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# PACAP activated adenylate cyclase in human sweat glands. An ultracytochemical study

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## SUMMARY

The ultracytochemical localization of adenylate cyclase (AC) was studied after stimulation with pituitary adenylate cyclase activating peptide (PACAP) in human sweat glands. PACAP stimulated AC in both eccrine and apocrine glands. In the secretory cells, enzymatic activity was associated with membranes involved in the secretory mechanism. In both glands, the cells of the excretory duct and myoepithelial cells presented AC activity. These localizations of enzymatic activity suggest a role for PACAP in regulating glandular secretion.

## INTRODUCTION

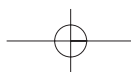
Pituitary adenylate cyclase activating peptide (PACAP), originally isolated from bovine hypothalamus (Miyata *et al.*, 1989), is a peptide that functions as a neurotransmitter, neuromodulator and neurotrophic factor in the central nervous system, although PACAP-like immunoreactivity has also been detected in peripheral organs (Arimura & Shioda, 1995). PACAP is a member of the VIP (vasoactive intestinal peptide) family and the N-terminal portion of PACAP has 68% homology with porcine VIP (Miyata *et al.*, 1989).

The biological effect of PACAP and VIP in target tissues is the activation of adenylate cyclase (AC) and production of cAMP. PACAP occurs in both 38 (PACAP 38) and 27 (PACAP 27) aminoacid forms and these peptides were found to be equipotent in the stimulation of AC (Miyata *et al.*, 1989).

Previous reports have demonstrated the activity of PACAP in exocrine and endocrine glands: PACAP controls secretion in rat adrenal medulla and in whole adrenal gland (Watanabe *et al.*, 1992; Yon *et al.*, 1994; Przywara *et al.*, 1996). PACAP has been detected in nerve fibers around seromucous glands, small glands in the lung, submandibular glands and in exocrine and endocrine pancreas, where it induces cAMP production and stimulates secretion (Arimura & Shioda, 1995). It is a potent stimulator of synthesis and release of melatonin from pineal gland (Simonneaux *et al.*, 1998).

In the human body, both eccrine and apocrine sweat glands are present. The eccrine glands, present everywhere, produce a watery secretion with some salt and few organic substances. The apocrine glands, located prevalently on the axillary and genital skin, produce a more concentrated secretion with odorant substances. A third type, the apoecrine glands, is found only in the adult axillae, where they represent less than 10% of all

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glands. These glands share some of the morphologic and functional characteristics of both eccrine and apocrine glands (Sato *et al.*, 1989).

Before the discovery of PACAP (Miyata *et al.*, 1989), VIP-immunoreactive nerve fibers were identified around the eccrine sweat glands (Vaalasti *et al.*, 1985) and the activity of AC stimulated by VIP was studied ultracytochemically in human eccrine glands (Tainio, 1987).

To our knowledge, after the discovery of PACAP, there have not been data available regarding the presence of PACAP and AC activity stimulated by this peptide in human sweat glands. Previous studies indicated that the activity of PACAP was much more potent than that of VIP (Miyata *et al.*, 1989). Moreover, in various tissues there are two types of PACAP receptors: PACAP- and VIP-shared sites and PACAP-specific sites (Arimura & Shioda, 1995). Therefore, the purpose of this study was to determine the localization of PACAP-stimulated AC activity in human eccrine sweat glands and in the apocrine glands given their different secretory pathways. The apoeccrine glands were not considered in the present study. To demonstrate the enzymatic activity, we employed an ultracytochemical method for electron microscopy.

## MATERIALS AND METHODS

Surgical specimens were obtained from 6 patients (3 males and 3 females) under local anaesthesia by mepivacaine hydrochloride. The patients ranged in age from 33 to 62 years. Samples from axillary skin were used for detecting both eccrine and apocrine sweat glands. The samples were fixed immediately after the excision for 20 min in cold (4°C) 1% glutaraldehyde in 0.1M cacodylate buffer containing 8% glucose, pH 7.4. Then samples were washed several times in the same buffer to remove the fixative. Under the stereomicroscope, thin sections were cut and stored at 4°C overnight before further processing.

### Ultracytochemical localization of AC activity

The present method was based on a previous ultracytochemical procedure for the detection of AC activity (Yamamoto *et al.*, 1998). Specimens were incubated in freshly prepared medium to demonstrate AC activity. The medium of incubation consisted of 80 mM Tris-maleate buffer pH 7.4, 8% glucose, 2 mM theophylline, 4 mM

MgSO<sub>4</sub>, 0.5 mM 5' adenylylimidodiphosphate sodium salt (App(NH)p), 2 mM Pb (NO<sub>3</sub>)<sub>2</sub>, 1 μM GTPγS, 10 mM ouabain (basal medium). Specimens were incubated in: (1) basal medium, (2) basal medium plus 10<sup>-8</sup>M PACAP 38 (complete medium).

In control experiments, samples were incubated in the complete medium minus App(NH)p, or they were preheated at 60°C for 20 min prior to incubation in the complete medium.

After incubation for 45 min at 37°C, the samples were rinsed several times in 0.1 M cacodylate buffer, and postfixed in 1% osmium tetroxide for 45 min. The samples were dehydrated in a graded series of ethanol and embedded in an Epon-Araldite mixture. After a brief staining in a saturated solution of uranyl acetate in 50% ethanol, ultrathin sections were examined in a Philips TEM 400 electron microscope.

For the ultracytochemical demonstration of the enzymatic activity, the method used allows the detection of a precipitate of enzymatically released imidodiphosphate and lead ions at the site of AC activity (reaction product) (Poeggel & Luppá, 1988).

App(NH)p and GTPγS, non hydrolyzable analogs of ATP and GTP respectively, were used. GTP is necessary for increasing PACAP stimulation of AC (Onali & Olianás, 1994). Theophylline was used as an inhibitor of phosphodiesterases, ouabain was included to inhibit Na<sup>+</sup>,K<sup>+</sup> ATPase.

PACAP, App(NH)p and GTPγS were obtained from Sigma, Milan, Italy.

## RESULTS

The morphologic characteristics of sweat glands in human axillae have been described (Sato *et al.*, 1989). The secretory portion of eccrine sweat glands consists of three types of cells: clear cells, dark cells and myoepithelial cells. The clear cells form between them intercellular canaliculi and the adjacent membranes interdigitate to form numerous short and narrow processes. These cells participate in transepithelial fluid and ion transport. The dark cells are filled with dense secretory granules surrounded by membrane. Peripheral to the secretory cells are myoepithelial cells. The excretory duct contain two layers of one single cell type. The secretory portion of apocrine sweat glands is composed of a single layer of secretory cells surrounded by myoepithelial cells. The cells do not form intercellular canaliculi. The cyto-

plasm contains numerous granules surrounded by membrane. Excretory duct is lined by several layers of small cuboidal cells.

Ultracytochemical evidence of PACAP-stimulated AC activity was demonstrated by the presence of an electron dense reaction product.

#### **Eccrine sweat glands**

In the clear cells, the reaction product of AC activity was associated with apical and lateral plasma membranes (Fig. 1a) and with membranes lining intercellular canaliculi (Fig. 1b). Some AC reaction product also showed a localization on intracellular membranes (Figures 1a,b). In the dark cells, AC activity was present on apical and lateral plasma membranes. Moreover, the reaction product of enzymatic activity had an intracellular localization; it was associated with membranes of the Golgi apparatus, and with membranes lining secretory granules (Figures 1a,c). In the control studies, samples incubated in the complete medium but without the substrate App(NH)p (Fig. 1d) or heat-inactivated tissue (not shown) did not reveal any enzymatic activity.

#### **Apocrine sweat glands**

In the secretory cells, the reaction product of AC activity had an intracellular localization; it was present on membranes of the Golgi apparatus and on membranes lining secretory granules. In addition, the apical plasma membrane showed some positivity (Fig. 2a).

#### **Eccrine and apocrine sweat glands**

In both eccrine and apocrine glands, the cells of the excretory duct showed enzymatic activity on plasma membranes (Fig. 2b), and myoepithelial cells showed AC activity with prevalent intracellular localization of reaction product, although some reaction product was associated with the plasma membrane (Fig. 2c).

No samples incubated in basal medium displayed ultracytochemical evidence of AC activity (not shown).

### **DISCUSSION**

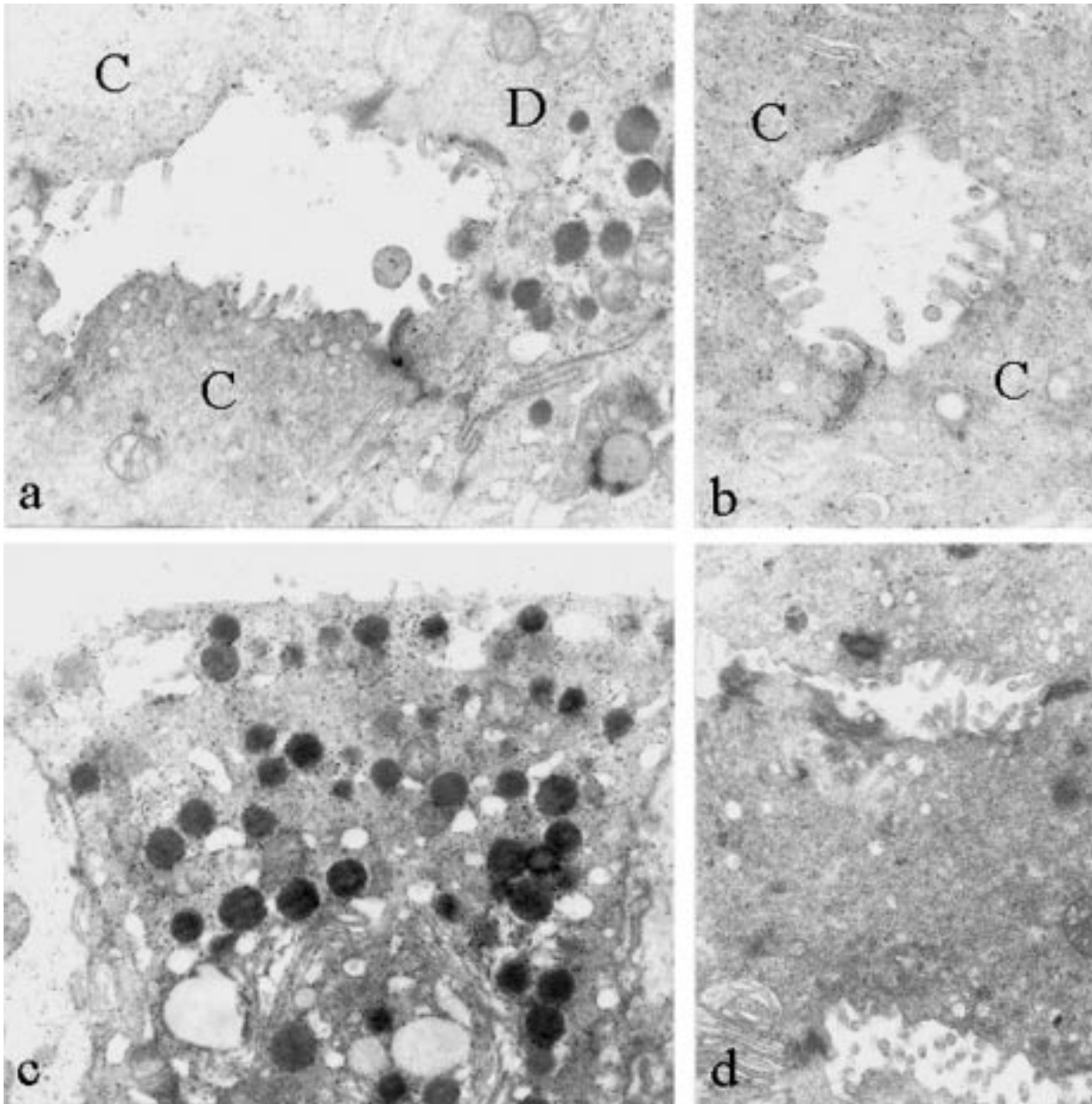
PACAP has been found in nerve fibers in several organs and double-immunostaining histochemistry revealed that PACAP immunoreactive nerve fibers constituted a subpopulation of the VIP immunoreactive fibers (Uddman *et al.*, 1993; Arimura and

Shioda, 1995). Before the discovery of PACAP (Miyata *et al.*, 1989), VIP immunoreactive nerve fibers were identified in human sweat glands (Vaalasti *et al.*, 1985) and, primarily in human eccrine axillary sweat glands, VIP-stimulated AC activity was seen on the plasma membrane of secretory cells and myoepithelial cells (Tainio, 1987). Successively, to our knowledge, no studies have been reported regarding the presence of PACAP immunoreactive nerve fibers, or PACAP-stimulated AC activity in either eccrine or apocrine sweat glands. The present ultrastructural observations reveal the cellular and subcellular distribution of AC activity in both of these glands in the presence of PACAP, known to activate this enzyme. In the present study, we demonstrated that exogenous PACAP could stimulate AC activity. In principle, the response to exogenously applied PACAP may be due to activation of AC that normally responds to PACAP present into nerve fibers and nerve endings present in sweat glands.

The absence of enzymatic reaction product in samples incubated in a medium devoid of PACAP suggests that the basal activity of the enzyme may be below the detection limit by the technique used in the present study. Moreover, here we provide morphological evidence that the presence of PACAP amplified the enzymatic activity over the level of the detection limit in these experimental conditions. One limit of the technique we used is that low amounts and/or activity of the enzyme may escape detection. Both  $Pb^{2+}$  and fixation have been reported to lower the AC activity (Cutler, 1983). However, a suitable amount of enzymatic activity still remains, and in the presence of 80 mM maleate and 2 mM  $Pb^{2+}$ , AC can still be stimulated by activators such as NaF or various specific hormones (Cutler, 1983). We used PACAP 38 at a concentration of  $10^{-8}$  M to produce the maximal stimulatory effect on AC activity. From the biochemical assays, increases in cAMP have been observed to be dose-dependent, and the maximal stimulatory effect of PACAP 38 was produced at a concentration of  $10^{-7}$  –  $10^{-8}$  M (Watanabe *et al.*, 1992).

Our data on the eccrine glands are partly in agreement with the result obtained by Tainio (1987) on ultracytochemical localization of AC activity stimulated by VIP. The reaction product of PACAP-stimulated AC activity was present on plasma membrane of the clear cells and myoepithelial cells.

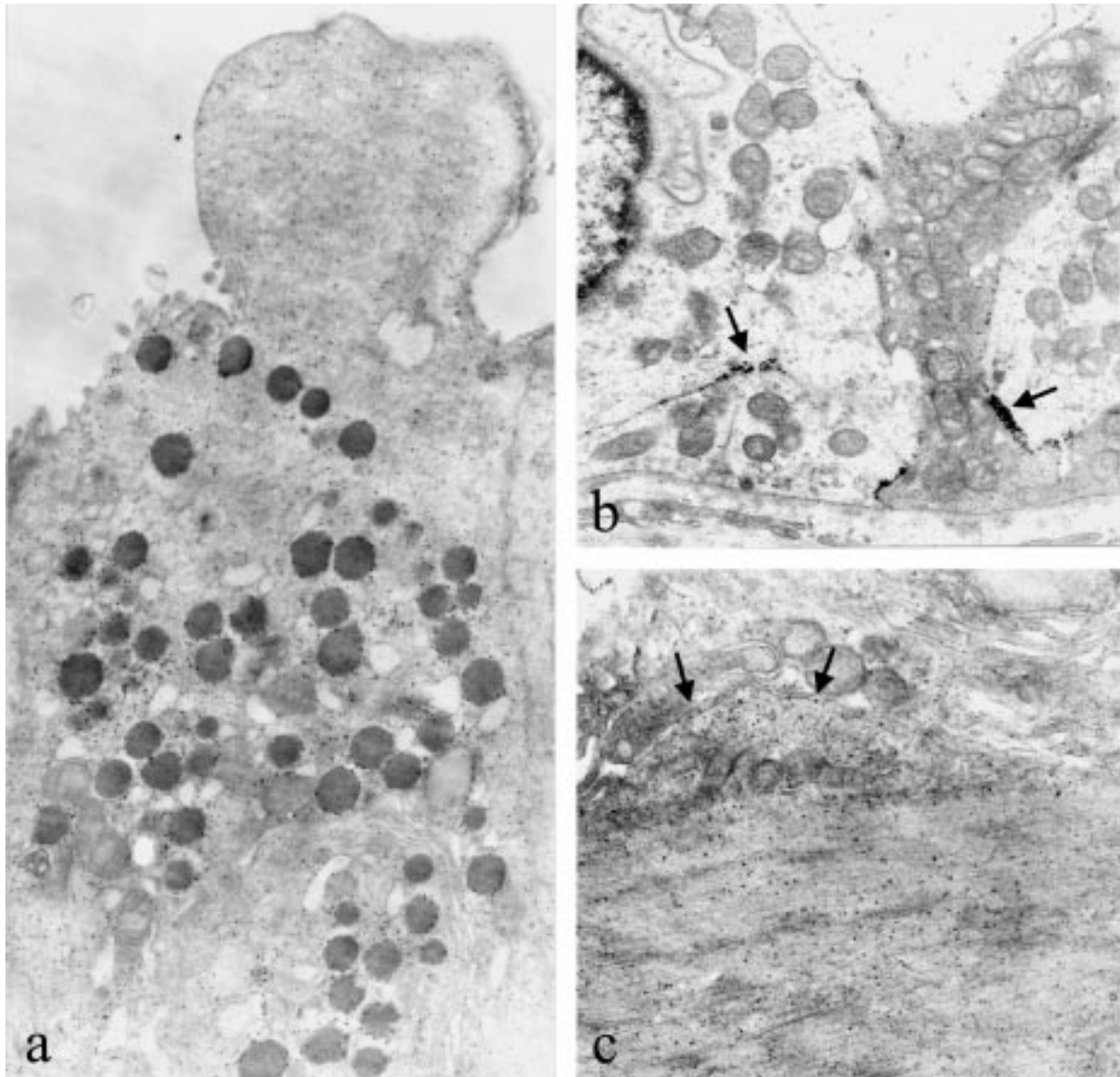
Previous studies on eccrine sweat glands suggest that VIP stimulates sweat secretion, by elevating



**Fig. 1** - Eccrine sweat glands. PACAP-stimulated AC activity. **a**: Clear cells (C) – Enzymatic activity is present on apical and lateral plasma membranes. Some deposits of reaction product are detected on intracellular membranes. Dark cell (D) shows AC activity on apical and lateral plasma membranes and on intracellular membranes lining secretory vesicles. x 18000. **b**: Clear cells (C) – Enzymatic activity is localized on adjacent plasma membranes and on membranes bordering an intercellular canaliculus. Some deposits of reaction product are detected on intracellular membranes. x 18000. **c**: Dark cell – Enzymatic activity is detected on Golgi membranes, membranes lining secretory vesicles and on the plasma membrane. x 12000. **d**: Control sample incubated in the presence of PACAP but without App(NH)p – Clear cells are devoid of enzymatic reaction product. x 18000.

cAMP concentration, and acts as a synergist for both acetylcholine-mediated and  $\beta$ -agonist-mediated sweat secretion, although the possible modulatory role of VIP remains to be elucidated (Sato *et al.*,

1989). Neither VIP nor norepinephrine seem to be involved primarily in physiological sweat secretion, but they seem to be important in the maximal accumulation of cAMP together with acetylcholine (Sato



**Fig. 2** - Apocrine sweat glands – PACAP-stimulated AC activity. **a:** Secretory cell – Enzymatic activity is present on membranes lining secretory vesicles, Golgi membranes and on apical plasma membrane. x 13000. **b:** Excretory duct – Cells show enzymatic activity on the plasma membrane (arrows). x 14000. **c:** Myoepithelial cells - Enzymatic activity has an intracellular localization. Some reaction product is present on the plasma membrane (arrows). X 20000.

& Sato, 1987; Sato *et al.*, 1989). Previous studies have indicated that PACAP is much more potent than VIP in stimulating AC, although PACAP is structurally similar to VIP (Miyata *et al.*, 1989). In the adrenal medulla, PACAP is an important non-cholinergic secretagogue (Watanabe *et al.*, 1992; Yon *et al.*, 1994; Przywara *et al.*, 1996); it is 1000 times more potent than VIP in catecholamine secretion, and VIP is very weak in stimulating cAMP pro-

duction in adrenal medulla cells as compared to PACAP (Watanabe *et al.*, 1992). The presence of PACAP-stimulated AC activity reported in the present study, suggests that this peptide could play a more important role than VIP in cAMP accumulation in eccrine sweat glands.

The reaction product of enzymatic activity was detected also on intracellular membranes of secretory and myoepithelial cells, in eccrine and apoc-

rine glands. The subcellular distribution of AC activity is in agreement with previous ultracytochemical studies (Poeggel and Luppá, 1988; Yamamoto *et al.*, 1998). Also immunocytochemical studies have demonstrated the presence of AC isoforms in the cytosol and on internal membranes (Mons *et al.*, 1995), and the reliability of the subcellular distribution of AC is increased. The subcellular distribution of AC on various internal membranes may reflect a compartmentalization of the enzyme to regulate a preferential specific function (Yamamoto *et al.*, 1998).

In dark cells of eccrine glands and secretory cells of apocrine glands, the activity of AC appears to be localized on intracellular membranes which participate directly in the formation and discharge of secretory products; this suggests that the enzyme may play a significant role in the secretory function of these cells. Finally, the presence of AC activity in myoepithelial cells is in agreement with the myorelaxant effect of PACAP (Mungan *et al.*, 1992).

In conclusion, the presence of AC activity suggests a role of PACAP in secretion in both eccrine and apocrine sweat glands although its modulatory role as well as that of VIP in sweat production requires further clarifications.

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