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Is PACAP the Major Neurotransmitter for Stress Transduction at the Adrenomedullary Synapse?

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Abstract

It has been known for more than a decade that the neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) is co-stored with acetylcholine in the splanchnic nerve terminals innervating the adrenal medulla. Both transmitters are robust secretagogues for catecholamine release from chromaffin cells. Here, we review the unique contribution of PACAP to the functioning of the splanchnic–adrenal synapse in stress. While acetylcholine is released across a wide range of firing frequencies, PACAP is released only at high frequencies of stimulation, and its role in the regulation of epinephrine secretion and biosynthesis is highly specialized. PACAP is responsible for long-term catecholamine secretion using secretory mechanisms different from the rapidly desensitizing depolarization evoked by acetylcholine through nicotinic receptor activation. PACAP signaling also maintains catecholamine synthesis required for sustained secretion during prolonged stress via induction of the enzymes TH and PNMT, and enhances transcription of additional secreted molecules found in chromaffin cells that alter further secretion through both autocrine and paracrine mechanisms. PACAP thus mediates chromaffin cell plasticity via functional encoding of cellular experience. These features of PACAP action at the splanchnic–adrenal synapse may be paradigmatic for the general actions of neuropeptides as effectors of stimulus–secretion–synthesis coupling in stress.

Keywords

Chromaffin cell; Neurotransmission; PACAP; Splanchnic–adrenal synapse; Stress

Introduction

The splanchnic–adrenal synapse was first examined in ultra-structural detail by Coupland, who observed that splanchnic nerve terminals on chromaffin cells contained both small clear

(presumably cholinergic) vesicles, and large dense-core vesicles of unknown content (Coupland 1965). Soon after, measurement of the co-release of epinephrine and chromogranins from the adrenal medulla in response to splanchnic nerve stimulation provided the first biochemical demonstration of secretion by exocytosis (Blaschko et al. 1967; Kirshner et al. 1966). The connection between the lumbar sympathetic afferents of the splanchnic nerve and the chromaffin cells of the adrenal medulla soon became one of the most studied ‘true synapses’ in the nervous system and — due to its critical physiological role in neuroendocrine homeostasis, neurochemical simplicity, and experimental accessibility — a model for the general principles of synaptic physiology throughout the nervous system. The splanchnic-adrenal synapse has recently re-emerged to illuminate a new principle — the release of different neurotransmitters from the same nerve terminal at low and high frequency that allows firing rate to encode qualitatively different post-synaptic effects during basal (low-frequency) and stress or emergency (high-frequency) conditions.

Normal or ‘homeostatic’ sympathetic tone encoded by low-frequency splanchnic nerve firing causes the adrenal medulla to supply catecholamine at a modest rate to set the homeostatic ‘rest and digest’ state (Klevans and Gebber 1970). In response to stress, however, splanchnic nerve firing rates (sympathetic tone) rises dramatically, and the adrenal medulla releases catecholamines at an elevated rate (Cannon 1929). At the most general organismic level, the neuroendocrine stress response can be defined operationally as ‘circuit and cellular responses to systemic or environmental perturbations outside of the normal physiological range’. The major circuits that mediate it are the hypothalamo-pituitary adrenocortical (HPA) and the hypothalamo-sympathoadrenal (HSA) systems. The HSA axis can be divided into the sympathetic nervous system (SNS) and the adrenomedullary hormonal system (AHS). In fact, the HPA and AHS are probably more closely coupled in differentiating and responding to stressful stimuli than are the AHS and SHS, and the ‘flight or fight’ (named by Walter Cannon) and ‘general adaptation’ (named by Hans Selye) syndromes generally associated with the SHS/AHS and HPA systems, respectively, have been recast to reflect this new understanding (Goldstein 2010; Goldstein and Kopin 2008).

On the cellular level, exposure to extracellular influences outside of the normal range can lead to hypoxia, protein damage, or changes in redox state that in turn affect metabolism and signaling. Typical cellular stress responses are manifested by heat-shock protein expression, changes in the endoplasmic reticulum (ER stress), and induction of glutathione and selenoprotein reductases (Kultz 2005). The significance of integrating the concepts of circuit and cellular responses to stress lies in the observation that the neurons and neuroendocrine cells arranged in circuits that *convey* the stress response do so by themselves being stressed — for example, by increased cellular calcium influx associated with increased neuronal firing rates. How organismic stress is conveyed at the cellular level, allowing hormone secretion, enzyme regulation and gene transcription, while at the same time the cells of neuroendocrine circuits protect themselves from the increased metabolic and oxidative burden occurring during ‘stress transduction’ are questions of compelling fundamental and practical interest.

The major cellular *messengers* in a stress circuit are the secretory products released from large dense-core vesicles (LDCVs). *Fast neurotransmitters* (ACh and, in some cases NE)

are released at all frequencies of stimulation from small synaptic vesicles (SSVs) and *slow neurotransmitters* (neuropeptides and in some cases catecholamines) are released only at high frequency stimulation from LDCVs. Thus, the release of LDCV content is synonymous with stress transduction, and the LDCV content released is the stress-transducing neurotransmitter, often a neuropeptide.

The concept that *dual* transmission in the autonomic nervous system is represented by preferential release from SSVs during low-frequency firing associated with basal functioning, and LDCVs during high-frequency firing associated with emergency responses, has been expressed in the slogan that ‘neuropeptides represent the language of stress’ (Hökfelt et al. 2003) and is depicted in Fig. 1. This concept has had some difficulty making its way into textbook knowledge about the autonomic nervous system, however. Medical and neuroscience students are aware that preganglionic inputs of the autonomic nervous system employ acetylcholine (ACh) as a neurotransmitter, and post-ganglionic sympathetics and parasympathetics employ norepinephrine and acetylcholine, respectively; it is less commonly known that a second critical level of neurotransmission, mediated mainly by the neuropeptides PACAP (preganglionic) and NPY (post-ganglionic sympathetic), is deployed during stress (Zukowska 2002). This is partly because neuropeptides were discovered later than biogenic amines; partly because the ‘duality’ of cholinergic versus noradrenergic transmission can be confounded with the ‘duality’ of fast versus slow transmission; and partly because the catecholamine NE is itself released both from SSVs (at low frequency from post-ganglionic sympathetic neurons) and LDCVs (at high frequency from postganglionic neurons and from chromaffin cells) (De Potter et al. 1997).

This review traces the development of the concept of dual transmission by ACh and PACAP at the splanchnic–adrenal synapse, considers its physiological and parapsychological implications for stress transduction at this synapse, and explores the implications of the modern dual neurotransmission concept for autonomic and central nervous system function and dysfunction.

Dual Transmission — Early Evidence and Implication of Peptidergic Transmission

In 1970, Mueller, Thoenen and Axelrod reported that the rate-limiting enzyme for catecholamine secretion from the adrenal medulla, tyrosine hydroxylase (TH), is induced to higher levels of activity when the innervating splanchnic nerve is stimulated in response to stress (Mueller et al. 1970). However, blocking the action of the only known neurotransmitter at this synapse, ACh, did not block the induction of TH elicited by stimulating the nerve. These authors suggested that a transmitter other than ACh might be involved in mediating the trans-synaptic increase in TH, thought to be necessary for the long-term homeostatic response of the adrenal gland to stress (Chuang and Costa 1974). The functional importance of this putative non-cholinergic transmitter was highlighted when it was shown to be required for the maintenance of continuous catecholamine secretion from the adrenal medulla during prolonged (several hours) of high-frequency splanchnic nerve stimulation (Wakade 1988).

A candidate for this non-cholinergic transmitter emerged in 1989 with the discovery by Miyata et al. (1989) of the pituitary adenylate cyclase-activating polypeptide (PACAP). Existing as an amidated peptide in both a short (27 amino acid) and long (38 amino acid) form, PACAP was initially discovered in a screen of hypothalamus for hypophysiotropic factors which, like previously discovered ones including GHRH, stimulated pituitary hormone secretion through elevation of cyclic AMP in the cells bearing the cognate G-protein coupled receptor (Arimura 1992; Miyata et al. 1989). PACAP is a member of the VIP-GHRH-glucagon family (part of the class II-liganded GPCR superfamily) (Martin et al. 2005). Subsequent studies demonstrated the presence of PACAP in both the central and peripheral nervous systems, in particular in splanchnic nerve terminals, with its receptors (mainly PAC1) on chromaffin cells themselves (Holgert et al. 1996). PACAP was proposed as a non-cholinergic transmitter at the splanchnic–adrenal synapse based on the ability of the exogenously applied transmitter to elicit catecholamine secretion in vivo, in isolated adrenal gland, and in cultured chromaffin cells (Przywara et al. 1996; reviewed by Hamelink et al. 2003).

This hypothesis was confirmed in 2002 by several lines of evidence. First, PACAP was localized to cholinergic terminals of splanchnic nerve, and to the corresponding cell bodies of the intermediolateral column of the spinal cord (Hamelink et al. 2002b), and not to sensory neurons in the adrenal medulla as previously suggested (Dun et al. 1996). The presence of both SSVs and extra-junctional LDCVs at this synapse at the ultrastructural level (Fig. 2), and the biochemical association of ACh with the former and neuropeptides with the latter (due to the requirement for processing for biological activity) is persuasive for dual transmission at varying frequency of neurotransmission across this synapse. Second, mice deficient in PACAP fail to recover from hypoglycemia after insulin administration, due to an inability to sustain epinephrine secretion from the adrenal medulla, representing both an attenuation of *secretion* and a failure to replete adenomedullary catecholamines through enhanced *biosynthesis*, even at reduced levels of secretion. The latter is due in turn to lack of induction of TH, the rate-limiting enzyme for catecholamine biosynthesis, in PACAP-deficient mice (Hamelink et al. 2002b). These results are consistent with the initial observation of Wakade and colleagues defining a non-cholinergic synaptic transmitter acting to release catecholamines under prolonged stimulation of the splanchnic nerve at high frequency for several hours (as would occur during prolonged hypoglycemia) (Wakade 1988). Finally, PACAP-deficient mice could be rescued from the otherwise fatal insulin shock with either exogenously administered glucose or PACAP-38 given intraperitoneally (Hamelink et al. 2002a, b), confirming the functional link between PACAP and recovery from hypoglycemia. Although not absolute proof that PACAP is the transmitter released at the splanchnic–adrenal synapse under high frequency activity, these lines of evidence all strongly support that conclusion.

A second compelling line of evidence implicating a second transmitter (whether PACAP or a related peptide) in addition to ACh to support sustained catecholamine release from the adrenal medulla is the observation that application of ACh does not mimic the effect of prolonged splanchnic nerve stimulation on catecholamine secretion (Klevans and Gebber 1970; Wakade 1998). The use of electrochemical amperometry allows precise real-time

monitoring of CA release from freshly prepared slices of rodent adrenal gland *ex vivo*, within time frames (seconds to minutes to hours) that are relevant to the physiological actions of the adrenal gland as a stress transducer *in vivo*. Application of ACh in this preparation elicits CA release that rapidly desensitizes over seconds to minutes, in contrast to prolonged splanchnic nerve stimulation, which does not. Furthermore, enhanced CA secretion elicited by 15 Hz splanchnic nerve stimulation is blocked by the PAC1 receptor antagonist PACAP(6–38), indicating that PACAP (or a related peptide) is required for sustained secretion (Kuri et al. 2009).

The secretagogue effect of PACAP is illustrated in Fig. 2. PACAP-evoked secretion occurs despite desensitization to cholinergic stimulation, a key aspect of the governing hypothesis for the proposal that PACAPergic transmission is necessary and sufficient for stress-driven catecholamine secretion from the adrenal gland *in vivo*. When challenged with ACh at physiological concentrations (30 μ M) the adrenal gland responds with a secretion that is immediate and robust, but rapidly desensitizes despite persistent ACh exposure. Later, during continued ACh desensitization, the same cell when stimulated with exogenous PACAP (1 μ M) responds with a rapid and robust catecholamine release. Exogenous PACAP evokes catecholamine secretion from chromaffin cells through a rapid influx of extracellular Ca^{2+} (O'Farrell and Marley 1997; Tanaka et al. 1996). PACAP-mediated exocytosis is independent of nicotinic ACh receptor (nAChR) function (Morita et al. 2002; Watanabe et al. 1992) or sodium-based action potentials (Mustafa et al. 2007). Thus, synaptic PACAP signaling bypasses the key sites of depolarization-initiated release that are triggered by nicotinic receptor stimulation, and linked to pronounced secretory desensitization.

Properties of PACAP-Induced Catecholamine Secretion and Current Flux in Chromaffin Cells — a Mechanism for Non-AP-Driven Exocytotic Secretion

Published studies have demonstrated that PACAP/PAC1 activation elicits a robust secretory response in chromaffin cells in virtually all mammalian species (reviewed by Hamelink et al. 2003; Mustafa and Eiden 2006). In rodent chromaffin cells, the ligand-bound PAC1 receptor initiates a non-canonical cAMP-dependent (Przywara et al. 1991) signaling pathway through activation of Epac (exchange protein directly activated by cAMP) (see Gerdin and Eiden 2007 and Ster et al. 2007). An emerging literature identifies a role for Epac in regulation of secretion from a variety of cell types (Kang et al. 2003). In chromaffin cells, as in dorsal root ganglion cells (Hucho et al. 2005), Epac activation leads to increased phospholipase C (PLC) activity and represents a functional linkage between cAMP and protein kinase C (PKC) activation, that ultimately elevates cytosolic Ca^{2+} and evokes catecholamine secretion (see Fig. 3a and Kuri et al. 2009 for a mechanistic description of this secretory pathway). Focal perfusion with the specific Epac activator, 8-pCPT-2'-O-Me-cAMP leads to catecholamine secretion from chromaffin cells that shares a common kinetic and pharmacological profile to PACAP stimulation (Kuri et al. 2009). Both 8-pCPT-2'-O-Me-cAMP and PACAP-evoked secretion are ultimately blocked by voltage clamp at -80 mV or by extracellular Ni^{2+} , Zn^{2+} and mibefradil, all consistent with Ca^{2+} influx through low voltage-activated (LVA) T-type calcium channels (Kuri et al. 2009; Perez-Reyes 2006; Przywara et al. 1996). Further characterization of PACAP-evoked secretion has

demonstrated that PAC1 activation does indeed lead to the recruitment of an LVA T-type calcium influx through a dual mode subthreshold depolarization (Kuri et al. 2009) and co-activation of silent T-type calcium conductance (Hill et al. 2011) leading to a sustained calcium influx to evoke catecholamine secretion. The signaling pathway leading to long-term (minutes to a few hours) PACAP-initiated secretion from bovine chromaffin cells and the dog adrenal medulla in vivo may employ additional types of calcium channels, whose activation moreover does not require cAMP (Geng et al. 1997; Hahm et al. 1998; Mustafa et al. 2010) (Fig. 3b). Finally, PKA-dependent catecholamine release may occur at even longer times (several hours) of secretion (Eiden et al. 1998). These findings highlight an often-unappreciated aspect of catecholamine secretion from the adrenal medulla: that secretion occurring in different temporal domains, from seconds to minutes to several hours may occur by molecularly distinguishable mechanisms, all of physiological relevance within a given temporal domain (Kuri et al. 2009; Mustafa et al. 2007; Wakade 1988). In all cases examined, however, PACAP acts as a potent secretagogue in a signaling path that is independent of the mechanism by which ACh acts in the same cell type. Moreover, the PACAP-evoked signaling cascade exhibits a long-term activation of cell secretory activity that is not susceptible to the negative feedback regulation associated with cholinergic excitation (i.e., nicotinic receptor inactivation followed by chromaffin cell depolarization block) making it particularly well suited to provide catecholamine release under a sustained stress response as originally described by Wakade (1988, 1998) (see summary in Fig. 3).

Induction of Catecholamine Biosynthetic Enzymes in Adrenal Medulla After Stress is PACAP-Dependent

Stress-induced splanchnic nerve firing in vivo not only enhances catecholamine release but activates TH through a post-translational, cAMP/PKA-dependent mechanism (Haycock 1996). TH enzymatic activation upon prolonged splanchnic nerve firing in vivo is abolished in PACAP-dependent mice (Hamelink et al. 2002b). In addition, several hours' exposure to PACAP up-regulates both TH and PNMT mRNA (Tönshoff et al. 1997) in cultured chromaffin cells. Likewise, the well-known induction of TH and PNMT mRNA in the adrenal medulla in response to restraint stress (Sabban et al. 1995, 2004) is largely PACAP-dependent (Stroth and Eiden 2010). The requirement for enhanced catecholamine biosynthesis to keep pace with secretion at high splanchnic nerve firing rates first remarked by Wakade in perfused adrenal gland ex vivo (Wakade 1988) is also observed in vivo. Prolonged splanchnic nerve firing induced by hypoglycemia does not result in adrenomedullary catecholamine depletion in wild-type mice, even in the face of massive catecholamine secretion from the gland, while in the PACAP-deficient mouse adrenomedullary catecholamine stores are significantly depleted after prolonged hypoglycemia, even though catecholamine secretion is greatly reduced compared to wild-type mice (Hamelink et al. 2002b).

PACAP and Adrenomedullary Cellular Plasticity — a Role Beyond Catecholamine Release

The complex signaling cascade initiated by PACAP engagement of the PAC1 receptor that results in catecholamine secretion and enhanced catecholamine biosynthesis also causes the parallel secretion and stimulation of biosynthesis of additional autocrine factors, paracrine factors and hormones from the adrenal medulla, including VIP, substance P, neurotensin, enkephalin, galanin and others. The mechanisms of neuropeptide secretion from the adrenal medulla are likely to be identical to those of catecholamines: although the extent of release from LDCVs of small and large molecules may differ kinetically due to the properties of the LDCV fusion pore (Perrais et al. 2004), there is no evidence to date that catecholamines and neuropeptides are stored in distinguishable LDCVs in chromaffin cells, albeit the mechanisms of their biosynthesis regulation may occur by distinct signaling pathways (Pruss et al. 1986).

Transcriptional regulation by PACAP of the cognate genes for other neuropeptides and neuroactive messenger molecules in chromaffin cells, likely occurs through a unique cAMP-dependent pathway, separate from cAMP-dependent modulation of secretion. As first characterized by Hamelink et al., PACAP causes a pronounced up-regulation of VIP protein in chromaffin cells (more than 500-fold elevation of VIP mRNA within hours) that is independent of PKC, and is mimicked by a combination of forskolin and elevated KCl, suggesting a dual calcium/cAMP signaling pathway. Intriguingly, this cAMP-dependent pathway is independent of PKA, in both bovine chromaffin and PC12 cells, and proceeds through activation of ERK1/2 (Hamelink et al. 2002a). The roles for this pathway in chromaffin cell gene regulation in stress remain to be fully elucidated, although it is the preferred pathway for activation of other neuropeptides besides VIP in chromaffin cells. It is also the required signaling pathway for the regulation of Stc1, a neuroprotective protein, in cultured cortical neurons (Holighaus et al. 2012), and is dependent on the specific isoform of the PAC1 receptor present on a particular neural cell (Holighaus et al. 2011).

Although stimulus–secretion–synthesis coupling was first demonstrated for ACh (Eiden et al. 1984), it is clear that PACAP is the major regulator of this phenomenon in the chromaffin cell, mainly because PACAP-induced release, during high-frequency splanchnic nerve firing, results in a far greater export of secretory molecules from the chromaffin cell per unit time than does the rapidly desensitizing secretion elicited by ACh. Since induction of neuropeptide expression after splanchnic nerve stimulation is quite long-lasting (Anouar and Eiden 1995; Fischer-Colbrie et al. 1992; Kanamatsu et al. 1986), it would be predicted that this function of splanchnic nerve stimulation is mediated by PACAP as well, although this remains to be demonstrated in the PACAP-deficient mouse. However, systematic analysis by microarray in PACAP-treated cultured chromaffin cells has revealed the prompt (within 6 h) induction of a large cohort of genes encoding secreted proteins, strongly suggesting a role for PACAP in stimulus–secretion–synthesis coupling at the splanchnic–adrenal synapse in vivo, and perhaps even in cellular plasticity potentially providing ‘stress memory’ in adrenomedullary function (Ait-Ali et al. 2010).

Summary and Future Perspectives

As recently as 2008, an authoritative review on mechanisms of catecholamine release at the adrenal medulla could be written without a single mention either of neuropeptides or PACAP, although it was opined that 'it could be interesting' to correlate patterns of splanchnic discharge and actual catecholamine release in vivo and in slice preparations to resolve clear discrepancies between acetylcholine effects on catecholamine secretion and the physiological consequences of splanchnic nerve stimulation on catecholamine release in vivo (de Diego et al. 2008). It now seems very likely, absent some key confirmatory experiments, that the answer to the question posed in the title of this review will be 'yes'. There is now sufficient evidence to accept at least provisionally the necessity if not the sufficiency of PACAP neurotransmission for stress signaling at the splanchnic–adrenal synapse, including both stress-mediated catecholamine secretion from the adrenal medulla, and stimulus–secretion–synthesis coupling for both catecholamine biosynthetic enzymes and other secretory proteins. Might its role be primarily *modulatory*, as suggested for catecholamine secretion from the guinea pig adrenal medulla (Inoue et al. 2000)? Although it is not yet formally proven that PACAP does not require priming by cholinergic signaling to cause catecholamine release upon splanchnic stimulation, this seems unlikely based on the copious evidence (reviewed by Hamelink et al. 2003) that PACAP at physiological concentrations causes catecholamine release from isolated chromaffin cells, under conditions in which acetylcholine is absent.

If PACAP is the sole transmitter for 'emergency signaling' for adrenomedullary catecholamine release, what is the physiological role of ACh at the splanchnic–adrenal synapse? The ability of nicotinic ACh signaling to respond rapidly and reversibly (due to desensitization) to fluctuations in excitation, makes this system ideal for tracking dynamic but less than 'all-or-none' changes in catecholamine secretion required for adaptation to intermittent hypoxia, cold, and exercise. The investigation of the relative roles of cholinergic and PACAPergic signaling across the range of physiological, paraphysiological and pathophysiological autonomic stimulation will be highly informative in testing this working hypothesis.

Is PACAPergic neurotransmission of equal importance for norepinephrine secretion in post-ganglionic sympathetic neurons and catecholamine secretion from chromaffin cells? Three decades ago (and 6 years before the structure of PACAP was elucidated by the Arimura laboratory) Ip and co-workers postulated that a non-cholinergic transmitter was responsible for induction of TH at the superior cervical ganglion under conditions of intense autonomic activation (10-Hz electrical stimulation of the cervical sympathetic trunk) (Ip et al. 1983). Since the discovery of PACAP, its role in regulation of catecholamine secretion and stimulus–secretion–synthesis coupling in cultured neonatal cultured sympathetic neurons has been thoroughly examined (Beaudet et al. 1998, 2000; Braas and May 1996, 1999; Brandenburg et al. 1997; May et al. 1998). However, PACAP's role in vivo as both a parasympathetic and sympathetic preganglionic neurotransmitter is not definitively established despite preliminary findings in heart, bladder and other autonomically innervated organs that are consistent with this concept (Girard et al. 2007; May and Vizzard 2009; Tompkins et al. 2007), perhaps because PACAP's function as a neurotrophic factor during

development has garnered somewhat greater attention than its role as an autonomic neurotransmitter role (Girard et al. 2002; May et al. 2010; Pavelock et al. 2007).

A brief comment on the role of PACAP in the central nervous system is merited since it illustrates that the stress transmitter concept championed by Hokfelt and colleagues is likely similarly valid throughout the nervous system. Several laboratories have now firmly implicated PACAP as a major transmitter regulating circadian function in the suprachiasmatic nucleus (co-released with glutamate from retinohypothalamic neurons) (Beaule et al. 2009; Colwell and Waschek 2001), hypothalamohypophysial ACTH secretion (Stroth et al. 2011), and anxiety responses in the bed nucleus of the stria terminalis (Hammack et al. 2010). The high density of expression of the PAC1 receptor in the dentate gyrus of the hippocampus, and in the cerebellum (Zhou et al. 2000), suggests that these systems as well may be ones in which PACAP, far from merely modulating transmission, is a primary neurotransmitter under conditions in which LDCV-associated transmitters are selectively deployed by high neuronal firing rates.

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Fig. 1.

Low- and high-frequency modes of neurotransmission: basal and stress signaling from neuroendocrine cells. **a** A diagram conceptualizing the dual transmitter hypothesis for synaptic transmission. Signaling events associated with low frequency neuronal activity are color coded green and those with sympathetic burst-mode firing are coded red. Low-frequency firing results in a modest Ca^{2+} influx that is limited to the active zone through a combination of Ca^{2+} channel localization and fast neuronal processes for Ca^{2+} buffering, sequestration and pumping. Due to this Ca^{2+} microdomain (Neher 1998) of suprathreshold Ca^{2+} (green dashed semicircle), small clear vesicles localized at the active zone are the only

secretory organelles that undergo fusion. However, during elevated sympathetic activity, burst-mode firing saturates the fast Ca^{2+} clearance processes and the micro-domain grows to encompass extrasynaptic large dense-core granules which are then too triggered to fuse, spilling their peptide transmitter into the synaptic cleft (*red dashed semicircle*). Postsynaptic features include a large surface area to sense both synaptic and extrasynaptic transmitter release. **b** The presynaptic morphology presented in panel **a** predicts an activity-dependent differential release probability for small clear vesicles and large dense-core vesicles. Together, their combined stimulus–secretion functions would result in a shift in synaptic transmitter burden with increased sympathetic firing

Fig. 2. Characteristics of PACAP-initiated catecholamine secretion and structural morphology of the splanchnic-adrenal synapse. **a** *Left*: a bright-field image of the in situ adrenal slice preparation demonstrates the amperometric recording configuration used to measure catecholamine release from chromaffin cells. *Right*: immunohisto-chemical staining shows that PACAP (green) is localized peripheral to chromaffin cells. The PACAP specific receptor (PAC1-R, red) is expressed in chromaffin cells (scale=10 μ m). **b** *Top*: an amperometric trace recorded from a chromaffin cell in situ that has been stimulated by focal perfusion with a Ringer containing 1 μ M PACAP causes an immediate catecholamine exocytosis. *Bottom*: ratiometric FURA signals demonstrate an immediate rise in intracellular Ca^{2+} upon PACAP stimulation. **c** Ultra-thin sections were cut from adult mouse adrenal

glands and imaged by transmission electron microscopy. (i) The nucleus of the cell is offset to the basal-lateral pole of the cell. The apical pole of the cell faces the capillary lumen. The innervating splanchnic terminal is indicated by the inset box. Several key features of the in situ morphology are indicated by symbols as explained in panel c, (ii). Scale bar=10 μm . (ii) The innervating cholinergic terminal of the splanchnic nerve from panel a at higher magnification. The terminal exhibits a morphology consistent with that conceptualized in Fig. 1a. The terminal is filled with many small clear vesicles as well as large dense-core granules (several are indicated by the *arrowheads*) Several preynaptic densities are outlined in dotted ovals and represent the synaptic active zones. The active zones are enriched with small clear vesicles while the large dense-core granules are extrasynaptic. Scale bar= 1 μm

Fig. 3.

Signaling pathways leading to PACAP-stimulated secretion and PACAP-mediated gene regulation (stimulus-secretion-synthesis coupling). A summary diagram based on the literature and representing a working hypothesis for **a** short-term (several minutes) and **b** long-term (minutes to hours) catecholamine secretion from chromaffin cells. **a** PACAP activation of the PAC1 receptor elevates cAMP, and in the short term, this elevation activates Epac, which together with activation of PLC to generate diacylglycerol and increase PKC activity, leads sodium influx through a tetrodotoxin-insensitive route. Data to date suggest this is through a phospho-activation of the sodium-calcium exchanger, although alternate molecular targets sharing a similar pharmacologic profile (i.e., Trp channel isoforms; see Beaudet et. al. 2000) remain to be definitively tested. The PKC activation also recruits a low voltage-activated T-type calcium conductance. The sodium influx, or alternatively calcium influx through 2-APB-sensitive channels, depolarizes the cell by approximately 20 mV, subthreshold for action potential activation, but sufficient to open the T-type calcium channels. A persistent phospho-dependent window current through the T-type channels provides the necessary calcium influx to elicit catecholamine release. Perturbation of any of these steps is sufficient to block acute PACAP-evoked catecholamine release. **b** Secretion dependent on L-channel opening (see Mustafa et al. 2007) is depicted as arising from calcium influx via 2-APB-sensitive channels causing partial depolarization leading to voltage-gated calcium channel opening and exocytosis. Question marks for steps involving PLC alert the reader to the possibility of non-specific action of the putative PLC inhibitor U73122 and the absence of non-pharmacological demonstrations of PLC involvement in long-term catecholamine secretion from chromaffin cells. In summary,

PACAP acts through parallel activation mechanisms to evoke catecholamine release: a rapid phospho-dependent recruitment of T-type calcium channels (**a**) and longer-term facilitation of L-type calcium channels (**b**). Even longer term (several hours), the cAMP elevation elicited by PAC 1 receptor activation may lead to both facilitation of L-type calcium channels and subsequent nifedipine-sensitive sustained catecholamine release as well as facilitation of release by mechanisms independent of voltage-gated calcium channels. It is the authors' hope that these two models be useful for calling attention to differences in regulation of short- and long-term secretion by PACAP, and providing a framework for interpretation of the existing experimental literature