and growth factors were found to have little effect, including fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, progesterone, estradiol, and testosterone (19, 138, 139). Other studies on VSMCs have shown that oxidative stress, induced by diethyldithiocarbamate, also increases adrenomedullin production (140). The regulation of fibroblast adrenomedullin gene expression is essentially the same as that of VSMCs (57), but there are some differences between vascular endothelial cells and VSMCs, notably in their response to thrombin and γ-interferon (59). γ-Interferon also increases adrenomedullin expression by rat astrocytes (32), while interleukin-1β, tumor necrosis factor-α, and dexamethasone stimulate cardiac myocytes to produce adrenomedullin (24, 141). An interesting observation on human aortic endothelial cells is the finding that shear stress down-regulates adrenomedullin gene expression (142).

In mouse and human macrophages, lipopolysaccharide, γ-interferon, tumor necrosis factor α, retinoic acid, and the phorbol ester phorbol 12-myristate 13-acetate increase adrenomedullin gene transcription and secretion (34–36). Synergistic effects were found when retinoic acid was added in combination with other effectors (36). It has also been shown that the antiestrogen, tamoxifen, induces adrenomedullin synthesis in endometrial macrophages (47).

Lipopolysaccharide is a potent stimulus to adrenomedullin secretion by macrophages (34–36), VSMCs (19), fibroblasts (57), and endothelial cells (59). It is clear that the induction of adrenomedullin transcription and synthesis by lipopolysaccharide and cytokines gives this peptide a significant role in sepsis and inflammatory states. However, more tissue-specific regulatory mechanisms also exist. In rat granulosa cells, for example, adrenomedullin gene expression is decreased by FSH treatment (44), and there is evidence that adrenomedullin is differentially regulated in renal mesangial and glomerular epithelial cells (37). In general, it ap-

pears that cAMP-mediated effects decrease adrenomedullin, while activation of the phospholipase C-protein kinase C pathway stimulates adrenomedullin (37, 143). Studies on transcriptional regulation of both human and rat adrenomedullin gene suggest that the effects of the cytokines are mediated by the NF-IL-6 regulatory element in the promoter region of the adrenomedullin gene (143, 144).

III. Receptors and Signal Transduction

Because the receptors involved in signaling the effects of adrenomedullin have already been reviewed in part (5, 6), in the present work we have concentrated on exciting recent findings relating to the molecular identity of adrenomedullin and CGRP receptors.

A. Do CGRP receptors mediate the effects of adrenomedullin?

Adrenomedullin receptors have always been closely associated with receptors for the related peptide, CGRP (145). CGRP receptors have been classified into two subtypes on the basis of the potency (pA_2) of the CGRP receptor antagonist fragment, $CGRP_{8-37}$ (146, 147). Some $CGRP_1$ receptors, at least in the rat, are antagonized by the fragment with a pA_2 of about 8.0 while $CGRP_2$ receptors require higher concentrations (pA_2 ~6.0) (148–150). $CGRP_2$ receptors have also been characterized by the ability of the CysACM analog (α-[acetimidomethyl-Cys^{2,7}]hαCGRP) to act as an agonist at these receptors but not at $CGRP_1$ receptors (151), but this has recently been disputed (152).

Initial pharmacology seemed to indicate that the vascular effects of adrenomedullin were directly mediated by a well characterized $CGRP_1$ receptor mechanism (153, 154). Nuki *et al.* (155) showed that the vasodilator effects of adrenomedullin and CGRP on the rat mesenteric vascular bed (a prototypic $CGRP_1$ preparation) could be blocked by $CGRP_{8-37}$. Similar effects of $CGRP_{8-37}$ on CGRP- or adrenomedullin-induced vasodilation were shown in the isolated rat heart preparation (156) and in the rat and hamster microvasculature (157). Since publication of these studies, a large body of evidence suggests that some adrenomedullin effects can be blocked by $CGRP_{8-37}$ (130, 157–164), but here we will consider only the receptors. Adrenomedullin certainly can bind with high affinity to and activate CGRP receptors in SK-N-MC neuroblastoma cells, commonly used as a model of $CGRP_1$ receptors (156, 162). In the study of Zimmermann *et al.* (162) adrenomedullin was only 7 times weaker in affinity than CGRP (IC_{50}= 0.3 *vs.* 2 nM). Adrenomedullin has also been shown to compete with ^{125}I-CGRP binding in rat lung and heart membranes (165), rat brain (166), SK-N-MC (156, 162, 167), L6 myoblasts (163), rat spinal cord (168), rat aorta (169), rat uterus (130), guinea-pig vas deferens (170), and rat hypothalamus (158). These data in general support the idea of high-affinity (low nanomolar) binding of adrenomedullin to all CGRP receptors with an affinity of about one-tenth to one-hundredth that of CGRP itself.

Interestingly, adrenomedullin has a low affinity (IC_{50}=129 nM) for ^{125}I-CGRP binding sites in guinea-pig vas deferens (170), a model of $CGRP_2$ receptors, indicating perhaps that adrenomedullin has a lower affinity at these receptors than at $CGRP_1$ receptors. Some caution should be used when interpreting the inhibitory effect of $CGRP_{8-37}$ on adrenomedullin actions as evidence of CGRP receptor involvement. In some studies very high concentrations of the antagonist are used, which may bind to specific adrenomedullin receptors (*e.g.*, Ref. 56; IC_{50} for $CGRP_{8-37}$ binding was 214 nM at adrenomedullin receptors in Rat-2 cells, where 10 μM CGRP inhibited cAMP elevation via specific adrenomedullin receptors) and cloud the interpretation. Binding studies showing an affinity of $CGRP_{8-37}$ for ^{125}I-CGRP sites similar to the concentrations used to inhibit adrenomedullin effects (*e.g.*, Ref. 163) are very useful although not always possible in animal experiments. *In vivo* experiments that show a lack of effect of $CGRP_{8-37}$ on adrenomedullin effects but antagonism of CGRP effects at the same concentration are convincing evidence for specific adrenomedullin effects (169, 171–175).

B. Are there specific adrenomedullin receptors?

Thus, CGRP receptors mediate at least some of the effects of adrenomedullin. However, later experiments using ^{125}I-adrenomedullin showed that specific adrenomedullin receptors existed. Eguchi *et al.* (176) demonstrated binding of ^{125}I-rat adrenomedullin to rat VSMCs and that this binding could be competed by rat adrenomedullin [dissociation constant (K_D) = 13 nM] and CGRP [inhibition constant (K_i) = 300 nM (176)]. This 23 times greater affinity for adrenomedullin over CGRP would not be expected for a CGRP receptor. Surprisingly, the rat adrenomedullin-mediated stimulation of cAMP levels seen in these VSMCs was inhibited by $CGRP_{8-37}$, albeit at high concentrations [IC_{50} = 300 nM (176)]. In another study on rat VSMCs, human adrenomedullin increased intracellular cAMP with an EC_{50} of 20 nM compared with 8.5 nM for CGRP with $CGRP_{8-37}$ blocking the action of adrenomedullin (IC_{50}=93 nM) (177). However, ^{125}I-human adrenomedullin binding in these cells (IC_{50}=73 nM) was not inhibited by either CGRP or $CGRP_{8-37}$ at concentrations up to 10 μM (177).

C. The pharmacology of specific adrenomedullin receptors

The data of Ishizaka *et al.* (177) and Eguchi *et al.* (176) indicate the presence of receptors with a higher affinity for adrenomedullin than CGRP, distinguishing these from any known CGRP receptor (148, 150). Examination of specific ^{125}I-adrenomedullin binding sites in rat tissues (165) showed high levels of specific binding in heart, lung, spleen, liver, skeletal muscle (soleus, diaphragm, and gastrocnemius), and spinal cord. CGRP receptors are also abundant in highly vascular tissues such as lung, heart, and spleen (145, 150). Apart from spinal cord, binding in the central nervous system (CNS) was low in contrast to CGRP binding, which is widespread and abundant in the brain (7, 145). Binding in adrenal and kidney membranes was low but as binding was measured in membrane preparations from whole tissues/glands, this in no way negates the large bodies of evidence for important roles for adrenomedullin in these tissues acting via highly localized receptors (5, 25, 178).

Binding sites in heart and lung were further characterized. These sites showed saturation dissociation and competition as would be expected of receptor binding sites. Rat and human adrenomedullin competed at both sites with rat adrenomedullin showing the greater affinity (Table 5). Competition by CGRP, amylin, and calcitonin was approximately 3 orders of magnitude less than rat adrenomedullin, indicating a high level of specificity. Other reports of ^{125}I-adrenomedullin binding also show a low affinity of CGRP at this site [VSMCs (176, 177), NG108–15 neuroblastoma-glioma cells (179), Swiss 3T3 mouse fibroblasts (57, 180), rat hypothalamus (158), rat spinal cord (168), rat blood vessels (169), L6 myoblasts (163), rat uterus (130), human brain (181), bovine endothelial cells (182), mouse astrocytes (183), human oral keratinocytes (184), rat-2 fibroblasts (56), rabbit kidney glomeruli (174), guinea-pig vas deferens (170), rat adrenal zona glomerulosa cells (25), and human skin cells (50)]. These results are summarized in Table 5. It is clear from the table that high-affinity (mean affinity = 6 nM) ^{125}I-adrenomedullin binding sites can be detected in tissues and cells from a number of species with differing methodologies. These sites all show low affinity for CGRP and, where measured, amylin

TABLE 5. [^{125}I]Adrenomedullin binding sites

Tissue or cell line	^{125}I-AM[a]	AM affinity	αCGRP affinity	Other competitors	Binding site density (B_{max})
Rat VSMC (176)	rAM	13 nM (K_d)	300 nM(K_i)	ND	B_{max} = 19,000 per cell
Rat VSMC (177)	hAM	73 nM (IC_{50})	>10,000 nM	$CGRP_{8–37}$ > 10,000 nM	ND
Rat VSMCs (185)	hAM	4 nM (K_D)	ND	$AM_{22–52}$ = 1,600 nM (K_i)	B_{max} = 29,000 per cell
Bovine aortic EC (182)	hAM (L)	10 nM[b]	>1,000 nM	ND	—
Rat lung membranes (165)	rAM (I)	1.3 nM (K_D)	>1,000 nM	hAM = 94 nM, sCT and AMY >1,000 nM	B_{max} = 2,800 fmol/mg[c]
Rat heart membranes (165)	rAM (I)	0.5 nM (K_D)	1,050 nM(IC_{50})	hAM = 4.2 nM, AMY = 240 nM, sCT >1,000 nM	B_{max} = 470 fmol/mg
Swiss 3T3 mouse fibroblasts (180)	rAM (I)	3.5 nM (IC_{50})	>1,000 nM	ND	B_{max} = 16,000 per cell
Swiss 3T3 mouse fibroblasts (57)	rAM (L)	0.6 nM (IC_{50})	510 nM(IC_{50})	hAM = 4.1 nM, $hAM_{22–52}$ = 900 nM, $CGRP_{8–37}$ = 60 nM	B_{max} = 12,000 sites per cell
Human breast cancer cell line MCF-7 (58)	hAM (P)	4 nM (K_D)	ND	ND	B_{max} = 50,000 per cell
Rat astrocytes (179)	hAM (CT)	0.27 nM (IC_{50})	23 nM(IC_{50})	AMY = 40 nM, sCT >1000 nM, $CGRP_{8–37}$ = 2.5 nM	ND
Rat hypothalamic membranes (158)	rAM (I)	0.54 nM (K_D)	>1,000 nM	$CGRP_{8–37}$ >1,000 nM	B_{max} = 214 fmol/mg
Rat aortic membranes (169)	rAM (I)	1.38 nM (K_D)	>1,000 nM	$CGRP_{8–37}$ >1,000 nM	B_{max} = 583 fmol/mg
L6 rat skeletal myoblasts (163)	rAM (I)	0.22 nM (IC_{50})	>1,000 nM	$CGRP_{8–37}$ = 601 nM	B_{max} = 950 fmol/mg
Rat spinal cord membranes (168)	rAM (I)	0.45 nM (K_D)	>10,000 nM	sCT and AMY >10,000 nM	B_{max} = 723 fmol/mg
Rat uterus membranes (130)	rAM (I)	0.08 nM (K_D)	>1,000 nM	$CGRP_{8–37}$ >100 nM	B_{max} = 21 fmol/mg
Rabbit kidney glomeruli (174)	rAM (CT)	0.45 nM (IC_{50})	>1,000 nM	$CGRP_{8–37}$ = 2,100 nM (IC_{50})	ND
Human skin cell lines (50)	hAM (P)	9 nM (K_D)[d]	>1,000 nM	$CGRP_{8–37}$, $AM_{22–52}$ and AMY >1,000 nM	B_{max} = 14,000 per cell
Human oral keratinocytes (184)	hAM (P)	8.2 nM (K_D)	>1,000 nM	$CGRP_{8–37}$ and AMY >1,000 nM	B_{max} = 466 fmol/mg
Human brain membranes[e] (181)	hAM (CT)	0.17 nM (K_D)	>1,000 nM	$CGRP_{8–37}$, $hAM_{22–52}$ = 300 nM	B_{max} = 723 fmol/mg
Rat zona glomerulosa cells (25)	rAM (P)	5.5 nM (K_D)	>50,000 nM	AMY >50,000 nM	B_{max} = 400 fmol/10^6 cells
Rat-2 fibroblasts (56)	rAM (I)	0.43 nM (K_D)	>1,000 nM	$CGRP_{8–37}$ = 214 nM	B_{max} = 50 fmol/mg
Guinea pig vas deferens membranes (170)	rAM (I)	1.2 nM (IC_{50})	224 nM(IC_{50})	AMY = 407 nM (IC_{50})	B_{max} = 643 fmol/mg
L1 rat adrenomedullin clone (4)	rAM (CT)	8.2 nM (K_D)	ND	ND	B_{max} = 680 fmol/mg
Human CRLR + RAMP-2 (186)	rAM (AM)	0.75 nM (IC_{50})	1,300 nM(IC_{50})	$CGRP_{8–37}$ = 75 nM, $AM_{22–52}$ = 15 nM (IC_{50})	B_{max} = 50,000 per cell
Rat CRLR + RAMP-2 in UMR-106 osteoblasts (187)	rAM (CT)	0.35 nM (K_D)	>1,000 nM	AMY >1,000 nM, $CGRP_{8–37}$ = 38 nM	ND

AM, Adrenomedullin, AMY, amylin, ND, not determined, IC_{50}, concentration inhibiting binding by 50%, K_D, dissociation constant, K_i, absolute inhibition constant, VSMC, vascular smooth muscle cell, EC, endothelial cell.

[a] The *letter in parentheses* denotes the method of iodination: L, lactoperoxidase; CT, chloramine T; I, iodogen or the source if purchased (P, Phoenix Pharmaceuticals Inc. or AM, Amersham Pharmacia Biotech).

[b] An exact IC_{50} could not be calculated.

[c] Femtomoles per mg membrane protein.

[d] CRL-7922 from normal skin.

[e] Cerebral cortex used in all measurements quoted.

and calcitonin, and therefore appear highly specific. The human adrenomedullin fragment, adrenomedullin$_{22-52}$, has been used as a specific adrenomedullin receptor antagonist (164, 185, 188–193) in a similar way that CGRP$_{8-37}$ is used for CGRP$_1$ receptors. In rabbit aortic endothelial cells [K_i for adrenomedullin-stimulated cAMP was 2.6 nM with no effect on CGRP-stimulated cAMP (188)] and rat cerebral blood vessels [5 μg/kg/min infusion inhibited adrenomedullin-mediated vasodilation (193)] adrenomedullin$_{22-52}$ was an effective antagonist. Some specificity was demonstrated by its lack of effect in T47 D cells [calcitonin receptor, 1000 nM (190)] or L6 myocytes [CGRP receptor, 1000 nM (192)]. However, in rat mesangial cells (IC_{50} for inhibition of adrenomedullin-stimulated cAMP: 70 nM compared with 50 nM for CGRP$_{8-37}$) and human neuroblastoma TGW cells [DNA synthesis stimulated by adrenomedullin (190)], there was no difference in potency between the effects of adrenomedullin$_{22-52}$ and CGRP$_{8-37}$. In rat VSMCs [half-maximal antagonism of adrenomedullin-stimulated cAMP was 4000 nM and K_i for binding 1600 nM (185)], adrenomedullin$_{22-52}$ was a very weak antagonist. Worse still, in rat cardiac cells [adrenomedullin-stimulated cAMP (189)] and the hindlimb vascular bed of the cat [vasodilation — effect of 30 nmol adrenomedullin$_{22-52}$ (194)], adrenomedullin$_{22-52}$ was inactive against adrenomedullin but inhibited CGRP effects. Thus, better antagonists need to be developed. It has been suggested that human adrenomedullin$_{26-52}$ is a more specific antagonist (195). The use of either rat or human adrenomedullin as radioligand appears not to affect the results and the two labels cross-react across species (170, 174, 177, 179, 182, 185, 186). We have found that human adrenomedullin is associated with a much higher nonspecific binding than rat adrenomedullin and therefore prefer this radioligand (nonspecific binding was 8% for rat adrenomedullin and 23% for human adrenomedullin in rat lung (A. A. Owji and D. M. Smith, unpublished observation). One obvious conclusion from this binding data is that circulating levels of adrenomedullin, approximately 3–6 pM in man (60, 62) and 3 pM in rat (81), cannot mediate the physiological effects of adrenomedullin by these specific receptors or by CGRP receptors, placing it firmly as a paracrine/autocrine factor.

Adrenomedullin seems to have little affinity for receptors for the other two members of the peptide family, calcitonin and amylin. Adrenomedullin has been shown to interact with the calcitonin receptor on human breast cancer T 47D cells on the basis that adrenomedullin-stimulated cAMP in these cells could be inhibited by the calcitonin receptor antagonist, salmon calcitonin$_{8-32}$ but not adrenomedullin$_{22-52}$ or CGRP$_{8-37}$ (190). However, very high concentrations of adrenomedullin (EC_{50}=132 nM) were required to stimulate cAMP, questioning the physiological role of such an interaction. In a comprehensive study, Vine *et al.* (167) showed little effect of human adrenomedullin *in vivo* on typical amylin (inhibition of soleus muscle glycogen synthesis, hyperlactemia) or calcitonin (hypocalcemia, inhibition of gastric emptying) effects. This correlated with weak competition in model amylin (rat nucleus accumbens membranes, K_i = 51 nM) and calcitonin (T 47D cells, K_i = 33 nM) receptor binding sites. Adrenomedullin also showed very low affinity for amylin binding sites (IC_{50}=0.33–18 μM depending on brain region) in an autoradiographic study of rat brain (166). Amylin binding sites in rat lung (196) have a higher affinity for adrenomedullin than amylin suggesting that these sites may be adrenomedullin binding sites (165). Very little receptor autoradiography has been performed using ^{125}I-adrenomedullin (probably because of technical problems with the radioligand). However, binding sites in the rat lung, kidney cortex, and liver have been shown by *in vivo* autoradiography with most binding localized to endothelial cells of small arteries (197). Binding sites in the human adrenal, specifically in the zona glomerulosa and capsular vessels, were demonstrated using *in vitro* autoradiography with only the vessel sites not competed by CGRP$_{8-37}$ (198).

One aspect of this binding data that remains puzzling is the lack of competition of ^{125}I-adrenomedullin binding by CGRP in tissues and cells that express both adrenomedullin and CGRP binding, since adrenomedullin will bind effectively to ^{125}I-CGRP sites. One explanation is that if the affinity of adrenomedullin for CGRP receptors is at least 10-fold less than for adrenomedullin receptors, then binding of the low concentration of ^{125}I-adrenomedullin radioligand to CGRP receptors may not be apparent in competition studies. Another possible explanation is that the ^{125}I-adrenomedullin is specific for adrenomedullin sites, whereas adrenomedullin binds to CGRP sites as well. This was supported by the lack of competition of nonradioactive iodoadrenomedullin with ^{125}I-CGRP (165). This requires further investigation, perhaps involving the development of new adrenomedullin probes such as fluorosceinated/biotinylated adrenomedullin. ^{3}H-adrenomedullin would be effective in tissues where receptor numbers are high, such as lung, but would be limited by its low specific activity. One possible improvement on this would be the use of higher specific activity metabolically labeled ^{14}C-adrenomedullin.

D. Receptor biochemistry: chemical cross-linking of adrenomedullin and CGRP receptors

Chemical cross-linking experiments using ^{125}I-rat adrenomedullin showed relative molecular weights (M_r) for adrenomedullin binding site-ligand complexes (in this section minor bands are shown in *italics* separated from the major band by a *slash mark*) in rat VSMCs of 120,000 and 70,000 (176) and in rat tissues of 83,000/*105,000* and 94,000 (165). In neither case were the labeled bands competed by CGRP. In further experiments specific adrenomedullin binding site-ligand complexes were demonstrated with M_rs, of 83,000 in rat 2 fibroblasts (56), of 84,000/*122,000* in rat spinal cord (199) of 76,000 in L6 myoblasts (163), and of 89,000/*105,000* in rat aorta (169). These complexes can be compared with cross-linked ^{125}I-CGRP sites that vary in M_r: 70,000/*110,000* in rat skeletal muscle, 55,000/*44,000* in rat liver (200), 70,000 and 120,000 in porcine ventricle (201), 75,000–90,000 in rat spleen (202), and 60,000–70,000 in rat VSMCs (203). Thus, on average, CGRP binding sites have an M_r of about 70,000 with adrenomedullin binding site about 85,000. This assertion should be treated with some caution as cross-linked bands are often broad, making comparison across studies difficult [*e.g.*, in rat liver Stangl *et al.* (202) found M_r = 74,000 and 68,000 compared with M_r = 55,000 by Chantry *et al.*

(200)]. Also the same receptor protein may be subject to large variations in size due to differential glycosylation in different tissues and species.

In rat spinal cord and L6 myoblasts, a direct comparison of the cross-linked adrenomedullin and CGRP bands was made (163, 199). In spinal cord ^{125}I-CGRP complexes showed an M_r= 74,000 and 61,000 compared with M_r = 84,000/*122,000* for ^{125}I-adrenomedullin complexes. Deglycosylation of ^{125}I-adrenomedullin complexes in spinal cord, heart, and lung resulted in a number of complexes with the lowest M_r being 52,000, 47,000, and 43,000, respectively (199). In L6 myoblasts ^{125}I-CGRP complexes showed an M_r = 82,000 compared with M_r = 76,000 for ^{125}I-adrenomedullin complexes. Thus, on the whole, although there is some evidence for CGRP receptors showing a lower M_r on SDS-PAGE than adrenomedullin receptors, this remains an outstanding question. Also a number of cross-linking studies of both adrenomedullin and CGRP binding sites (165, 169, 176, 199, 200) show second bands of higher mol wt than the major band, indicating the possibility of a further complex in addition to that of the ligand and binding site. On the whole, these second bands are not large enough to be receptor dimers (204).

E. Receptor biochemistry: molecular characterization of adrenomedullin and CGRP receptors

Examination of the pattern of binding of ^{125}I-adrenomedullin in rat tissues led us to reconsider the role of the L1 orphan receptor (205) [also known as G10d from rat liver (206), a 395-amino acid seven-transmembrane receptor, GenBank accession number L04672], which is expressed in lung, adrenal, heart, and spleen (4). When transfected into COS-7 cells, this receptor bound ^{125}I-adrenomedullin (K_D = 8.2 nM) and gave adrenomedullin-mediated increases in intracellular cAMP (ED_{50} = 7 nM) that were only inhibited by high concentrations of $CGRP_{8-37}$ (K_i = 1 μM) (4). The human homolog of this receptor was then cloned but not expressed and found to show 73% similarity by amino acid sequence, which is not high for a species homolog (404 amino acids, EMBL accession number Y13583) (207). The identification of both rat and human sequences as adrenomedullin receptors has recently been questioned (208). No binding of rat or human ^{125}I-adrenomedullin followed transfection of either sequence into COS-7 cells, despite the presence of mRNA and expression of the protein at the cell surface (208). The most closely related receptor to L1 is a dog 7-transmembrane receptor called RDC-1 (49) (GenBank accession number X14048). Expression of this receptor in COS-7 cells gave a pharmacology typical of a $CGRP_1$ receptor with CGRP-stimulated cAMP generation (EC_{50} = 3 nM) potently inhibited by $CGRP_{8-37}$ (209). Adrenomedullin also stimulated cAMP levels with an EC_{50} of 100 nM, as would be expected of a $CGRP_1$ receptor. Binding studies showed a similar affinity for CGRP and $CGRP_{8-37}$ (9 and 13 nM, respectively) (209). RDC-1 mRNA expression is high in vascular tissues such as lung and liver, which express high levels of ^{125}I-CGRP binding (49).

An interesting study from Luebke *et al.* (210) showed the expression cloning of a hydrophilic 146-amino acid protein from guinea pig organ of Corti, which conferred CGRP receptor activity on *Xenopus* oocytes (210) (GenBank accession number U50188). This protein, called receptor component protein (RCP), is expressed in human and mouse mainly in testis, with smaller amounts in human in prostate, ovary, small intestine, and spleen (211). RCP conferred CGRP (10 nM) effects on oocytes but not calcitonin or amylin (100 nM) effects. Using *in situ* hybridization in the guinea pig CNS, RCP was shown to be abundant in the cerebellum and hippocampus (212). Adrenomedullin effects via RCP have not been tested, but the limited distribution of RCP means it can only account for a small subset of adrenomedullin receptors at best unless other RCPs are yet to be cloned.

The other side of the CGRP/adrenomedullin receptor story relates to another orphan receptor called calcitonin receptor-like receptor (CRLR). This was originally cloned by two groups, Legon and co-workers (213) (GenBank accession number X70658) using a PCR strategy amplifying rat hypothalamic mRNA with primers based on the porcine calcitonin and opossum PTH receptors and Chang *et al.* (214), who also described the identification of a CRF receptor (GenBank accession number L27487). The full sequence of the human homolog of CRLR from cerebellum was reported by Fluhmann *et al.* (215) (GenBank accession number U17473). Human CRLR, expressed mainly in lung, heart, and kidney, is a 461-amino acid seven-transmembrane protein with 91% homology to its rat homolog and 51% similarity to the human calcitonin receptor. This receptor expressed in COS-7 cells did not bind any member of the calcitonin family of peptides and was considered an orphan receptor. However, in 1996 Aiyar and co-workers (216) showed that hCRLR stably transfected into human embryonic kidney (HEK) 293 cells exhibited the pharmacology of a $CGRP_1$ receptor (CGRP K_d = 19 pM, $CGRP_{8-37}$ pA_2 = 7.57, CysACM-CGRP ineffective up to 1 μM) (216). Adrenomedullin showed binding and stimulation of cAMP, albeit weak, in these cells. These results were confirmed using the rCRLR stably transfected into HEK 293 cells (217) and later the porcine CRLR as well (218). CRLR mRNA is extremely abundant in the rat lung [as is specific ^{125}I-adrenomedullin binding (165)] and was shown by *in situ* hybridization studies to be associated with blood vessels (213). CRLR protein was also shown by immunocytochemistry to be associated with vascular endothelial cells (217). This fits well with a role for adrenomedullin as a pulmonary vasodilator (see *Section IV.A*) and the presence of adrenomedullin binding on endothelial cells (197) but disagrees with the previous localization by *in situ* PCR to alveolar cells (216).

The question now became what was the factor in HEK 293 cells that was not present in COS-7 cells that allowed CGRP receptor expression? The surprising answer was provided by Foord's group using a *Xenopus* oocyte/cystic fibrosis transmembrane regulator system, similar to that used to clone RCP, where increases in intracellular cAMP can be detected as chloride currents. They cloned a receptor-activity modifying protein (RAMP-1) of 148 amino acids with a single transmembrane domain that conferred $CGRP_1$ receptor activity (no response to adrenomedullin, amylin, or calcitonin but inhibited by $CGRP_{8-37}$) to the oocytes (186). When transiently cotransfected with hCRLR into HEK 293 cells, RAMP-1 conferred ^{125}I-CGRP binding properties to these cells, but no binding was seen with either RAMP-1 or CRLR

alone. The pharmacology of this combination was very similar to that of CGRP receptors in SK-N-MC cells (186). The failure of expression of CGRP binding by CRLR in COS-7 cells is therefore probably due to the lack of endogenous RAMP-1. The action of RAMP-1 was shown to involve transport of CRLR to the cell surface. ^{125}I-CGRP could be cross-linked to proteins of M_r = 66,000 (CRLR) and 17,000 (RAMP-1). This M_r of 66,000 compares well with that reported for CGRP receptors in tissues (see previous section). Cross-linking of CGRP to RAMP-1 allows for the intriguing possibility that RAMP-1 forms part of the binding site for CGRP. CRLR is present in HEK 293 cells as a M_r = 58,000 glycosylated receptor, which in the presence of RAMP-1 is further glycosylated to M_r = 66,000, consistent with RAMPs acting to transport CRLR.

Two further members of the RAMP family that did not confer CGRP receptor activity were also identified, but overall the three RAMPs showed 31% identity in amino acids (186). The three RAMP mRNAs have widespread and different distributions in human tissues. RAMP-2 and RAMP-3 also facilitated expression of CRLR on the cell surface but as a M_r = 58,000 glycoprotein. Coexpression of CRLR and RAMP-2 in oocytes or HEK 293 cells resulted in a typical specific adrenomedullin receptor pharmacology with no effects of CGRP (186). Thus, the RAMP hypothesis offers an extremely interesting explanation of CGRP/adrenomedullin receptor pharmacology, *i.e.*, CRLR/RAMP-1 = $CGRP_1$ and CRLR/RAMP-2 = adrenomedullin.

Since the discovery of RAMPs, some aspects of the hypothesis have been confirmed. Kamitani *et al.* (219) showed that RAMP-2 is expressed in human VSMCs and endothelial cells, and a combination of RAMP-2/CRLR transfected into HeLa EBNA or 293 EBNA cells led to adrenomedullin-stimulated cAMP with no effect of CGRP. Transfection of RAMP-1/CRLR conferred CGRP and adrenomedullin-stimulated increases in cAMP, which differs from the oocyte studies by McLatchie *et al.* (186) but agrees with the binding data for $CGRP_1$ receptors (see above). Muff *et al.* (188) showed that rabbit endothelial cells express RAMP-2 and CRLR and that adrenomedullin stimulated cAMP (EC_{50}=0.18 nM). When these cells are transfected with RAMP-1, they then express CGRP-stimulated cAMP (EC_{50} = 0.41 nM) which is inhibited by $CGRP_{8-37}$ (100 nM), indicating that CRLR can be converted from an adrenomedullin-specific receptor to a CGRP receptor by a dominant effect of RAMP-1. This effect was further investigated by the same group using rCRLR and RAMPs expressed in UMR-106 rat osteoblast-like cells and COS-7 cells (187). UMR-106 cells transiently transfected with CRLR express ^{125}I-adrenomedullin binding, which was enhanced by RAMP-2 cotransfection. Here ^{125}I-CGRP binding required transfection of RAMP-1 but was unaffected by RAMP-2. Similar results were shown in COS-7 but, as expected, since COS-7 lack RAMPs, adrenomedullin binding required RAMP-2 cotransfection. The amino terminus of the RAMPs has been shown to be the major factor controlling glycosylation and ligand binding using chimeric RAMP-1/2 proteins (220). RAMPs 2 and 3 appear indistinguishable in terms of CRLR glycosylation and adrenomedullin binding in this study. Thus, the RAMP hypothesis appears correct but has not yet been totally proven, and some aspects of it need to be investigated. What is the purpose of RAMP-3/CRLR if it yields an identical pharmacology with RAMP-2/CRLR? In rat tissues, adrenomedullin receptors appear by cross-linking to be equal to or larger than CGRP receptors (see previous section), whereas the RAMP hypothesis predicts that CGRP receptors should be larger than adrenomedullin receptors. Do partners other than CRLR exist for RAMP, *e.g.*, the calcitonin receptor (221)? Do CRLR and RAMP-1/2 account for all adrenomedullin and CGRP binding?

There has been little study of whether the presence of RDC-1, L1, and CRLR correlate with CGRP/adrenomedullin binding. In the rat brain, the distributions of the three putative receptors were compared by *in situ* hybridization (222). RDC-1 mRNA was mainly associated with the dentate gyrus, hippocampal CA3, choroid plexus, and blood vessels. L1 was very weakly expressed except in cells of the pia mater. CRLR was expressed in the caudate putamen and the central and basolateral amygdaloid nuclei. These data match well in some areas but not at all in others with CGRP or adrenomedullin binding in the rat brain (145, 181, 223). None of the three mRNAs was present in spinal cord (222) despite high levels of CGRP and adrenomedullin binding (168). All three mRNAs were expressed in adult rat heart and neonatal cardiac myocytes, with RDC-1 being most abundant followed by CRLR with L1 being of low abundance (224). In rat aortic VSMCs, RDC-1, but not L1 or CRLR, mRNA was detected using specific RNase protection assays (225). Binding experiments using ^{125}I-adrenomedullin and fragments of adrenomedullin and CGRP showed two subtypes of adrenomedullin receptor in astrocytes and NG108–15 cells (179). Chemical cross-linking of ^{125}I-adrenomedullin binding sites in rat tissues also shows heterogeneity of mol wt (165, 168). Thus, there are indications that CRLR/RAMP and/or L1/RDC-1 do not account for all CGRP/adrenomedullin binding sites.

F. Signal transduction pathways activated by adrenomedullin

It is now clear from a vast number of studies that the major effect on adrenomedullin-stimulated cells is an elevation of cAMP (25, 33, 38, 39, 44, 56, 58, 59, 156, 162–164, 174, 176, 177, 179, 180, 182–185, 189, 226–246). This is typical of the calcitonin family of peptides, all of which have been shown to elevate cAMP levels in various tissues and cells (8, 150). It should not be forgotten that this was the property that was used to discover adrenomedullin (1). Also, all of the cloned receptors, regardless of whether they are actually adrenomedullin/CGRP receptors *in vivo*, are associated with increased cAMP when transfected into cells (4, 186, 188, 209, 216, 217). Thus, the initial mechanism of action of adrenomedullin (and CGRP) is in most cases via G-protein linked receptor activation of Gs, adenylyl cyclase, and protein kinase A (PKA) (163). In this section we will examine other possible mechanisms and some consequences of elevated cAMP. Most of the studies on adrenomedullin signaling have been performed using primary cells (especially VSMCs) or cell lines. Since adrenomedullin will bind to both specific adrenomedullin and CGRP receptors and these are often expressed together in cells, the failure to define which

receptor is actually mediating the effect is a problem. This can be addressed by use of inhibitors such as $CGRP_{8-37}$ or $adrenomedullin_{22-52}$, but these are not especially potent or specific, and better antagonists would greatly advance this work. At present, the only conclusive results are obtained by careful use of antagonists, use of cells expressing only one type of binding, or use of transfected cells. Of course the transfection approach has problems associated with heterologous expression and also the doubt surrounding which receptor should actually be transfected.

The effects of adrenomedullin on calcium signaling mechanisms have been investigated since endothelial NO has been implicated in adrenomedullin-mediated vasodilation (see *Section IV.A*), and logically this should be the result of an increase in intracellular calcium ($[Ca^{2+}]_i$) activating endothelial cell nitric oxide synthase (NOS). In the bovine aortic endothelial cell-specific ^{125}I-adrenomedullin binding (IC_{50} = 10 nM) and adrenomedullin-mediated cholera toxin-sensitive increases in cAMP were observed (EC_{50}=0.17 nM) (182). Here adrenomedullin also directly increased $[Ca^{2+}]_i$ (EC_{50} = 3 nM) with an initial peak followed by a prolonged increase. The initial effect was blocked by thapsigargin, and the prolonged effect by EGTA and nifedipine. Pretreatment of cells with U-73122, the phospholipase C (PLC) inhibitor, but not its inactive analog U-73343, blocked all calcium responses to adrenomedullin. Similarly, cholera toxin, but not H89 (PKA inhibitor) or pertussis toxin, blocked all effects. As expected for a PLC-mediated effect, adrenomedullin (100 nM) increased the intracellular levels of ITP. Adrenomedullin also increased intracellular cGMP. This very detailed study offers an interesting account of how adrenomedullin might increase NO production and thereby vasodilation. One omission in this study was the effects of CGRP and the action of $CGRP_{8-37}$ on the adrenomedullin effects. Another problem is that Barker *et al.* (247) were able to show increases in cAMP but unable to show any $[Ca^{2+}]_i$ effects in bovine endothelial cells . Adrenomedullin did not affect $[Ca^{2+}]_i$ but did increase cAMP in Swiss 3T3 cells that express only specific adrenomedullin receptors and not CGRP receptors (180). Similar results were obtained using cultured rat astrocytes (179). Adrenomedullin was shown to decrease $[Ca^{2+}]_i$ and calcium sensitivity in porcine coronary artery strips, possibly by a direct cAMP-mediated mechanism (248). In favor of adrenomedullin increasing $[Ca^{2+}]_i$ are results using KG-1C human oligodendroglial cells where adrenomedullin and CGRP both increased cAMP and $[Ca^{2+}]_i$ (228). Unfortunately the effects of $CGRP_{8-37}$ or ^{125}I-ligand binding were not investigated. Also, in the perfused rat heart, adrenomedullin enhanced cardiac contractility by a mechanism that was independent of cAMP but involved changes in $[Ca^{2+}]_i$ (249). In the L6 skeletal muscle cell line, both adrenomedullin and CGRP receptors were present, but increases in intracellular cAMP were mediated only via CGRP receptor binding (163).

Reports of effects of adrenomedullin on growth and mitogenesis (see *Section IV.B*) have led to investigation of the regulation of mitogen-activated protein kinase (MAPK) by adrenomedullin. In rat glomerular mesangial cells, adrenomedullin increased cAMP and PKA but inhibited proliferation (both of quiescent and platelet-derived growth factor (PDGF)-stimulated cells) and MAPK activity (241). Also in mesangial cells, adrenomedullin (and other agents that increased cAMP) inhibited endothelin-1 (ET-1)-stimulated MAPK (but not, in this case, basal levels) and MAPK kinase (250) and stimulated expression of a MAPK phosphatase (251). Chini *et al.* (230) also showed that adrenomedullin reduced PDGF-stimulated MAPK activity and mitogenesis in rat VSMCs, effects that were blocked by the PKA inhibitor, H89. However, in quiescent rat VSMCs, adrenomedullin increased DNA synthesis, cell proliferation, tyrosine phosphorylation, MAPK activity, and expression of the immediate-early gene, c-*fos*. These effects could be blocked by $CGRP_{8-37}$ and the tyrosine kinase inhibitor, genistein, but not by cAMP or PKA antagonists, indicating a cAMP-independent effect (252). Interestingly, in these VSMCs, adrenomedullin had no effect on $[Ca^{2+}]_i$ or ITP. In the Rat-2 fibroblast cell line, which expresses specific adrenomedullin but not CGRP receptors, adrenomedullin stimulated cAMP and inhibited basal and PDGF-stimulated MAPK (56).

Adrenomedullin has also been shown to activate other signal transduction mechanisms including K^+-ATP channels (253) and c-*fos* expression (229, 254). Adrenomedullin augmented interleukin-1β-stimulated NO synthesis in rat VSMCs by a cAMP-dependent mechanism (239). Desensitization of adrenomedullin receptors has not been widely investigated, but Iwasaki *et al.* (227) showed that adrenomedullin pretreatment caused a loss of adrenomedullin-stimulated adenylyl cyclase activity in rat aortic VSMCs. Drake *et al.* (226) showed that in SK-N-MC cells, preexposure to CGRP or adrenomedullin desensitized the cells to a subsequent CGRP stimulus, but preexposure to CGRP or adrenomedullin did not affect a subsequent exposure to adrenomedullin. This is interesting since if CRLR is responsible for cAMP stimulation with both CGRP and adrenomedullin in SK-N-MC cells, then they would be expected to give similar desensitization patterns. It seems then that we still need to learn a lot more about adrenomedullin signaling before its mechanisms of action in each of its different roles can be deduced.

IV. Biological Actions of Adrenomedullin

Although the first paper on adrenomedullin described the cardiovascular effects of this peptide (1), it is now known that adrenomedullin is rather more than simply a vasodilator. Adrenomedullin has been shown to have a remarkable range of actions, from regulating cellular growth and differentiation, through modulating hormone secretion, to antimicrobial effects.

A. Vascular actions

In rat, cat, sheep, and man, intravenous infusion of adrenomedullin results in a potent and sustained hypotension (101, 174, 255–262), mainly via NO generation in the vasculature (261, 263, 264) and is comparable to that of CGRP (1, 265). Initial studies of the hemodynamic effects of human adrenomedullin used anesthetized rats (266, 267). Acute or chronic administration of adrenomedullin resulted in a significant decrease in total peripheral resistance accompanied by a fall in blood pressure. This is concomitant with a rise in

heart rate, cardiac output, and stroke volume (266–269). Similar effects are seen in both conscious (270) and hypertensive rats (266, 268). The hypotensive effect of adrenomedullin on mean arterial pressure in the anesthetized rat is not inhibited by $CGRP_{8-37}$, suggesting this effect is not mediated via CGRP receptors (169). The vascular beds in which adrenomedullin is effective are listed in Table 6.

Responses to rat and human synthetic adrenomedullin have been studied in the systemic vasculature as well as regional vascular beds, and there is a marked variation in these responses among species. Adrenomedullin lowered vascular resistance in lung, heart, kidney, and adrenal gland (266). In rat mesenteric vascular beds preconstricted with methoxamine, administration of adrenomedullin resulted in a dose-dependent decrease in perfusion pressure and arterial pressure (155). This vasodilator response was attenuated by $CGRP_{8-37}$, suggesting that the CGRP receptor may mediate, at least in part, the vasodilator response to adrenomedullin in the mesenteric vasculature (155, 161, 265). In canine renal arteries, adrenomedullin-induced vasorelaxation occurs in the absence and presence of the endothelium (195) and coincides with an increase in cAMP levels. These effects were blocked by $CGRP_{8-37}$. In contrast, the relaxant effect in dog veins, which is endothelium dependent, is not dependent on cAMP production, NO synthesis, or oxygen free radical generation (195). $CGRP_{8-37}$ has no effect on canine venous tone. Adrenomedullin injection into the human forearm causes a prolonged rise in forearm blood flow (259).

Adrenomedullin is vasodilatory in the systemic vascular system of the cat (171, 255, 287, 290), an effect that is antagonized by $CGRP_{8-37}$. However, in the hindlimb vascular bed of the cat, adrenomedullin induces a vasodilatory effect not altered by the $CGRP_1$ receptor antagonist (171, 194, 275). The mechanism by which adrenomedullin reduces vascular resistance in the cat hindlimb circulation is not clear, but it is possible that adrenomedullin may relax vascular smooth muscle by inducing an increase in cAMP levels (176, 177, 185, 291). It is also possible that vasodilation induced by adrenomedullin may be mediated via NO release or arachidonic acid metabolism from the endothelium. It has been previously shown that NO mediates responses to adrenomedullin in the renal vascular bed of the dog and the rat pulmonary and hindquarter vascular beds (261, 272, 278). However, in the cat, NOS inhibitors were without effect and, in fact, duration of the vasodilator response to adrenomedullin was significantly increased after administration of rolipram, a type IV phosphodiesterase inhibitor, suggesting a cAMP-mediated mechanism of action (287, 290).

In pig renal artery smooth muscle strips stimulated by phenylephrine, adrenomedullin caused a fall in tension that was concomitant with a decrease in $[Ca^{2+}]_i$ (273). Using the intact canine kidney it has been demonstrated that adrenomedullin caused an increase in renal blood flow (RBF) that was attenuated by NOS inhibitors (263). Similar effects were seen in isolated perfused rat kidneys (264, 274) and in rabbits (174). In all cases $CGRP_{8-37}$ was without effect.

The feline model has been used in investigating the physiology and pharmacology of penile erection (292, 293). Champion and co-workers (287–289) have used this model to study the effects of adrenomedullin on the erectile response. Adrenomedullin caused significant dose-dependent increases in intracavernous pressure and penile length when injected directly into the corpus cavernosum. Responses to adrenomedullin were comparable to those induced by intracavernous injection of a standard triple-drug combination composed of papaverine, phentolamine, and PGE_1. The mechanism of the erectile response to adrenomedullin is unclear but is unlikely to be NO dependent since administration of N^G-nitro-L-arginine methyl ester (L-NAME) was without effect.

In the intact cat pulmonary vascular bed, adrenomedullin has no effect on resting arterial pressure; however, in the presence of a thromboxane A_2 agonist, adrenomedullin caused a decrease in pulmonary arterial pressure (276, 277). Using the isolated perfused rat lung preparation, it has been shown that human adrenomedullin causes decreases in preconstricted vascular tone with no effect on resting tone (173). Neither $CGRP_{8-37}$ nor NOS inhibitors had any effect, suggesting that in the lung at least, vasodilation occurs via an NO-independent mechanism. This finding, however, contradicts a study using pulmonary arterial rings whereby NO produced by the vascular endothelium was required for relaxation in response to adrenomedullin (294). In conditions of hypoxia, the vasodilatory effect appears to be mediated via PG synthesis rather than NO production (294).

PGs have been implicated in another regional vascular system. Adrenomedullin is 16 times more potent than PGI_2 as a vasodilator in the uterine circulation. Using nonpregnant, oophorectomized ewes, Friedman *et al.* (279) infused either adrenomedullin (0.01 to 3 μg/min) or PGI_2 (0.03 to 10 μg/min) for a period of 5 min. At the doses administered there were no changes in heart rate, cardiac output, or blood

TABLE 6. Vasodilatory actions of adrenomedullin

Vascular bed	Species	$CGRP_{8-37}$ inhibition? (Y/N)	NO implicated? (Y/N)	Ref.
Mesenteric	Rat	Y	Y	(155, 161, 265)
	Cat	Y		(271)
Renal artery	Dog	Y		(195, 272)
	Pig	N		(263, 273)
Renal vein	Dog	N		(195, 272)
Perfused kidney	Rat	N	Y	(264)
	Dog	Y	Y	(274)
Forearm	Human	Y		(259)
Hindlimb	Cat	N	Y	(171, 194, 275)
Hindquarter	Rat	N	Y	(261)
Pulmonary	Cat[a]	N		(276, 277)
	Rat	N	Y	(278)
	Rat[a]	N		(173)
Uterus	Sheep	Y	Y	(279)
Coronary artery	Pig	Y	Y	(156, 248, 280)
Tibia	Dog[a]			(281)
Cerebral artery	Dog	Y		(282)
	Rat	Y		(23)
Cerebral arterioles	Rat	Y		(283)
Cerebral microvessels	Cat	N		(284)
Pial artery	Cat			(285)
	Rat			(286)
Penis	Cat	Y	Y	(287–289)

[a] In these vascular beds vasodilation was only observed after preconstriction.

pressure; however, there was a significant and dose-dependent increase in uterine blood flow. Clearly, local levels of adrenomedullin, released from endothelial cells in the uterine vasculature, may result in significant vasodilation, suggesting that adrenomedullin may play a role in pregnancy-associated vasodilation (295).

Adrenomedullin appears to have direct effects on the heart and coronary circulation. As mentioned above, canine heart and cultured rat cardiac myocytes express adrenomedullin immunoreactivity and mRNA. The vasodilatory effect of adrenomedullin on porcine coronary arteries is mediated via CGRP receptors and is abolished upon removal of the vascular endothelium (156, 248, 280). Szokodi *et al.* (249) reported that adrenomedullin exhibits inotropic effects by increasing heart contractility via a specific adrenomedullin, Ca^{2+}-dependent mechanism in the rat heart. The same group, however, had previously demonstrated that the inotropic effect of adrenomedullin was inhibited by $CGRP_{8-37}$ (296). In rabbit cardiac myocytes, adrenomedullin has a negative inotropic effect which appears to be mediated via NO (297).

Kato and co-workers (281) designed studies to investigate the effect of adrenomedullin on the pressor response to exogenous norepinephrine using the isolated canine tibia as a model for bone circulation. Bolus administration of adrenomedullin (1 nmol) reduced the pressor response to norepinephrine significantly, and this effect was long lasting. The effect was not altered by indomethacin, which blocks PG synthesis. However, infusion of L-NMMA blocked the effect of adrenomedullin on norepinephrine-induced pressor responses, suggesting a role for NO in this action (281, 298).

Until recently, the effects and mechanisms of action of adrenomedullin in cerebral vessels have been poorly defined. Baskaya *et al.* demonstrated that the vasodilator effects of adrenomedullin on conducting arteries of the cerebral circulation were inhibited by $CGRP_{8-37}$ and that prior administration of CGRP prevented subsequent adrenomedullin-induced relaxation (282). This effect was not mediated via NO or vasodilator prostanoids, but because intracisternal administration of adrenomedullin caused an increase in cAMP levels in CSF, these authors speculated that vasodilation in response to adrenomedullin involved an adenylyl cyclase-dependent mechanism. A study of the effect of adrenomedullin on cat cerebral parenchymal microvessels reported that adrenomedullin caused a significant increase in cerebral blood volume, but had no effect on maintaining the resting tone of intracerebral parenchymal vessels (284). These effects were not significantly inhibited by $CGRP_{8-37}$ but were attenuated by $adrenomedullin_{22-52}$. A recent study by Lang *et al.* (285) demonstrates that adrenomedullin-induced dilation of pial arteries is not subject to tachyphylaxis, but involves the opening of both ATP-sensitive and Ca^{2+}-dependent K^+ channels. The precise physiological role of adrenomedullin in the cerebral circulation remains unclear. However, its clinical significance may be related to a role in the mechanism of injury from focal cerebral ischemia (283, 286, 299).

B. Growth and development

Adrenomedullin was originally purified from a human adrenal tumor (1). Cuttitta's group extended their initial observation of adrenomedullin and L1 receptors in pulmonary tumors (43, 300) to study the expression of adrenomedullin in human tumor cell lines in general (58). This opened up the possibility of adrenomedullin being an autocrine/paracrine growth factor in tumors and possibly normal cells. A neutralizing antiadrenomedullin monoclonal antibody was growth inhibitory to these cells, which also showed both ^{125}I-adrenomedullin binding (L1 receptor mRNA was also present) and adrenomedullin-stimulated cAMP. In Swiss 3T3 cells adrenomedullin increased DNA synthesis in a dose-dependent manner by a mechanism involving specific adrenomedullin receptors and increased cAMP/PKA (180). These findings have been confirmed, and Swiss 3T3 cells were shown to produce correctly processed adrenomedullin, which is regulated by cytokines and growth factors (57). In normal and malignant skin, adrenomedullin and the L1 receptor (263) were detected and adrenomedullin increased ^{3}H-thymidine uptake (50). Adrenomedullin also stimulated DNA and cAMP synthesis in human oral keratinocytes (184). The effect on DNA synthesis was inhibited by an adenylyl cyclase inhibitor (SQ22, 36) and mimicked by $(Bu)_2$cAMP (184). In quiescent rat VSMCs, adrenomedullin and CGRP stimulated DNA synthesis and cell proliferation (252). These results provide strong evidence for a growth-promoting effect of adrenomedullin, possibly mediated via cAMP.

However, agents that increase cAMP are often associated with inhibition of cell proliferation. In human normal glial cells and glial cell tumors, adrenomedullin suppressed cell growth and increased intracellular cAMP (183, 301). Growth of human and rat astrocytomas and human glioblastomas, as well as cultured glioblastoma-derived cell lines, was inhibited by adrenomedullin (33, 183, 301). However, this contrasts with studies using C6 gliomal cell cultures. Moody *et al.* (229) reported that adrenomedullin exerted mitogenic effects on these cells that correlated with increases in cAMP and c-*fos* expression. In rat mesangial cells, adrenomedullin suppressed mitogenesis by a cAMP-dependent mechanism (241, 251, 302). A similar result was obtained in TGW human neuroblastoma cells where the inhibition was blocked by both $CGRP_{8-37}$ and $adrenomedullin_{22-52}$ (191). In bovine aortic endothelial cells, addition of a monoclonal antibody to adrenomedullin increased DNA synthesis and cAMP, but no effect was seen in rat mesangial or VSMCs, even though all three cell types released adrenomedullin (38). Using rat VSMCs, adrenomedullin was shown to inhibit serum-stimulated ^{3}H-thymidine uptake, which could be blocked by $CGRP_{8-37}$ (303). These effects indicate an inhibition of growth by adrenomedullin as might be expected with increased intracellular cAMP, but some of these results are contradictory to those above (9, 252). This needs to be investigated further in vascular cells to establish whether adrenomedullin is a vasodilator and inhibitor of proliferation, which might counteract the effects of vasoconstrictor/proliferators such as endothelin and angiotensin II.

In addition to the possible antiproliferative effects of ad-

renomedullin, it may also inhibit coronary artery smooth muscle cell migration (232, 304), perhaps with the two effects combining to inhibit vascular remodeling. Adrenomedullin has also been shown to inhibit hypertrophy in cultured neonatal cardiac myocytes [inhibition of angiotensin II stimulated ^{14}C-phenylalanine incorporation (23)] and in the right ventricle of pulmonary hypertensive rats [right ventricle weight of monocrotaline-treated rats (305)]. Adrenomedullin has also been shown to be angiogenic in the chick chorioallantoic membrane assay and to increase human umbilical vein endothelial cell number (47). This finding, combined with the tamoxifen induction of the adrenomedullin gene in endometrial stromal cells, may indicate a role for adrenomedullin in uterine growth and vascularization (47). Adrenomedullin has also been proposed as an important factor in embryogenesis and differentiation (36, 53, 306–308) and as an apoptosis survival factor for rat endothelial cells (233). Taken together, although there is some debate on the exact effects of adrenomedullin, there is little doubt that these findings indicate a role for adrenomedullin in cell and tumor growth, and this might be expected to be a productive area of research in the near future.

C. Endocrine effects

1. The pituitary. In 1995, two groups described the effects of adrenomedullin on the pituitary. In the first study primary cultures of rat anterior pituitary cells were exposed to adrenomedullin and accumulation of ACTH in the medium measured (309). Adrenomedullin inhibited ACTH release from these cells in a dose-dependent manner and also attenuated CRH-stimulated ACTH production. It appeared that adrenomedullin did not affect basal or CRH-stimulated cAMP responses in these cells, which suggested that adrenomedullin was exerting its effect through an adenylyl cyclase-independent mechanism. Samson *et al.* (309) also demonstrated the ability of angiotensin II to antagonize the actions of adrenomedullin. In this report, the workers did not observe any changes in levels of LH or GH from isolated pituitary cells in response to adrenomedullin. The second study by Parkes and May (260) describes the effects of intravenous infusion of adrenomedullin into conscious sheep. These workers observed a significant reduction in plasma ACTH levels from 50 pg/ml to 21 pg/ml, which continued to fall to a level of 14 pg/ml 1 h after cessation of the infusion. Taken together, these studies suggest that adrenomedullin has a role in inhibiting ACTH release.

2. The adrenal gland. Like other regulatory peptides present in the adrenal gland (310, 311), adrenomedullin affects the secretory activity of the adrenal cortex in both rat and human. Yamaguchi *et al.* (312) studied the effect of adrenomedullin on aldosterone production in the rat *in vivo*. Secretion of aldosterone was stimulated by placing rats either on a sodium-deficient diet or by performing a bilateral nephrectomy. In both cases adrenomedullin was administered by injection, and adrenal renin activity and aldosterone concentration were measured. These workers demonstrated that adrenomedullin significantly inhibited aldosterone production in response to either of these manipulations, but had no effect on adrenal or PRA, plasma corticosterone, or K^+ levels. The results of such experiments are difficult to interpret since many different factors interact to maintain aldosterone levels. For example, pituitary peptides have a significant influence in the response to sodium depletion, and these were not measured in the study by Yamaguchi *et al.* (312). As adrenomedullin inhibits ACTH secretion in the sheep (260), it would be expected that this may have been a factor in the aldosterone studies.

Previously this group has reported that adrenomedullin significantly inhibited aldosterone secretion in response to angiotensin II, K^+, and the calcium ionophore, A23187, from dispersed rat adrenal zona glomerulosa cells, but that aldosterone stimulated by either ACTH or $(Bu)_2$cAMP was not affected (313). These data are entirely consistent with an agonist working through cAMP generation as we have previously shown that agonists activating cAMP-dependent and inositol trisphosphate-dependent pathways are mutually antagonistic in the rat adrenal gland (314).

Several different adrenal tissue preparations have been used to investigate the effects of adrenomedullin on steroid secretion. These studies have proven to be rather contradictory. The first studies, using collagenase-dispersed zona glomerulosa cells, demonstrated the inhibitory effect of adrenomedullin on angiotensin II-stimulated aldosterone secretion in rat and man (315, 316). This was blocked by $CGRP_{8-37}$, suggesting that adrenomedullin exerts this effect via a $CGRP_1$ receptor in this tissue, but the high concentration of $CGRP_{8-37}$ used (1 μM) may favor nonselective effects. Other studies, using intact capsular tissue, to which the zona glomerulosa cells adhere, or slices of human adrenal tissue have shown a stimulatory effect of adrenomedullin on aldosterone secretion (236, 298, 316). Most recently it has been demonstrated that adrenomedullin, acting through specific adrenomedullin receptors, stimulates zona glomerulosa cells to produce aldosterone (25, 317). In addition, it has been reported that CGRP and adrenomedullin exert opposite effects on aldosterone secretion (317). Thus it is likely that the differences between responses of different rat zona glomerulosa cells are dependent on whether adrenomedullin is acting through CGRP or adrenomedullin receptors in this tissue.

Using the isolated perfused *in situ* rat adrenal preparation developed in our laboratory, it has been shown that adrenomedullin causes an increase in perfusion medium flow rate (160, 298, 318). In intact rats adrenomedullin also causes an increase in adrenal blood flow (266). The observation that adrenomedullin acts as a vasodilator in the adrenal vascular bed, as in other tissues, strongly suggests that adrenomedullin can stimulate corticosterone secretion by the rat adrenal. Indeed, studies on the intact perfused rat adrenal preparation suggest this is the case (298, 315, 316). Studies of the direct action of adrenomedullin on collagenase-dispersed rat zonae-fasciculata/reticularis cells *in vitro* suggest that adrenomedullin does not stimulate corticosterone secretion (298). This suggests that the observations in the perfused adrenal preparation are likely to be secondary to vascular events. However, infusion of adrenomedullin (100 μg/h) into sheep results in a 55% decrease in cortisol production with a significant increase in PRA (260). In this study there was also a 58% decrease in ACTH levels, and it is likely that

the decrease in cortisol levels was a consequence of the drop in ACTH, rather than a direct effect on the adrenal gland (260).

We, and others, have demonstrated that adrenomedullin is abundant in adrenal medullary cells (25, 178), and it appears to be cosecreted with catecholamines in response to nicotinic receptor stimulation (112). In cultured bovine adrenomedullary cells, adrenomedullin does not affect basal catecholamine release, but increases Ca^{2+} efflux, presumably by stimulating Na^{+}/Ca^{2+} exchange (319). Masada *et al.* (320) have recently reported the results of their study of adrenomedullin on adrenal catecholamine release in dogs. In this study, catecholamine release was evoked by splanchnic nerve stimulation and by injecting cholinergic agonists into the adrenal gland of conscious dogs via the phrenicoabdominal artery. Adrenomedullin (1–10 ng/kg/min) had no effect on basal catecholamine output; neither did it alter catecholamine levels in response to splanchnic nerve stimulation or acetylcholine administration. Even at high doses (100 ng/kg/min), adrenomedullin did not affect catecholamine levels in response to nerve stimulation even though blood pressure was significantly lowered and there was an increase in adrenal plasma flow. However, the possibility exists that endogenous adrenomedullin has a maximal effect on adrenal catecholamine release physiologically and, therefore, masks the effects of exogenous adrenomedullin, since adrenomedullin levels in the adrenal medulla have been demonstrated to be more than 30-fold higher than other tissues (6).

3. Reproductive effects. Reproduction is a complex and finely programmed process involving extensive tissue remodelling and changes in blood flow (321, 322). The mechanisms underlying normal functioning of reproductive tissue are being unraveled; however, it is clear there is cross-talk involving endocrine, paracrine, and autocrine factors. In light of the known roles of adrenomedullin, it has been suggested that it may have important regulatory roles in reproductive tissues. Adrenomedullin has been shown to be present throughout the female reproductive tract/system (46, 323). Studies from our laboratory tested the effect of adrenomedullin on galanin-stimulated contractile responses of rat uterine muscles (130). Adrenomedullin significantly attenuated contractility in response to galanin, suggesting a role for adrenomedullin in uterine function. This may have some relevance in the events that occur in late pregnancy for example (63, 77, 128, 324).

As described above, plasma levels of adrenomedullin are elevated in normal pregnancy. The physiological significance of this secretion remains to be established, but a number of studies provide some indications. Marinoni *et al.* (45) suggested that adrenomedullin may be involved in the process of adaptation of the vascular system to pregnancy. The presence of adrenomedullin in placenta and fetoplacental tissues (45, 306, 307, 325) supports a role for adrenomedullin in control of vascular tone at the local level to regulate uteroplacental-fetal circulation. Furthermore, adrenomedullin has been demonstrated to inhibit PDGF- and thrombin-induced ET-1 production (245). In the placenta ET-1 is localized to trophoblasts and endothelial vessels of villi (326). Taken together, these findings suggest adrenomedullin may modulate vascular tone by inhibiting/stimulating vasoactive agents during normal and pathological states of pregnancy, such as preeclampsia and intrauterine growth restriction. In this light, a very recent study by Makino *et al.* (327) investigated the effect of adrenomedullin during preeclampsia. In this study an animal model of preeclampsia was used. Other workers have previously demonstrated that L-NAME-treated pregnant rats show preeclampsia-like symptoms consisting of hypertension, intrauterine growth restriction, proteinuria, and renal glomeruli injury (328, 329). There is also increased fetal mortality (327, 330). Makino and coworkers (327) demonstrated that infusion of adrenomedullin (3–10 pmol/h) reversed hypertension and decreased pup mortality induced by L-NAME when given to rats during late gestation (day 14 of pregnancy), but not in animals in early gestation or in nonpregnant rats. Furthermore, adrenomedullin appeared not to affect basal blood pressure or pup mortality in normal pregnant animals nor did it have any effect on L-NAME-induced hypertension after delivery. These findings suggest that adrenomedullin may have an important regulatory role in the utero-placental cardiovascular system.

Expression of adrenomedullin and its receptor have been found in mouse mammary glands (48). Apart from effects on growth, further studies are required to determine the role of adrenomedullin in ductal homeostasis, immune/innate defense, and neonatal nutritional supplement.

4. The pancreas. Mulder *et al.* (28) first reported the stimulatory effects of adrenomedullin (1–100 nM) on insulin secretion from isolated rat islets (in the presence of either 3.3 or 8.3 M glucose). In direct contrast to this, Martínez *et al.* (54) clearly demonstrated the inhibitory role of adrenomedullin on insulin secretion *in vitro*. These results were further strengthened by the observations that a blocking adrenomedullin antibody neutralized both the endogenous and exogenous effects of adrenomedullin on insulin secretion. These workers then went on to study the *in vivo* effects of adrenomedullin: adrenomedullin attenuated and delayed the insulin response to oral glucose challenge, resulting in initial elevated glucose levels. The vasodilatory effect of adrenomedullin may also have some influence on insulin secretion by elevating pancreatic perfusion rate, but this remains to be proven. However, the existence of a constitutively inhibitory tone in pancreatic islets may play a role in the homeostasis of this organ.

D. Renal effects

Circulating adrenomedullin can affect renal function, and evidence exists for a role for locally produced adrenomedullin in tubular function. The first reported studies on renal function involved intrarenal arterial perfusion in anesthetized dogs (274). Adrenomedullin administration had no effect on heart rate or mean arterial blood pressure, but increased RBF, urine output, and urinary Na^{+} excretion in a dose-dependent manner, indicative of direct preglomerular and postglomerular arteriolar effects (274). Subsequent studies found this effect to be mediated via an endothelial, NO-dependent mechanism (263, 264). In the anesthetized rat, intrarenal adrenomedullin infusion leads to increases in RBF,

arterial conductance, glomerular filtration rate (GFR), Na^+ excretion, and urine flow (264, 291, 331). These effects are not inhibited by $CGRP_{8-37}$. Bolus administration of adrenomedullin peripherally significantly lowers mean arterial pressure and raises RBF, GFR, and urine flow; the latter three responses were significantly attenuated in the presence of L-NAME (264, 332).

Studies have been carried out recently in a rat model of heart failure. In this series of experiments, Nagaya and coworkers (333) demonstrated that intravenous infusion of a low dose of adrenomedullin to normal rats or those with heart failure led to significantly increased urine volume and Na^+ excretion without changing GFR, RBF or any other hemodynamic parameter (333). High-dose adrenomedullin infusion decreased mean arterial pressure and increased cardiac output in both rat groups. They also showed that adrenomedullin significantly reduced right ventricular systolic pressure in heart failure rats with pulmonary hypertension. In addition, adrenomedullin did not increase urinary cGMP levels, suggesting that the renal actions of adrenomedullin may not be mediated totally by the NO pathway in these rats (67, 238, 263).

Contrary to this, Rademaker *et al.* showed that intravenous administration of adrenomedullin increased Na^+ excretion without an increase in urine flow or creatine clearance in an ovine model of heart failure (334). The discrepancy between these studies may be explained by the differences in renal perfusion pressure; however, it is unlikely that circulating levels of adrenomedullin regulate renal function physiologically. This is due to the fact that the threshold levels for cardiovascular actions are much lower than those required for renal effects (21, 258). Recently, however, it has been suggested that neutral endopeptidase (NEP) can potentiate the renal natriuretic and diuretic actions of intrarenal adrenomedullin infusion (335). NEP is a membrane-bound metalloproteinase that cleaves endogenous peptides at the amino side of the hydrophobic residues. This ectoenzyme is localized in a number of tissues but is found predominantly in the kidney (336, 337). Substrates for NEP include bradykinin, AVP, and substance P, and the study described by Lisy *et al.* (335) concludes that adrenomedullin is also a substrate for this ectoenzyme. Inhibiting systemic NEP raises plasma adrenomedullin levels significantly, supporting the conclusion that adrenomedullin is a substrate for NEP. NEP inhibition also potentiates an increase in Na^+ excretion in the absence of an increase in GFR or further increases in RBF in response to exogenous adrenomedullin. This indicates that a decrease in tubular Na^+ resorption is the mechanism for natriuresis. The identification of adrenomedullin in the inner medullary ducts correlates with the ability of this tissue to increase its permeability to water in response to adrenomedullin (39).

There is also evidence for a role for adrenomedullin in mesangial cell biology. In addition to its capability to modulate mesangial cell contraction, adrenomedullin also inhibits ET-1 production in response to PDGF in these cells (241). This mirrors the inhibitory effect adrenomedullin has on PDGF-induced MAPK and cell proliferation (241, 251, 302). Parameswaran *et al.* (338) have shown that adrenomedullin stimulates hyaluronic acid (an extracellular matrix component) release from cultured rat mesangial cells via p38 kinase and phosphatidylinositol-3-kinase pathways. These data imply there may be a role for adrenomedullin in the pathophysiology of mesangial cell proliferation and matrix biology. Adrenomedullin may also have a role in protecting the kidney glomeruli from inflammatory reactions or immune injuries. It has been demonstrated that the proinflammatory cytokines, tumor necrosis factor-α and interleukin-1β, stimulate adrenomedullin production from mesangial cells and because it is known that adrenomedullin attenuates the generation of free radicals in these cells and in macrophages, parallels have been drawn (230).

Adrenomedullin could also play a regulatory role in the endocrine function of the kidney. Whole animal studies, described by Jensen *et al.* (234) suggest that adrenomedullin elevated plasma renin levels in rats, a response thought to be secondary to the hypotensive action of adrenomedullin. Subsequent studies, using the isolated perfused kidney preparation, suggest that, in the absence of changes in perfusion pressure or renal nerve activity, adrenomedullin stimulates intrarenal renin release. Renin release was shown to be from juxtaglomerular granulosa cells (234).

E. Other peripheral effects

1. Gastric function. The central actions of adrenomedullin on gastric function are described below. Adrenomedullin has been shown to have profound effects on gastrointestinal motor and secretory functions in several species and experimental models. In conscious rats, intravenous injection of adrenomedullin (150–600 pmol) decreased gastric emptying of a noncalorific meal in a dose-dependent manner. These actions were reversed by administration of $CGRP_{8-37}$ (339). Attenuation of gastric emptying appears to be unrelated to cardiovascular changes since the doses of adrenomedullin required to inhibit gastric emptying are lower than those necessary to evoke changes in cardiovascular parameters (266, 339, 340). Motility effects of adrenomedullin have not been studied, but phasic and tonic intraluminal pressure in the gastric corpus may be affected (341).

Peripheral infusion of adrenomedullin into conscious rats with gastric cannulae inhibits both basal and pentagastrin- and 2-deoxy-D-glucose-stimulated gastric acid secretion (342). In contrast, in conscious rats with pylorus ligation, a bolus intravenous injection of adrenomedullin stimulated gastric acid output due to increases in the volume of secretion and elevated pepsin levels (339, 340). This discrepancy may be due to the different experimental models used.

Very recently, a study by Fukuda *et al.* (343) investigated the effects of adrenomedullin on gastric mucosa integrity. Gastric mucosal restitution involves the rapid reestablishment of epithelial integrity as part of the important mechanism of host defense/protection. In this study rat and human gastric mucosa were damaged by applying various concentrations of NaCl (with low concentrations of NaCl causing less mucosal damage and high NaCl causing extensive damage to the submucosa). Restitution was assessed by measuring the transmucosal potential difference and it was found that 1 μM adrenomedullin had a significant restitutional effect at low concentrations (0.5–1.0 M) of NaCl. These workers also studied the effect of ad-

renomedullin on Na^+ absorption and Cl^- secretion in rat colonic mucosa, but found adrenomedullin had little effect on ion transport. Adrenomedullin did, however, cause relaxation of colonic smooth muscle contraction in response to KCl. This remained persistent particularly when using high (100 nM) concentrations of adrenomedullin.

2. Bone. Studies by Montuenga and co-workers (306, 307) have supported a role for adrenomedullin beyond that of the cardiovascular system and fluid homeostasis. Expression of adrenomedullin, and its receptor, was seen in osteoblasts during the later stages of rodent embryogenesis and in maturing chrondocytes of fetal mice. Further evidence for a physiological role of adrenomedullin in bone metabolism comes from Cornish *et al.* (344). In a series of elegant experiments, these workers have demonstrated that adrenomedullin acts on fetal and adult rodent osteoblasts to increase cell growth comparable to those of known osteoblast growth factors such as transforming growth factor-β. Also, adrenomedullin increased protein synthesis *in vitro* and the area of mineralized and unmineralized bone *in vivo*. Taken together, these data suggest that adrenomedullin might play a paracrine regulatory role in skeletal growth throughout life. This has important implications clinically; for example, one of the major challenges in osteoporosis research is to develop a therapy that increases bone mass via osteoblastic stimulation.

3. Lung. In addition to causing pulmonary vasodilation, adrenomedullin inhibits bronchoconstriction induced by histamine or acetylcholine (294). This suggests there may be a relevant role for the increase in circulating levels of adrenomedullin seen during acute attacks of asthma (93, 345). The adrenomedullin-relaxant response of the vasculature may also provide a protective role, for example, in the pulmonary circulation of patients with pulmonary hypertension (119, 346). Additionally, adrenomedullin may have an anti-inflammatory role in the lung. Macrophages secrete neutrophil chemoattractants in response to chemotaxis as part of the inflammatory process, particularly in the lung (347). Adrenomedullin significantly inhibits alveolar macrophage release of neutrophil chemoattractants in response to lipopolysaccharide, in a dose-dependent manner (244).

4. Innate immunity/mucosal defense. Studies from our laboratory, and others, have shown that adrenomedullin is expressed in key mucosal surfaces, such as the skin, lung, gut, and oral cavity (348, 349). The epithelium provides a first line of defense against potentially pathogenic microorganisms. Many antimicrobial peptides with a broad spectrum of activity have been identified in the mucosal epithelia of mammals (350, 351). Data obtained from our laboratory (349) and that of Walsh *et al.* (348) provide evidence that adrenomedullin also has antimicrobial properties against both Gram-positive and -negative bacteria isolated from skin, oral cavity, respiratory tract, and the gut. The concentration of adrenomedullin required to kill/inhibit bacterial growth is higher than those levels found in the circulation; however, in certain circumstances, such as sepsis, elevated plasma adrenomedullin levels obtained during this condition may contribute to the response to bacterial challenge.

F. CNS effects

Adrenomedullin and its receptor exist in the CNS and its cellular components (see above). Focal brain ischemia is the most common event leading to stroke in humans, and a role for adrenomedullin in this condition has been suggested. Using the rat focal stroke model of middle cerebral artery occlusion (MCAO), Wang *et al.* (286) demonstrated increases in adrenomedullin mRNA expression in the ischemic cortex. This occurred 3 h after MCAO and remained elevated for up to 15 days. Using immunocytochemistry, adrenomedullin was localized to the ischemic neuronal processes. These workers also demonstrated that administration of adrenomedullin (8 nM) before and after MCAO led to an increase in the degree of focal ischemia injury (286, 299). This study contrasts with one reported by Dogan *et al.* (193). Using spontaneously hypertensive rats pre- and postinfusion of adrenomedullin (1 μg/kg/min) attenuated the reduction in regional blood flow after MCAO and decreased the degree of ischemic brain injury. These workers concluded that adrenomedullin may have a role in preventing ischemic brain injury by increasing collateral circulation. Furthermore, adrenomedullin administration into the CNS has a number of effects. The first such study was published in 1995 by Parkes and May (260). Adrenomedullin (100 μg/h) was administered to sheep for 60 min by intracerebroventricular (icv) infusion. No significant changes were observed in any cardiovascular parameter measured, although there was a trend for heart rate and cardiac output to decrease. There was no effect on plasma ACTH, cortisol, basal AVP, or renin (260, 352). However, an inhibitory effect on stimulated AVP release was observed (260). In contrast, Charles *et al.* recently described the infusion of adrenomedullin (3.3 pmol/kg/min for 90 min) into the lateral cerebral ventricle of healthy sheep (353). These workers found that plasma AVP levels rose by 50%, while plasma levels of ACTH and cortisol increased 3- to 4-fold in response to adrenomedullin. No changes in arterial pressure, heart rate, or cardiac output were observed, in accordance with the findings of Parkes and May (260, 352–354).

Yokoi *et al.* (355) showed that icv administration of adrenomedullin into conscious rats attenuated AVP increases induced by giving large volumes of saline (hyperosmolarity) or by reducing plasma volume by giving polyethylene glycol (hypovolemia). Since icv injection of adrenomedullin cannot reach the posterior pituitary (356), the exact site(s) of adrenomedullin action is not clear. However, the paraventricular nuclei and supraoptic nuclei are the most likely sites of action; previous studies have shown adrenomedullin immunoreactivity and binding sites exist here (109, 357). Under conditions of volume excess it makes sense physiologically for adrenomedullin to have an inhibitory effect on AVP production consistent with its renal actions.

For many vasoactive peptides, CNS actions tend to go hand-in-hand with their peripheral effects (358). For example, the calcitonin family of peptides and atrial natriuretic factor are known to exert natriuretic and diuretic effects and also act within the CNS to reduce food and water intake (158, 350, 359–363). The hypothalamus has been identified as the main target for inducing anorexia (364), and it is known that

adrenomedullin, and its receptor, are found here. Based on these observations it was predicted that adrenomedullin actions in the CNS would match the peripheral effects of inhibiting water drinking (365) and salt appetite (366). In the former study, Murphy and Samson reported that adrenomedullin had significant inhibitory effects on pharmacologically induced (icv administration of angiotensin II or hyperosmotic challenge) or physiologically induced (overnight dehydration) water drinking in the rat (365). These actions were exerted with no significant effect on mean arterial blood pressure lending further support to a CNS site of action. Water uptake by rats is not affected by icv administration of adrenomedullin; however, adrenomedullin significantly attenuated saline drinking in response to isotonic hypovolemia in a dose-dependent manner (366). The CNS actions of adrenomedullin on water drinking and salt appetite are the first biological effects shown to have physiological relevance. Use of antiadrenomedullin antibodies to passively neutralize endogenous adrenomedullin results in an exaggerated intake of water and salt (366). Very recently, an attempt has been made to determine whether endogenous, brain-derived adrenomedullin plays a role in salt appetite in rats. Using antisense oligonucleotide technology, Samson and co-workers (367) tried to compromise adrenomedullin production in the brain. This antisense approach significantly reduced production of adrenomedullin; however, it did not result in a decrease of anterior pituitary levels of adrenomedullin, probably due to the diffusion of substances from the site of injection (lateral ventricle). With limited data available it appears antisense injection had an effect on the early phase of saline drinking, *i.e.*, exaggerated drinking was observed.

Intracerebroventricular injection of adrenomedullin also causes a dose-dependent reduction in feeding in rats (158). This effect was attenuated by administration of $CGRP_{8-37}$ indicating that the adrenomedullin action is mediated, in part, by CGRP receptors. Low doses of adrenomedullin administration, which reduced food intake, had no effect on blood pressure when given icv, similar to other studies in the rat described by Samson and Murphy (366) and in sheep (260). In fact, larger doses of icv adrenomedullin administration are required to induce hypertension than to attenuate food or water intake in rats, suggesting that the receptors mediating hypertension are further from the ventricular system (158, 365, 366).

Intracerebroventricular administration of adrenomedullin has also been shown to prevent reserpine-induced gastric ulcers in rats in a dose-related manner (368). It appears, however, that this action is mediated via CGRP receptors since administration of $CGRP_{8-37}$ abolishes this effect. This is in agreement with studies by Martínez *et al.* (339) who reported that adrenomedullin and CGRP inhibit gastric emptying in rats by acting on the autonomic nervous system via a common mechanism. When given intravenously, adrenomedullin does not modify reserpine-induced gastric damage, lending further support for a central mode of action for adrenomedullin.

The hypotensive effect of adrenomedullin in the periphery is not paralleled in the brain. In fact icv administration of high doses of adrenomedullin provokes hypertension and increased heart rate in conscious, unrestrained rats (369, 370). These CNS hypertensive effects have also been reported from studies using rats anesthetized with urethane (364) but not inactin-anesthetized animals (365). The central hypertensive actions of adrenomedullin in conscious rats are dose dependent and not antagonized by $CGRP_{8-37}$ (370). Direct effects of adrenomedullin on sympathetic nerve activity in conscious rats were observed (370), and these studies are consistent with the observations of Takahashi *et al.* (364) who showed, in anesthetized rats, that icv adrenomedullin induced an increase in preganglionic sympathetic discharge. Electrical activity of neurons within the area postrema of the medulla are directly affected by adrenomedullin in brain slice preparations (371, 372).

The temporal aspects of CNS-induced hypertension by adrenomedullin parallel those seen after administration of angiotensin II (369, 373). This suggests that there is a common mechanism underlying the hypertensive action of both these peptides in the brain. For example, phentolamine blocks the actions of both adrenomedullin and angiotensin II in the CNS, lending further support for adrenomedullin and angiotensin II to act within the brain to stimulate sympathetic nervous system function. Additionally, this type of mechanism can explain the CNS effect of adrenomedullin-induced inhibition of gastric emptying (339).

The central hypertensive actions of adrenomedullin may be cardioprotective in that it acts to protect against major cardiovascular collapse such as events encountered during sepsis. To understand completely the mechanism by which CNS administration of adrenomedullin controls cardiovascular parameters, further studies must be carried out to determine whether there is a physiological role of adrenomedullin in this process and what factors regulate adrenomedullin transcription in the brain.

V. Unresolved Issues and Future Perspectives

Adrenomedullin has been shown to be virtually ubiquitous, being produced by a great number of different cell types, in all tissues of the body, with the possible exception of the thyroid and thymus. Comparison of plasma adrenomedullin concentrations with its receptor affinity clearly suggests that adrenomedullin is not a conventional hormone. Plasma adrenomedullin is increased in a variety of pathological conditions, usually, it appears, as a compensatory response to cardiovascular changes. The one situation where plasma adrenomedullin concentrations may reach those levels required for receptor activation is in septic shock, where adrenomedullin may cause cardiovascular change. It may be predicted that the application of knockout technology to the adrenomedullin gene may influence every tissue in the body. It may also be necessary to apply modifications of the technology to distinguish adrenomedullin from other adrenomedullin gene products, such as PAMP.

Adrenomedullin binding has been demonstrated in most cell types and tissues of the body. The effects of adrenomedullin may be mediated by both specific adrenomedullin binding sites and by CGRP binding sites. The actions of adrenomedullin have been inhibited using

$CGRP_{8-37}$ and $adrenomedullin_{22-52}$, a $CGRP_1$ receptor antagonist and an adrenomedullin receptor antagonist, respectively. However, neither of these peptides is a very potent inhibitor, and some doubts have been expressed as to their specificity. What is clearly required to further investigate the physiology of adrenomedullin receptors are potent and specific antagonists, preferably small molecule, nonpeptide antagonists.

Two receptor clones have been proposed to have specific adrenomedullin binding properties, L1 and the CRLR-RAMP2 combination. There are, however, situations in which neither of these candidates appears to account for specific adrenomedullin binding. Clearly there are receptors for adrenomedullin that remain to be cloned.

Adrenomedullin has a range of biological actions including vasodilatation, cell growth, regulation of hormone secretion, natriuresis, and antimicrobial effects. Its mechanism of action, however, remains unclear. cAMP is the second messenger in the majority of adrenomedullin actions, but other systems must be involved. The role of NO remains to be elucidated, as does the mechanism of the growth-stimulatory effect of cAMP.

Clearly there are many questions remaining in this field. The answers are likely to contribute, not only to our understanding of adrenomedullin biology, but also to our fundamental understanding both of receptor biology and the regulation of cellular growth.

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Announcement: Recent Progress in Hormone Research

The Publications Committee of The Endocrine Society decided last year that, despite the discontinuation of the Recent Progress in Hormone Research meeting, the annual proceedings journal of the same name will be maintained due to its continuing popularity. Articles will be invited, many of which will be drawn from The Endocrine Society's Annual Meeting.

Although the articles published in *Recent Progress in Hormone Research* (RPHR) differ significantly from those published in *Endocrine Reviews*, the Publications Committee has taken the extra step of ensuring the most effective coordination of editorial office efforts by linking RPHR with *Endocrine Reviews.* The RPHR Editor will work with the Editor-in-Chief of *Endocrine Reviews* to identify and solicit the most appropriate articles for RPHR and will use the *Endocrine Reviews* editorial office as a base for correspondence. In addition, the RPHR Editor will use the *Endocrine Reviews* Editorial and Advisory Boards for consultation.

Anthony R. Means has been appointed the first RPHR Editor under this structure. His appointment is for three years, after which time the *Endocrine Reviews* Editor-in-Chief will have the option of re-appointing him for another three years or recommending another individual.

Any questions, comments, or recommendations regarding this new structure for RPHR are encouraged and should be sent to Marc Caron (endoreviews@endo-society.org) or Tony Means (tony.means@duke.edu).