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INVITED REVIEW

PACAP signaling in stress: insights from the chromaffin cell

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Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP) was first identified in hypothalamus, based on its ability to elevate cyclic AMP in the anterior pituitary. PACAP has been identified as the adrenomedullary neurotransmitter in stress through a combination of ex vivo, in vivo, and in cellula experiments over the past two decades. PACAP causes catecholamine secretion, and activation of catecholamine biosynthetic enzymes, during episodes of stress in mammals. Features of PACAP signaling allowing stress transduction at the splanchnicoadrenomedullary synapse have yielded insights into the contrasting roles of acetylcholine's and PACAP's actions as first messengers at the chromaffin cell, via differential release at low and high rates of splanchnic nerve firing, and differential signaling pathway engagement leading to catecholamine secretion and chromaffin cell gene transcription. Secretion stimulated by PACAP, via calcium influx independent of action potential generation, is under active investigation in several laboratories both at the chromaffin cell and within autonomic ganglia of both the parasympathetic and sympathetic nervous systems. PACAP is a neurotransmitter important in stress transduction in the central nervous system as well, and is found at stress-transduction nuclei in brain including the paraventricular nucleus of hypothalamus, the amygdala and extended amygdalar nuclei, and the prefrontal cortex. The current status of PACAP as a master regulator of stress signaling in the nervous system derives fundamentally from the establishment of its role as the splanchnicoadrenomedullary transmitter in stress. Experimental elucidation of PACAP action at this synapse remains at the forefront of understanding PACAP's role in stress signaling throughout the nervous system.

Keywords Acetylcholine · Catecholamine · NCS-Rapgef2 · PAC1 · Sympathoadrenal axis · Sympathetic nervous system

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Introduction

Coupland identified the features of the splanchnicoadrenomedullary synapse of the rat in 1965 [16].[1] This synapse remains a paradigm, both by similarity and difference, for the study of chemical neurotransmission in the nervous system. In large part, this is because the purpose of the release of acetylcholine from the splanchnic nerve to the chromaffin cell,

[1] References for some earlier work relevant to this review can be found in the bibliographies of the reviews of the literature cited herein.

like that of acetylcholine release from nerve to muscle at the neuromuscular junction (and unlike that of acetylcholine release at parasympathetic and sympathetic ganglia and in the brain), is physiologically obvious: to effect a stimulus to the chromaffin cell resulting in expression of its primary function; to release catecholamines into the general circulation, analogous to the ability of acetylcholine released from the motor neuron to excite muscle contraction. In fact, the naming of calcium-dependent release of catecholamines in response to acetylcholine as "stimulus-secretion coupling" by Douglas [18] pays tribute to the discovery of acetylcholine-stimulated, calcium-dependent muscle contraction which is called "stimulus-contraction coupling" [69]. Following the characterization of the morphological features of the splanchnico-adrenomedullary synapse by Coupland [16] and of cholinergic neuroeffector junctions of the somatic nervous system [39], the mechanisms of acetylcholine-induced calcium influx at both cholinergic neuroeffector junctions and synapses proceeded synergistically and rapidly. Much of our knowledge about the action of acetylcholine released from the splanchnic nerve, acting through ionotropic nicotinic receptors, on the release of catecholamines from the adrenal medulla, has come from the laboratory of Antonio Garcia [32], to whom this volume of Pflüger's Archiv is dedicated.

As our understanding of cholinergic neurotransmission and its role in catecholamine release progressed (see [6] and references therein), three additional themes of synaptic transmission to the chromaffin cell came into view. One of these is that chromaffin cells also express muscarinic cholinergic receptors, which are metabotropic and allow calcium mobilization through G_q coupling [47], thus raising the question as to the relative roles of ionotropic and metabotropic cholinergic signaling in effecting catecholamine secretion. The second is that catecholamines are released from large dense core vesicles by the process of exocytosis, along with a cohort of proteins including the biosynthetic enzyme for catecholamine biosynthesis, dopamine beta-hydroxylase and the protein chromogranin A (which makes up 40% of the weight of the chromaffin granule and 10% of the protein content of the chromaffin cell) ([21, 89, 90] and references therein) and later, a bevy of neuropeptides including enkephalins, NPY, galanin, and others [3, 27, 29, 30, 88]. Importantly, the question was raised that if secretion exhausted an important set of proteins from the cell, there must be a coupling not only between stimulus and secretion, but between stimulus and replenishment of the biosynthetic enzyme complement of the cell exhausted by protein exocytosis. The notion emerged that a process of coupling between stimulus, secretion, and protein synthesis, termed by some as "stimulus-secretion-synthesis coupling," and ascribed to first messenger signaling not only to the cell membrane stimulating exocytosis, but also to the nucleus, stimulating gene transcription, must exist [1, 15]. Finally, hints began to appear that, on the presynaptic side, a second neurotransmitter other than acetylcholine may be released from splanchnic nerve terminals [62]. Early work by Ip and Zigmond, and Wakade and colleagues suggested that this second transmitter could be a peptide augmenting cholinergic stimulation of secretion and regulating the production of catecholamine biosynthetic enzymes and other proteins secreted from the chromaffin cell ([75] and references therein). This review describes the discovery that the neuropeptide PACAP, itself first identified as a brain hypophysiotropic factor, is co-stored with acetylcholine and released from the splanchnic nerve upon stimulation, and is in fact the principal neurotransmitter responsible for enhanced catecholamine release from the chromaffin cell during stress. It is dedicated, as are the other contributions to this issue of the journal, to Antonio Garcia and his pioneering work to understand acetylcholine's actions at the chromaffin cell.

PACAP as the adrenomedullary transmitter in stress

The pituitary adenylate cyclase-activating polypeptide (PACAP) was identified by Miyata and Arimura in 1989, as a 38-amino-acid neuropeptide present in extracts of the ovine hypothalamus, causing elevation of cyclic AMP in perfused hemi-pituitary glands [61]. In the years since, PACAP has been found in numerous deuterostomes, both chordate and echinoderm, and acts at three distinct receptors PAC1, VPAC1, and VPAC2 (the last two also activated by the related peptide VIP) all coupled to adenylate cyclase activation through heterotrimeric G_s-coupled receptors [4, 5]. PACAP has been identified as the adrenomedullary neurotransmitter in stress through a combination of ex vivo, in vivo, and in cellula experiments over the past two decades [75, 78, 79].

Following the chemical identification of PACAP [61] and its availability as a synthetically derived pharmacological reagent, several reports indicated PACAP's ability to cause catecholamine secretion, via a PACAP receptor expressed by the chromaffin cell (reviewed in [38, 63]). In 2009, it was reported that the PACAP antagonist PACAP (6-38) inhibited maximum catecholamine secretion, measured by in situ amperometry, elicited by high-frequency electrical stimulation of the splanchnic nerve in mouse adrenal slices ex vivo, and was without effect on basal catecholamine secretion elicited by low-frequency splanchnic nerve stimulation [40, 51]. We reviewed the status of PACAP as an adrenomedullary neurotransmitter based on the existing evidence in 2012 and identified a series of experiments that, in our view, would establish PACAP's status as the "stress transmitter" at the splanchnico-adrenomedullary synapse [75]. For example, the necessity for PACAP expression in an adrenomedullary response to insulin-induced hypoglycemia (catecholamine secretion; adrenomedullary tyrosine hydroxylase induction) adequate for survival due to compensatory glucogenesis was

established in PACAP-deficient mice [37]. However, evidence that the necessary PACAP was actually that released from the axon terminals of the splanchnic nerve innervating the chromaffin cell [14] was lacking. This evidence was later supplied in the form of ex vivo experiments performed in adrenal slices from wild-type and PACAP-deficient mice [79].

Establishing PACAP as the transmitter responsible for catecholaminesecretion in stress at the mouse splanchnico-adrenomedullary synapse ex vivo, and for both catecholamine secretion and biosynthetic enzyme induction in vivo, was an important step in convincingly positing that PACAP, rather than acetylcholine, is the principal physiologically relevant transmitter for stress transduction at this endocrine gland in mammals. It may also have profound importance for our understanding of the basic physiology of the autonomic nervous system, long thought to be primarily regulated by only one neurotransmitter (acetylcholine), two neuroeffectors (acetylcholine and norepinephrine), and one hormone (epinephrine) [12]. The remainder of this review and commentary focuses on four questions that are begged by the demonstration of PACAP as the splanchnico-adrenomedullary transmitter in stress. How does PACAP cause catecholamine secretion from chromaffin cells? How does PACAP regulate gene transcription related to maintenance of the secretory competence of the chromaffin cell? Does PACAP have a neurotransmitter role in sympathetic ganglia similar to its actions at the splanchnicoadrenomedullary synapse? Finally, what is, in fact, the essential role of acetylcholine in controlling chromaffin cell function?

How does PACAP cause catecholamine secretion?

There is a plethora of studies that contribute to understanding the actions of PACAP on the electrical activity and secretory function of the chromaffin cell. These can be conceptually evaluated in a number of ways. First, there are studies conducted in cell lines of adrenomedullary origin, such as PC12 and NS-1 cells. These are conveniently manipulated in culture, so that hypotheses about the necessity for specific molecules of secretion and signaling for biosynthesis can be easily and robustly verified. However, generalizing these hypotheses to the chromaffin cell itself requires important caveats, not all of them obvious. Studies in chromaffin cells in culture are more physiologically relevant, but in turn beg the question of how generalizable experiments conducted using mouse, rat, or bovine chromaffin cells are to the other species and to the adrenal medulla in situ. Here, it is worth noting the meticulous contributions of the Garcia Lab and other laboratories to establishing the variation in the type and abundance of the various voltage-gated ion channels across mouse, rat, cow, and human adrenal chromaffin cells and how important these studies have been in establishing a firm appreciation for species variation in calcium-dependent catecholamine secretion [32, 33, 54]. These differences may reflect evolutionary flexibility in the use of specific molecules to solve the same biological problem (catecholamine secretion) or evolutionary differences in the fitness contribution of the adrenal medulla from one species to another. In studies conducted in chromaffin cells, special note must be made of those that examine the regulation of calcium per se and those that examine the regulation of calcium-dependent downstream events that drive the professional activities of the cell including catecholamine secretion. Many apparent conundrums centering on alternative mechanisms of calcium-dependent secretion are resolvable on the basis of whether or not a particular PACAP-dependent effect on calcium influx, mobilization, sequestration, or re-distribution actually affects catecholamine secretion or chromaffin cell gene regulation under conditions likely to obtain in vivo. For this reason, we have focused here on studies in which secretion or gene regulation is examined under nominally physiological conditions.

It is well established that acetylcholine causes cell depolarization necessary for calcium entry and exocytosis, via action potentials generated by cation influx through the nicotinic receptor followed by activation of voltage-dependent sodium channels, cell depolarization, opening of voltage-gated calcium channels, and calcium-dependent large dense-core vesicle exocytosis [32, 68]. Secretory effects of acetylcholine on chromaffin cells are absolutely dependent upon the opening of voltage-gated sodium channels, as evidenced by blockade of ACh-induced catecholamine release by tetrodotoxin (TTX), and absolutely dependent on calcium influx [58]. It is equally well established, though perhaps less well appreciated, that PACAP-stimulated secretion does not depend upon the opening of voltage-gated sodium channels [64], but only upon the entry of extracellular calcium through a variety of voltage-gated calcium channels in cultured bovine chromaffin cells [64, 66] (see Fig. 1 and [83, 86].

Kuri et al. have proposed a possible mechanism for PACAP's effects on catecholamine secretion from rodent chromaffin cells in slice preparations based on electrophysiological and pharmacological investigations featuring its dependence on (a) cAMP, (b) PKC (however see Vitale et al. ([87]) for data suggesting that stimulation of PKC may not affect calcium entry into chromaffin cells), (c) T-type or other "low-threshold" calcium channel opening, and finally calcium influx through canonical (combination of L, P, N, or Q) high-capacity voltage-gated calcium channels [51]. The model is not dispositive for the specific components involved, but rather constrained by removal from the model of components (such as voltage-gated sodium channels and intracellular calcium mobilization rather than calcium influx) *not* required for PACAP-stimulated secretion, and the proposal of components with known properties consistent with inclusion in the model. Epac, for example, has been suggested to enhance depolarization-induced secretion from PC12 cells when expressed exogenously in them, however, what percentage of PACAP-induced release in chromaffin cells occurs by this mechanism is not yet clearly defined (see Fig. 1, [67, 71]).

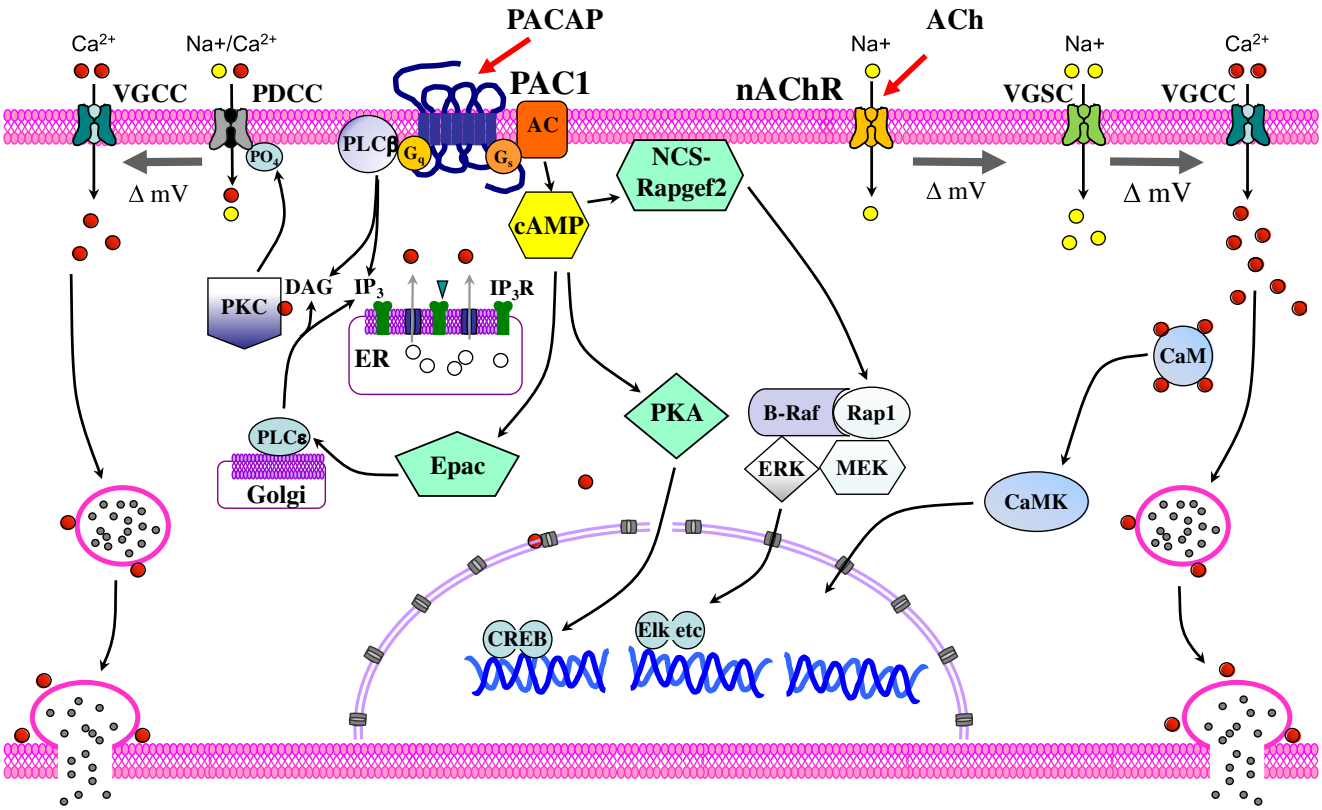

Fig. 1 Schematic illustration of chromaffin cell-signaling pathways leading to secretion of catecholamines by cholinergic and PACAPergic stimulation. Signaling for secretion is indicated for each neurotransmitter as it is known or hypothesized to act on the mammalian chromaffin cell. PACAP-dependent signaling requires a phospho-dependent depolarizing cation conductance (PDCC) for activation of VGCC and secretion. The best characterized features of PDCC are that it requires phosphorylation by PKC for its activation and it is sensitive to inhibition by benzamil [51]. The molecules most consistent with this pharmacological and functional profile are the sodium-calcium exchanger [51] and TRPP3 [17]. As comprehensively as possible, the authors have depicted the pathways for which the available evidence is strongest and most consensual across mammalian species, albeit features proven for some may not be established for all, and especially may not be established clinically (i.e., in human; and see text). Scheme adapted from [63]

This model (incorporated into Fig. 1 here) clearly suggests that PACAP-induced catecholamine secretion from chromaffin cells does not require and is not accompanied by action potential generation, and the negative evidence supporting this is the TTX-insensitivity of PACAP-induced catecholamine and neuropeptide release from the chromaffin cell [51, 66, 83]. It is worth noting that a cell depolarization of around 20 mV in cells resting at −55 to −60 mV would be sufficient to trigger sustained or "burst firing" [36, 86] even in the absence of available Nav channels. Chromaffin cells in isolation have a variable resting potential between −80 and −50 mV [26]. PACAP-evoked depolarization may be suprathreshold for action potential firing in those cells resting near the depolarized end of this range, and enhancement of burst firing, even if it does not occur in chromaffin cells, may be a mechanism of PACAP action in other excitable cell of the autonomic as well as the central nervous system.

A cAMP/Epac-dependent signaling mechanism, whereby Epac activates a non-classical target, PLCepsilon, leading to PKC activation, has been reported in HEK293 cells [48, 70]. Since these initial observations, Epac-dependent and PKC-dependent signaling pathways for hormone secretion have been reported to play a role in glucose-induced insulin release [19] and glucagon-induced ghrelin release from stomach [31] and of course in the chromaffin cell itself (40, 50). Further investigation in this arena is of great importance for comprehensive understanding of the physiology of adrenomedullary function and possibly of the autonomic nervous system in general (vide infra). We include Epac as a potential actor in PACAP-stimulated catecholamine secretion from chromaffin cells, as depicted in Fig. 1.

How does PACAP regulate chromaffin cell gene transcription?

It has been long appreciated that activation of catecholamine secretion from the adrenal medulla following psychogenic, hypoglycemic, and several other types of stress results in increased firing of the splanchnic nerve [49], and that prolonged

stimulation of secretion (for up to 6 h, the time of a prolonged episode of glucose deprivation) of catecholamines results in the secretion of the entire contents of the adrenal medulla [56]. The fact that the adrenal medulla retains its full complement of catecholamine despite this high secretory rate means that the adrenal medulla is capable of re-synthesizing its entire catecholamine content during an episode of stress responding, and this in turn implies a dramatic up-regulation of the catecholamine biosynthetic capacity of the gland. This is accomplished by upregulation of at least two biosynthetic enzymes for epinephrine production, tyrosine hydroxylase, and phenylethanolamine N-methyltransferase ([76–79] and references therein). Thoenen, Mueller, and Axelrod first remarked that upregulation of TH in adrenal in vivo after stress [62] was abolished by splanchnic nerve transection, but not by nicotinic or muscarinic blockade, concluding that "it is possible that in the adrenal medulla either another cholinergic receptor or another neurotransmitter is involved in mediating the transsynaptic increase in tyrosine hydroxylase." Later, Ip and Zigmond postulated that this peptide could be the vasoactive intestinal polypeptide (VIP) [42, 43], and indeed Wakade added support to this idea by demonstrating the release of VIP into adrenal perfusates upon splanchnic nerve stimulation and the effects of VIP on catecholamine secretion from the rat adrenal [55, 57]. Immunohistochemistry for PACAP using carefully validated antibodies (PACAP and VIP have similar structures and a propensity for immunological cross-reactivity) allowed the localization of PACAP rather than VIP in mouse cholinergic splanchnic nerve terminals [37]. The PACAP knockout mouse used to validate PACAP immunohistochemistry was also employed to show that PACAP is required not only for catecholamine secretion but also for TH induction following hypoglycemic stress. Subsequent experiments demonstrated that both systemic and psychogenic stress (e.g., restraint stress) upregulation of TH and PNMT mRNA in adrenal was PACAP-dependent [76, 78, 79].

The signaling pathways by which PACAP regulates catecholamine biosynthetic enzyme gene expression, like those activated for secretion, remain surprisingly ill-defined [2, 81, 82]. Our own group has identified three separate sensors for cAMP elevated by PACAP in NS-1 and chromaffin cells responsible for gene activation; these are protein kinase A, Epac, and the novel neuronal/endocrine-specific cAMP sensor NCS-Rapgef2 [46]. Which of these pathways, if any, actually regulate TH and PNMT gene regulation associated with adrenal stress responding in vivo is unknown, although a PKA-independent pathway is suggested by experiments on TH and PNMT induction in cultured bovine chromaffin cells by PACAP [79]. The possibility exists that Epac, PKA, and NCS-Rapgef2 are all involved in gene regulation by PACAP in chromaffin cells; this includes not only the catecholamine biosynthetic enzymes, but galanin and other neuropeptides that are significantly upregulated in expression in adrenal medulla by stress [3, 28–30]. The NCS-Rapgef2-mediated pathway has been associated with galanin gene regulation in chromaffin cells, through an extracellular signal-regulated kinase (ERK)-dependent mechanism [22].

What is the role of ACh at the adrenomedullary synapse?

PACAP deficiency appears to completely block high-frequency splanchnic nerve-stimulated release of catecholamines ex vivo and to attenuate it in vivo sufficiently to negate catecholamine-dependent glucogenesis (and survival) after insulin shock. It is predicted that acetylcholine alone is released from splanchnic terminals at low splanchnic firing rats and both acetylcholine and PACAP at the higher rates characteristic of stress responding [75]. We have not adduced evidence for a co-transmitter role for acetylcholine in either the secretory or the gene regulatory actions of PACAP in chromaffin cells. However, it is difficult to approximate the actual concentrations of these two transmitters that reach their receptors at the splanchnicoadrenomedullary synapse. A perhaps overlooked role for acetylcholine in adrenomedullary function is the priming of adequate levels of stored catecholamines in the adrenal gland by tonic low-frequency stimulation from the splanchnic nerve. Splanchnic innervation itself, even in the absence of stress leading to increased firing rate, does not appear to be required for maintenance of catecholamine levels [73]. However, another role of acetylcholine-evoked calcium influx may be to maintain vesicle populations in a "ready-release" mode [74] to allow the higher rates of CA release required for stress response. Finally, the role of tonic catecholamine release itself, even in the absence of stress, is physiologically important, with the adrenal medulla and post-ganglionic sympathetic neurons supplying hormonal and synaptic catecholamines for cardiac and vascular regulation both at rest and during stress [50].

Does PACAP have a role in ganglionic transmission in sympathetic and parasympathetic neurons?

A critical unanswered question is whether or not PACAP functions as a stress-specific ganglionic transmitter in the sympathetic and parasympathetic nervous systems. May and colleagues have adduced significant evidence, employing developing sympathetic neurons in culture, that PACAP has multiple roles in regulation of post-ganglionic sympathetic neurons in culture, including the regulation of biosynthesis and release of neuropeptides co-stored with norepinephrine in these cells [7, 8, 10, 11, 59]. Zigmond and colleagues have

likewise postulated a trophic role for PACAP in the developing sympathetic nervous system [91]. Investigation of the role of PACAP in stress responding that is largely sympathetic rather than adrenal, for example cold stress [34, 35], would be of great interest. Parsons and colleagues have studied PACAPergic effects on cardiac tissue, i.e., on postganglionic parasympathetic neurons of the atrium [41, 60]. What do the mechanisms of PACAP-evoked membrane currents have to tell us about PACAPergic secretory mechanisms? Can clearly defined effects of PAC1 receptor stimulation on afterhyperpolarization potential modulation in cardiac tissue, for example, inform PACAPergic signaling at the chromaffin cell, or is this signaling private to individual cell types in the parasympathetic and sympathetic nervous systems? In sum, it appears that the accumulating evidence justifies consideration of PACAP as the pre-ganglionic transmistter in stress at both sympathetic and parasympathetic ganglia based on cell culture and ex vivo experiments, with a final demonstration in vivo (in PACAP-deficient mice, for example) as the sine qua non for general acceptance of this concept (see Fig. 2).

Role of PACAP in stress signaling in the central nervous system

PACAP is required for the stress response originating in the central nervous system as well. PACAPergic nerve terminals heavily invest corticotropin-releasing hormone (CRH)-positive neurons of the paraventricular nucleus (PVN). These neurons are the central final common pathway for activation of the hypothalamo-pituitary-adrenocortical (HPA) axis that is the hallmark, along with activation of the hypothalamo-sympathoadrenomedullary (HSA) axis, of the stress response [13, 72]. However, it was initially observed that cortisol levels across the diurnal cycle, as well as CORT (cortisol in human; corticosterone in rodents) elevation accompanying hypoglycemic stress, were unaffected by PACAP deficiency [37]. It was later learned, however, that PACAP does play an important role in regulation of the HPA axis in stress, but this role is restricted to psychogenic (allostatic) HPA axis activation, and not systemic (homeostatic) HPA axis activation [52, 65, 85]. One to 3 h of restraint stress causes an approximate doubling of CRH mRNA in the PVN, and this is completely abrogated in PACAP-deficient mice [80]. Chronic psychological stress caused by either daily restraint, or daily social defeat for 14 days, results in elevated CORT, increased anxiety, and depressive behavior in the mouse; all of these effects of chronic psychogenic stress are greatly attenuated in PACAP-deficient mice [52, 65]. An interesting feature of the PACAP dependence of CORT (cortisol in human; corticosterone in rodents) elevation associated with psychogenic stress responding is that graded (1, 2, or 3 h) daily "doses" of restraint stress reveal a dissociation between the effects of stress on CORT elevation and on behavior (e.g., stress-induced appetite suppression). Thus, even a single episode of restraint, for one, two, or three hours, results in acute CORT elevation and appetite suppression detected as significant weight loss after 24 h. While both CORT elevation and appetite suppression elicited by 2 or 3 h of restraint are blunted in PACAP-deficient mice, only appetite suppression, but not CORT elevation, is blunted in PACAP-deficient mice elicited by 1 h of

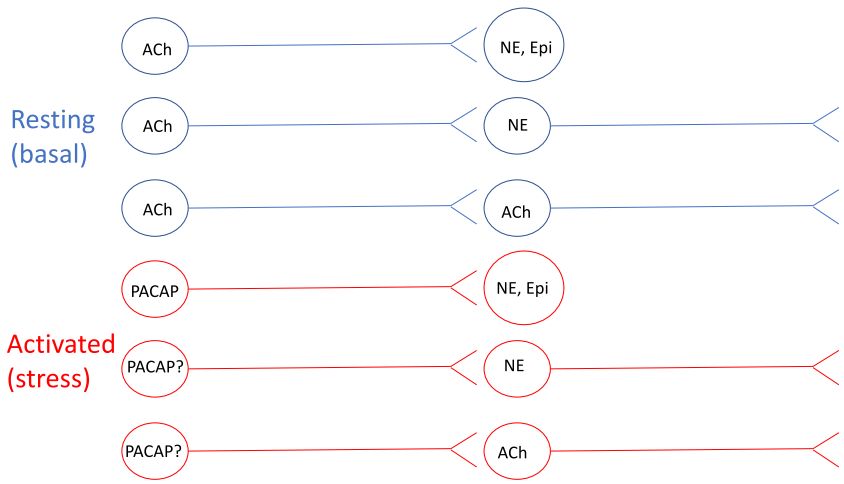

Fig. 2 The neurotransmitters of the autonomic nervous system. Neurotransmission under basal and activated (rest and stress) conditions are shown in blue and red, respectively. ACh acetylcholin; Epi epinephrine/adrenaline; NE norepinephrine, noradrenaline; PACAP pituitary adenylate cyclase-activating polypeptide. Neurotransmitter actions of PACAP at sympathetic and parasympathetic synapses in stress, in vivo, are still only hypothesized (indicated as ?). Note that the PACAPergic component of the autonomic nervous system is most strongly supported by work in rodent species in vivo and in the bovine adrenal medulla in culture, although there is a significant body of work supporting PACAPergic splanchnicoadrenomedullary neurotransmission for primate species as well (see text). Of course, other non-adrenergic, non-cholinergic (NANC) neurotransmitters, including ATP, exist in the autonomic nervous system (see [12] for review)

restraint [44, 45]. We have interpreted these findings as indicative of an effect of PACAP on CRH biosynthesis, via elevation of CRH mRNA, in PVN, but not on CRH secretion into the portal circulation to the pituitary gland (to release ACTH, thus elevating CORT). This implies that PACAP action at the CRH neuron, unlike that at the chromaffin cell, involves stimulus-transcription, but not stimulus-secretion coupling. A further implication is that PACAP has differential sites of action for stress-induced HPA axis activation than for stress-induced appetite suppression. In this case, PACAP is likely to mediate stimulus-secretion coupling, as triggering of behavior effects after only a single hour of restraint stress is unlikely to be mediated via a transcriptional regulatory mechanism alone. Thus, at this locus, PACAP's actions are more like those occurring at the chromaffin cell.

Future prospects based on work in chromaffin cells

The chromaffin cell has provided a neuroendocrine experimental system that has revealed the role of PACAP, at the cellular and organismic levels, in transduction of the stress response. Regarding the signaling mechanisms employed by this Gs-coupled GPCR for its secretory and biosynthetic activities, the chromaffin cell has also been a rich source for discovery of signaling components and pathways employed during PACAP signaling. Notably, the neuroendocrine-specific cAMP sensor NCS-Rapgef2 linking PACAP signaling through PAC1 and Gs to activation of the MAP kinase ERK was first identified in bovine chromaffin cells [22, 24]. Equally fundamental to understanding the role of PACAP in stress signaling in the autonomic nervous system is the elucidation of the mechanism(s) whereby PACAP, through the PAC1 receptor, causes cell depolarization leading to calcium influx, the secretion of catecholamines from the adrenal medulla and presumably norepinephrine from post-ganglionic sympathetic neurons, and of acetylcholine from post-ganglionic parasympathetic neurons (see Fig. 2). Figure 1 summarizes the signaling pathways leading to catecholamine secretion from mammalian chromaffin cells. The figure prominently features the role of phospho-dependent depolarizing cation conductances (PDCCs) activated by PKC after engagement of the PAC1 receptor in chromaffin cells. It is noteworthy that a clear exposition of whether or not Gq engagement by PAC1 is also a driver for PKC activation leading to phosphorylation of PDCCs in the chromaffin cell.

Pharmacological evidence for a primary involvement of PKC in catecholamine secretion derives from the mouse adrenal ex vivo [51], the cultured bovine chromaffin cell [83], and indirectly from PMA pharmacological effects manifested as enhanced secretion of enkephalin from bovine chromaffin cells [20]. A plethora of evidence adduced in the cardiac ganglion preparation ex vivo suggests that PACAP profoundly affects current input-response characteristics via a pathway involving cAMP and MAPK activation [84]. Whether this is related to a general mechanism for PACAP's secretogogue action, or whether these two cell types affect secretion by distinct mechanisms is an important question for fully understanding the neurochemistry and physiology of the autonomic nervous system. It has implications as well for PACAP signaling in stress at central synapses; current evidence suggests that PACAP acts as a secretogogue at some, as yet undefined central synapses, while in the paraventricular nucleus, PACAP, via the PAC1 receptor, appears to affect corticotropin-releasing factor synthesis (vide infra) without apparent secretogogue action.

Significant progress in understanding PACAP-dependent signaling to the nucleus has occurred using PC12 cells and their high-content analysis-adapted variant, the NS-1 cell [22]. In particular, the downregulation of expression of specific signaling proteins using siRNA and CRISPR technology has been helpful in first identifying signaling proteins of interest by their deletion and then developing a pharmacology for them with high-content analytical screening [23, 25]. However, it is somewhat remarkable that precise delineation of how this critical autonomic neurotransmitter causes catecholamine release from the chromaffin cell, either in culture or in vivo, is still incomplete. Such investigations are important not only in their own right but also to establish appropriate pharmacological tools with which to ascertain whether or not the mechanisms of PACAP-stimulated secretion are universal across multiple types of PACAP-receptor bearing neuroendocrine cells, or highly specific to chromaffin cells, or even to chromaffin cells of only some mammalian species. In this regard, the work of Garcia and colleagues, over several decades [9, 33, 53, 54] in delineating the mechanisms and features of acetylcholine-induced catecholamine release from chromaffin cells is a worthy reminder that the chromaffin cell yields biological information perhaps slowly, but surely.

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