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REVIEW

Neuroendocrine Communication in the Frog Adrenal Gland

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INTRODUCTION

The adrenal gland is composed of two main tissues, of distinct embryologic origin, united in a single organ: the adrenocortical tissue derives from the mesoderm while chromaffin cells originate from the ectoderm. In addition, the adrenal gland contains a dense network of nerve terminals from various sources: (i) efferent fibers from spinal ganglia; (ii) afferent fibers from the splanchnic nerve, from nerve bundles coursing along the walls of blood vessels and from neurons located in the subcapsular space [95].

It has long been considered that, in the adrenal gland of mammals, the cortex and the medulla are two independent glands regulated by distinct mechanisms. However, the fact that both tissues are activated under stress conditions, and their anatomical association in the same organ favour the view that the two entities may be involved in physiological interactions. In support of this hypothesis, it has been demonstrated that glucocorticoids control the expression of various enzymes implicated in catecholamine biosynthesis [93, 98]. More recently, it has also become evident that various neurotransmitters and neuropeptides synthesized by chromaffin cells may regulate the activity of adrenocortical cells [7, 8, 30, 36]. In particular, it has been shown that dopamine inhibits while (nor)adrenaline stimulates the secretion of corticosteroids [1, 23]. In addition, adrenomedullary cells contain various regulatory peptides, including arginine vasopressin [82], enkephalins [65, 83], neuropeptide tyrosine [66, 83] and corticotropin-releasing factor [9]. All these peptides have been shown to modulate the activity of adrenocortical cells [3, 37, 63, 68, 69, 82, 85], which has led to the concept of neuroendocrine regulation of the adrenal cortex [31, 95].

In mammals, however, the zonation of the adrenal gland on the one hand and the centripetal direction of the blood flow on the other hand make it unlikely that bioactive compounds secreted by medullary cells can actually participate in the regulation of adrenocortical cells. In contrast, the peculiar arrangement of the adrenal gland of amphibians, which consists of intermingled chromaffin and corticosteroidogenic cells, favours interactions between the two types of tissues [20, 102]. Moreover, the adrenal gland of amphibians, in very much the same way as its mammalian counterpart [10, 40], receives a rich innervation from multiple origins [24, 59, 64, 83, 99]. Therefore, the amphibian interrenal tissue represents a very suitable model in which to investigate the paracrine and neuroendocrine mode of regulation of adrenocortical cells.

NEUROENDOCRINE REGULATION OF ADRENOCORTICAL CELLS BY CHROMAFFIN CELLS

Frog chromaffin cells contain a number of regulatory factors which have been visualized by immunohistochemistry

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and characterized by analytical methods. Several of these factors have been shown to modulate corticosteroid secretion by the adrenal tissue *in vitro*.

Catecholamines

In amphibians, as in other vertebrates, chromaffin cells express the enzymes of the pathways of catecholamine biosynthesis. In particular, the presence of tyrosine hydroxylase has been demonstrated by immunocytochemistry in all chromaffin paraneurons [58]. The occurrence of phenylethanolamine-N-methyltransferase (PNMT) has been found in a subpopulation of cells which represent 77% of the whole chromaffin tissue [58]. These data indicate that all frog chromaffin cells can synthesize dopamine and noradrenaline, and that a large proportion of these cells can convert noradrenaline into adrenaline. In agreement with these data, high-performance liquid chromatography (HPLC) analysis of frog adrenal extracts combined with electrochemical detection has revealed that large amounts of adrenaline and, to a lesser extent noradrenaline, are contained in the tissue, whereas the concentration of dopamine is much lower [58]. Concurrently, *in vitro* studies have shown that noradrenaline and adrenaline are secreted by frog adrenal slices and that the release of both catecholamines is significantly stimulated by depolarizing pulses of potassium [58].

The effect of catecholamines on corticosteroid secretion has been investigated by using a perifusion system technique [72, 74]. Dopamine induces a dose-dependent inhibition of corticosterone and aldosterone release ($ED_{50}=10^{-6}$ M). Although noradrenaline and adrenaline also inhibit corticosteroid output, their potency is 100 to 2,000 times lower than that of dopamine. Exposure of dispersed adrenal cells to dopamine results in a biphasic response consisting of a brief stimulation followed by a sustained inhibition (Fig. 1). The stimulatory phase is mediated by a D_1-like receptor subtype, while the inhibitory phase is caused by activation of a D2 receptor subtype [74]. Dopamine-induced inhibition of corticosteroid secretion is associated with a reduction of prostaglandin biosynthesis [71] and an inhibition of phosphatidylinositol breakdown [73], suggesting that, in the frog adrenal gland, the D_2 receptors are negatively coupled to both phospholipase A_2 and phospholipase C.

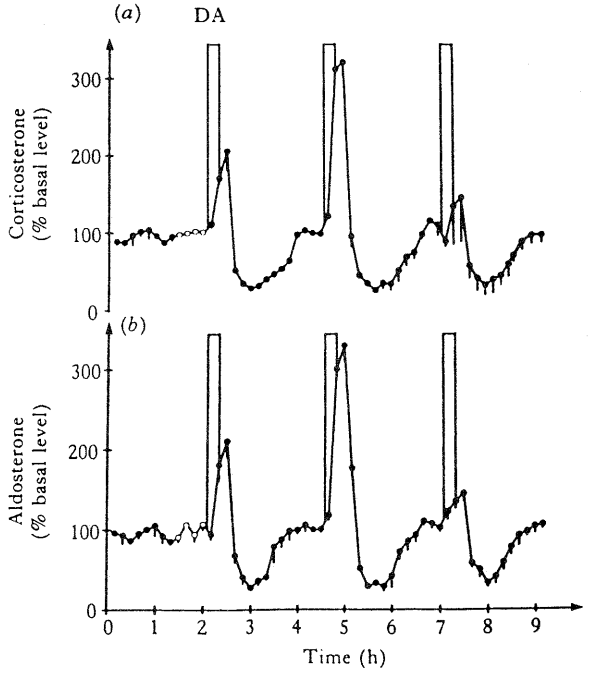

FIG. 1. Effect of equimolar doses of dopamine (50 μM) on the secretion of (a) corticosterone and (b) aldosterone by enzymatically dispersed frog adrenocortical cells. The profiles represent the mean ± S.E.M. secretion pattern of three independent experiments. Reproduced from [74] by permission from the Journal of Endocrinology Ltd.

In summary, catecholamines and more specifically dopamine, synthesized and released by chromaffin cells appear to regulate the secretion of steroids in adrenocortical cells through D_1- and D_2-like receptors.

Serotonin

The presence of serotonin in the adrenal gland has been demonstrated in several mammalian species including mouse [84], rat [38, 39, 94] and human [60]. In amphibians the occurrence of serotonin has been shown by autoradiography [28] and immunocytochemistry [18]. Double immunostaining studies have revealed that serotonin is present exclusively in PNMT-containing chromaffin cells [18], indicating that serotonin is colocalized with adrenaline. At the electron microscopic level, serotonin appears to be sequestered in chromaffin vesicles located at the periphery of the cells [18]. Serotonin has also been characterized in frog adrenal extracts by HPLC analysis combined with electrochemical detection (Fig. 2). The presence of large amounts of the oxidized metabolite 5-hydroxyindolacetic acid (5-HIAA) in the tissue extracts indicates that serotonin is rapidly metabolized in the adrenal tissue. The origin of serotonin in the frog adrenal gland has been investigated by pulse-chase techniques [21]. The ability of frog adrenal slices to convert L-tryptophan into serotonin demonstrates that the indoleamine is synthesized locally. It was also found that adrenal cells release substantial amounts of serotonin and possess a serotonin-uptake mechanism [21]. It thus appears that frog chromaffin cells, which have the ability to synthesize, release, uptake and degrade serotonin, behave like authentic serotonergic neurons [14].

In vitro studies have shown that serotonin is a potent stimulator of corticosteroid secretion in amphibians [19]. The fact that serotonin can stimulate corticosteroid release from acutely dispersed frog adrenal cells, a preparation in which the cytoarchitecture of the interrenal tissue is disrupted, indicates that the indoleamine exerts its stimulative effect through a direct action on adrenocortical cells [41]. Using various agonists and antagonists, it has been demonstrated that the action of serotonin is mediated through typical 5-HT_4 receptors whose activation produces an elevation of cAMP [15, 41, 42]. Interestingly, the stimulatory effect of

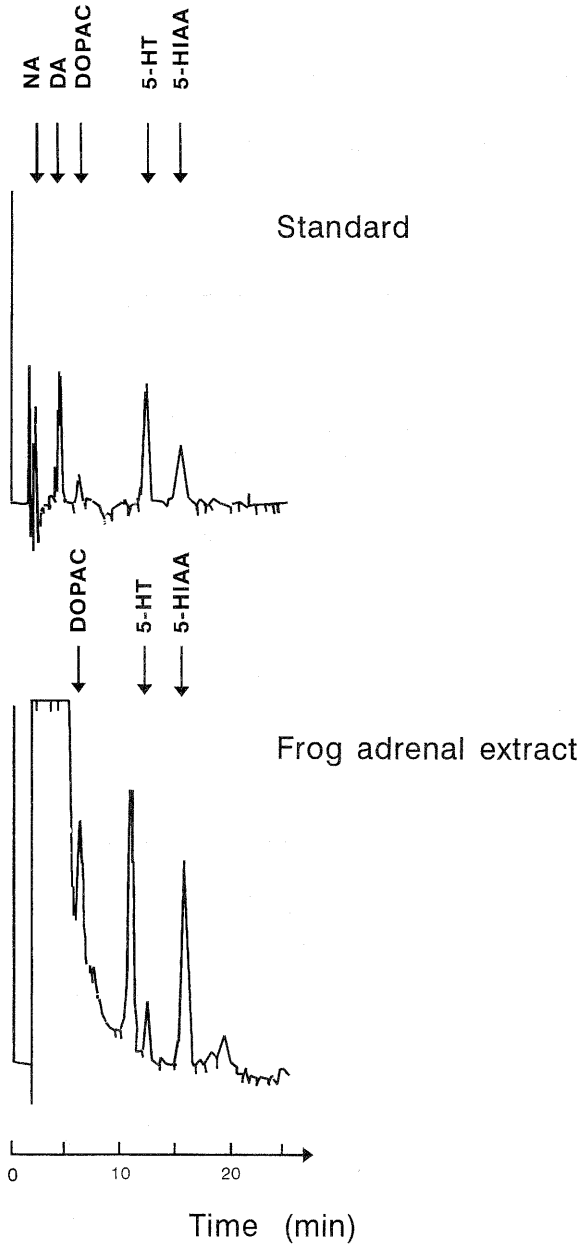

FIG. 2. Characterization of 5-HT in adrenal gland extracts by combining reversed-phase HPLC analysis and electrochemical detection. The tissue extract and the synthetic standards were chromatographed in the same conditions. Reproduced from [18] with permission from Elsevier Science Publishers BV.

serotonin on the human adrenal cortex is also mediated through 5-HT_4 receptors [60–62].

In summary, serotonin is synthesized, released and metabolized by frog chromaffin cells and the indoleamine stimulates steroid secretion in adrenocortical cells through activation of a 5-HT_4 receptor subtype positively coupled to adenylate cyclase.

Vasotocin

The neuropeptide arginine vasotocin causes stimulation of ACTH release from frog pituitary corticotrophs [92]. Concurrently, the presence of arginine vasopressin has been documented in the adrenal gland of rat [80], hamster, guinea pig, cow [34] and human [82]. The occurrence of vasotocin has been demonstrated in chromaffin cells of the frog adrenal gland by immunohistochemistry [49]. Labelling of consecutive sections with antisera against vasotocin and tyrosine-hydroxylase revealed that all chromaffin cells express vasotocin [49]. In contrast, no labelling of adrenal cells was observed using an antiserum against mesotocin. At the electron microscopic level, vasotocin-like immunoreactivity appears to be strictly contained in chromaffin granules [49], suggesting that the peptide can be released together with catecholamines during stress.

The possibility of a role of vasotocin in the regulation of amphibian adrenal steroidogenesis has been studied *in vitro*. Vasotocin was found to stimulate both corticosterone and aldosterone secretion from perifused frog adrenal slices [49, 51]. The other neurohypophysial nonapeptides, i.e. vasopressin, oxytocin and mesotocin, were also able to enhance corticosteroid secretion, but vasotocin was by far the most potent stimulator of steroidogenesis ($ED_{50}=5\times10^{-10}$ M). The use of various agonists and antagonists revealed that the action of vasotocin is mediated through receptors related to both mammalian V2 and oxytocin receptors [50]. The transduction pathway associated with the stimulative action of vasotocin involves both polyphosphoinositide breakdown [50] and cytosolic calcium mobilization [52] (Fig. 3).

In summary, vasotocin contained in chromaffin cells causes stimulation of corticosteroid secretion through activation of a V2/oxytocin receptor subtype positively coupled to phospholipase C.

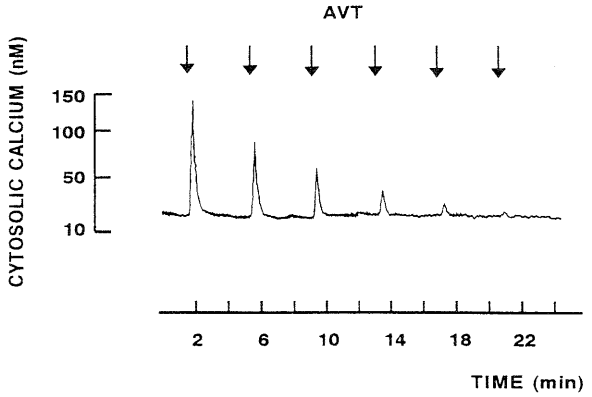

FIG. 3. Effects of repreated pulses (5 sec) of AVT (10^{-7} M) on $[Ca^{2+}]_i$ in adrenocortical cells. Arrows indicate the onset of AVT application. Reproduced from [52] with permission from the Endocrine Society.

Vasoactive intestinal peptide

Immunohistochemical studies have shown that frog chromaffin cells contain a peptide related to vasoactive intestinal peptide (VIP) [55]. At the electron microscope level, VIP appears to be sequestered in chromaffin vesicles [56]. VIP has recently been isolated from the frog brain and the

sequence of frog VIP is identical to that of chicken VIP [12].

VIP causes stimulation of corticosteroid secretion from perifused frog adrenal slices [55, 57]. Cytochalasin B suppresses the response of frog adrenal tissue to VIP, indicating the involvement of the microfilament network in the mechanism of action of the peptide [75]. The doses of VIP required to cause a significant effect on steroid secretion are fairly high. The weak potency of the peptide cannot be accounted for by species specificity since porcine and frog (chicken) VIP are equally active [55]. Whether VIP is involved in the acute control of corticosteroidogenesis or whether the peptide exerts modulatory effects therefore remains unknown. The fact that VIP stimulates growth of the mouse fetus [33] favours a trophic rather than a neuroendocrine role for this peptide.

NEUROENDOCRINE REGULATION OF ADRENOCORTICAL CELLS BY NERVE FIBERS

Acetylcholine

It is well known that the adrenal gland of mammals is innervated by cholinergic axons originating from the splanchnic nerve [16]. Whether cholinergic fibers are also present in the adrenal gland of amphibians is still debated. In particular, acetylcholinesterase could not be detected in the adrenal parenchyma of *Rana catesbeiana* [47]. In contrast, acetylcholinesterase activity has been observed in *Discoglossus pictus* [29] and *Triturus cristatus* [67].

Acetylcholine is a major stimulatory factor of chromaffin cells in mammals [96] whereas acetylcholine does not stimulate catecholamine secretion from the frog adrenal gland [58]. In contrast, acetylcholine is a potent stimulator of corticosteroid secretion in amphibians [6]. The effect of acetylcholine on frog adrenocortical cells is typically mediated through muscarinic receptors [6]. Several lines of evidence indicate that prostaglandins are involved in the mechanism of action of acetylcholine: (i) the effect of acetylcholine is blocked by indomethacin, a cyclooxygenase inhibitor; (ii) acetylcholine stimulates the synthesis of PGE_2 and PGI_2, and this effect precedes the increase in corticosteroid secretion [17]. In addition, the stimulatory effect of acetylcholine is totally blocked when the frog adrenal tissue is treated with cytochalasin B, indicating that microfilaments play a pivotal role in the steroidogenic response to acetylcholine [25]. Consistent with this observation, it has recently been shown that microfilaments are required for the incorporation of inositol into membrane phospholipids in the frog adrenal gland [26].

In summary, acetylcholine stimulates adrenocortical cells (but not chromaffin cells) in the frog adrenal gland, and the action of acetylcholine is mediated through muscarinic receptors positively coupled to phospholipase A_2 and/or phospholipase C activity.

Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide that results from alternative splicing of the primary transcript of the gene encoding calcitonin [2]. The structure of CGRP has recently been determined from frog brain and intestine extracts [12]. The sequence of frog CGRP shows only two amino acid substitutions (Val^{22}→Met and Gly^{23}→Ala) compared with chicken CGRP. Such a

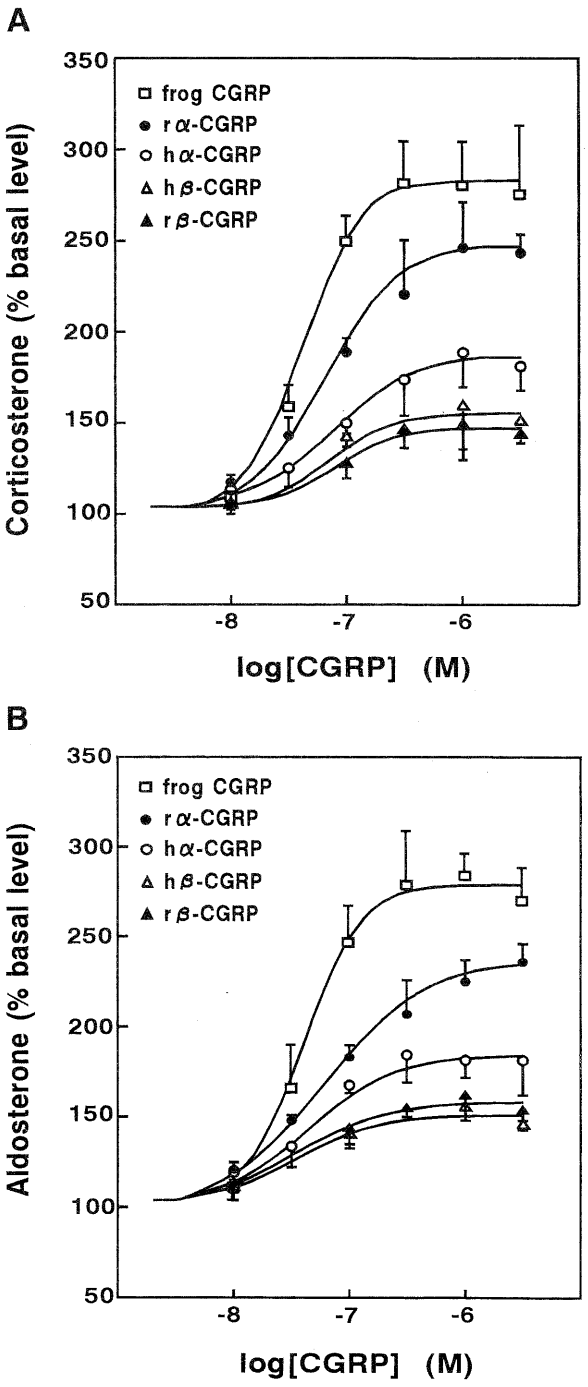

FIG. 4. Semilogarithmic plot comparing the effects of different forms of CGRP on the secretion of corticosterone (A) and aldosterone (B) by perifused frog adrenal slices. Graded concentrations of each neuropeptide were administered as 20-min pulses to perifused interrenal fragments. Reproduced from [24] with permission from the Endocrine Society.

high degree of conservation is consistent with important physiological roles for CGRP. The occurrence of CGRP has been described in chromaffin cells and nerve fibers in the rat adrenal gland [46, 48]. Using an antiserum against rat α-CGRP, the presence of a network of positive fibers has been recently detected in the adrenal gland of the frog *Rana ridibunda* [24]. The CGRP-like immunoreactivity is contained in varicose fibers contacting islets of interrenal cells and in thin fibers coursing in the walls of blood vessels. HPLC analysis of frog adrenal gland extracts revealed that endogenous CGRP co-eluted with synthetic frog CGRP, indicating that the immunoreactive peptide corresponds to mature CGRP [24].

The possible involvement of CGRP in the regulation of corticosteroid secretion from the frog adrenal gland has been investigated *in vitro*. Synthetic frog CGRP induces a dose-related stimulation of corticosterone and aldosterone secretion ($ED_{50}=10^{-8}$ M). The potency of rat and human α-CGRPs is slightly lower than that of frog CGRP ($ED_{50}=6.3\times10^{-8}$ M and 7.5×10^{-8} M, respectively). Rat and human β-CGRP are appreciably less efficient than frog α-CGRP in stimulating corticosteroidogenesis in frog (Fig. 4). Prolonged infusion of CGRP causes a rapid increase in corticosteroid release, followed by a gradual decline of steroid secretion, suggesting the occurrence of a desensitization phenomenon. CGRP also stimulates corticosterone and aldosterone secretion from enzymatically dispersed frog adrenocortical cells, indicating that the peptide exerts a direct stimulatory effect on corticosteroid secretion [24].

In summary, CGRP is contained in two types of fibers innervating the frog adrenal gland and the peptide seems to be involved in the local regulation of corticosteroidogenesis.

Tachykinins

Three molecular forms of tachykinins have recently been isolated in the frog *Rana ridibunda* (Fig. 5): (i) ranakinin (a substance P-related peptide), (ii) neurokinin B (NKB) whose structure is identical to that of mammalian NKB [81] and (iii) the neurokinin A (NKA)-related peptide ([Leu^3, Ile^7]NKA) [97]. Ranakinin and NKB are expressed in brain tissue whereas [Leu^3, Ile^7]NKA is found in the gut. Using antibodies against substance P and NKA, we have detected a dense network of positive fibers in the frog adrenal gland [59]. In contrast, no fibers could be detected using antibodies to NKB. Immunocytochemical studies at the ultrastructural level have shown that the immunoreactive tachykinins are sequestered in secretory vesicles, indicating that the peptides can be released in the vicinity of adrenal cells [59]. The immunoreactive peptides have been characterized by HPLC analysis and RIA detection: two major molecular forms, co-eluting respectively with synthetic ranakinin and [Leu^3, Ile^7]NKA, have been identified; NKB-like peptides have not been detected in frog adrenal extracts [59].

The effect of tachykinins on corticosteroid secretion has been studied using perifused frog adrenal slices. For concentrations ranging from 10^{-8} to 10^{-4} M, substance P induces a dose-dependent stimulation of corticosterone and aldosterone secretion [59]. In mammals, three types of tachykinin receptors, termed NK-1, NK-2 and NK-3, have been identified on the basis of their pharmacological properties and molecular characteristics ([35] and [53] for review). The NK-3-preferring agonist [Pro^7]NKB is the most effective stimulator of steroidogenesis. However, RK which is a dual agonist for mammalian NK-1/NK-2 receptors [5, 81] is also very active in stimulating corticosteroid secretion from the

								Phe	*X*	Gly	Leu	Met-NH_2
Substance P		Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met-NH_2
Ranakinin		Lys	Pro	Asp	Pro	Glu	Arg	Phe	Tyr	Gly	Leu	Met-NH_2
Physalaemin		pGlu	Ala	Asp	Pro	Asn	Lys	Phe	Tyr	Gly	Leu	Met-NH_2
NKA			His	Lys	Thr	Asp	Ser	Phe	Val	Gly	Leu	Met-NH_2
[Leu^3,Ile^7]NKA			His	Lys	Leu	Asp	Ser	Phe	Ile	Gly	Leu	Met-NH_2
Eledoisin		pGlu	Pro	Ser	Lys	Asp	Ala	Phe	Ile	Gly	Leu	Met-NH_2
Kassinin	Asp	Val	Pro	Lys	Ser	Asp	Gln	Phe	Val	Gly	Leu	Met-NH_2
NKB			Asp	Met	His	Asp	Phe	Phe	Val	Gly	Leu	Met-NH_2

FIG. 5. A comparison of the primary structures of different tachykinins.

frog adrenal gland [59]. The stimulatory effect of substance P is blocked by the cyclooxygenase inhibitor indomethacin, suggesting that arachidonic acid metabolites are involved in the mechanism of action of tachykinins in the frog adrenal gland. In support of this hypothesis, the substance P-induced stimulation of prostaglandin E_2 and prostacyclin release precedes by 10 min the increase in corticosteroid secretion [59]. Recent studies indicate that tachykinins do not directly stimulate adrenocortical cells [45]. In fact, tachykinins appear to stimulate adrenochromaffin cells which, in turn, release some corticotropic factor(s) that are responsible for the activation of corticosteroid secretion.

In summary, the frog adrenal gland is innervated by fibers containing two distinct tachykinins: ranakinin and [Leu^3, Ile^7]NKA. These peptides stimulate corticosteroid secretion through a novel type of receptor coupled to activation of the arachidonic acid cascade.

Atrial natriuretic factors

The term atrial natriuretic factors (ANF) designates a family of vasodilatory, diuretic and natriuretic peptides, which have been initially characterized in the mammalian atria [4, 88]. In atrial cardiocytes, ANF is stored as a 126 amino acid precursor polypeptide [91]. In contrast, the major biologically active and circulating form of ANF corresponds to the 28 amino acid C-terminal fragment of the precursor [90]. The presence of ANF-like peptides has been demonstrated in the heart and brain of *Rana ridibunda* [76–79] and *Hyla japonica* [27] and the primary structure of the peptide has been determined in *Rana ridibunda* [54] and *Rana catesbeiana* [86]. Specific ANF receptors have been characterized in bovine [22], rat [32] and frog adrenal gland [43, 44]. Immunohistochemical studies have shown the occurrence of beaded ANF-positive fibers innervating the frog adrenal gland parenchyma [64].

The possible role of ANF in the regulation of corticosteroid release from the frog adrenal gland has been studied *in vitro*. ANF does not modify the spontaneous secretion of corticosteroids but it significantly attenuates the stimulatory effect of ACTH and angiotensin II on corticosterone and aldosterone secretion [64].

In summary, both "hormonal" ANF supplied by cardiocytes and "neurohormonal" ANF released by nerve terminals in the vicinity of adrenocortical cells may modulate the response of frog interrenal tissue to various corticotropic stimuli.

Pituitary adenylate cyclase-activating polypeptide

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a recently discovered neuropeptide which belongs to the VIP/secretin/glucagon superfamily [70]. In mammals, PACAP exists in two amidated molecular forms with 38 (PACAP38) and 27 (PACAP27) amino acids. PACAP has been isolated from frog brain extracts and the structure of the peptide appears to be strikingly similar to that of its mammalian counterpart with only one amino acid substitution [11]. PACAP-immunoreactivity and PACAP-specific binding sites have been detected in the human and rat adrenal medulla [87, 89]. Immunohistochemical studies have shown that, in the frog, PACAP is present in fibers innervating the adrenal gland [99]. Specifically, two types of processes are found in the adrenal tissue: thick varicose fibers running between adrenal cells and thin processes located in the walls of the blood vessels irrigating the gland. Immunocytochemical data at the electron microscopic level indicate that PACAP-like-immunoreactivity is sequestered in electron-dense vesicles within nerve endings contacting both adrenocortical and chromaffin cells [100]. HPLC analysis of adrenal extracts combined with RIA quantification revealed that the predominant molecular form present in the frog adrenal tissue corresponds to PACAP38.

In vitro experiments have demonstrated that synthetic frog PACAP38 (fPACAP38) increases steroid secretion from frog adrenal slices in a dose-dependent fashion [101]. Interestingly, the peptide appears to be more potent in stimulating aldosterone ($ED_{50}=0.74\pm0.02\ \mu M$) than corticosterone release ($ED_{50}=2.16\pm0.04\ \mu M$). VIP, which is structurally related to PACAP, is about ten times less potent than fPACAP38 in elevating steroidogenesis while the [Des-His^1]-fPACAP38 analogue is 100 times less effective [99]. fPACAP38 causes stimulation of corticosteroid secretion from enzymatically dispersed adrenal cells, indicating that the peptide acts directly on adrenocortical cells to induce steroid release. Cytoautoradiography, performed on cultured adrenal cells, showed that both adrenocortical and chromaffin cells express PACAP-binding sites (Fig. 6). Biochemical characterization of the recognition sites revealed the occurrence of specific and high-affinity ($Kd=0.66\pm0.03$ nM) type I PACAP receptors [100]. In the frog adrenal gland, PACAP activates two second messenger systems: fPACAP38 provokes a dose-related increase in cAMP synthesis by adrenal slices and elevates cytosolic calcium levels in adrenocortical and chromaffin cells [100].

In summary, PACAP contained in nerve fibers innervating the frog adrenal gland, exerts a direct stimulative effect on adrenocortical cells. Concurrently, PACAP may also influence steroid secretion through stimulation of release of corticotropic factors from chromaffin paraneurons. In the frog adrenal tissue, PACAP stimulates the adenylate cyclase and the cytosolic calcium transduction pathways.

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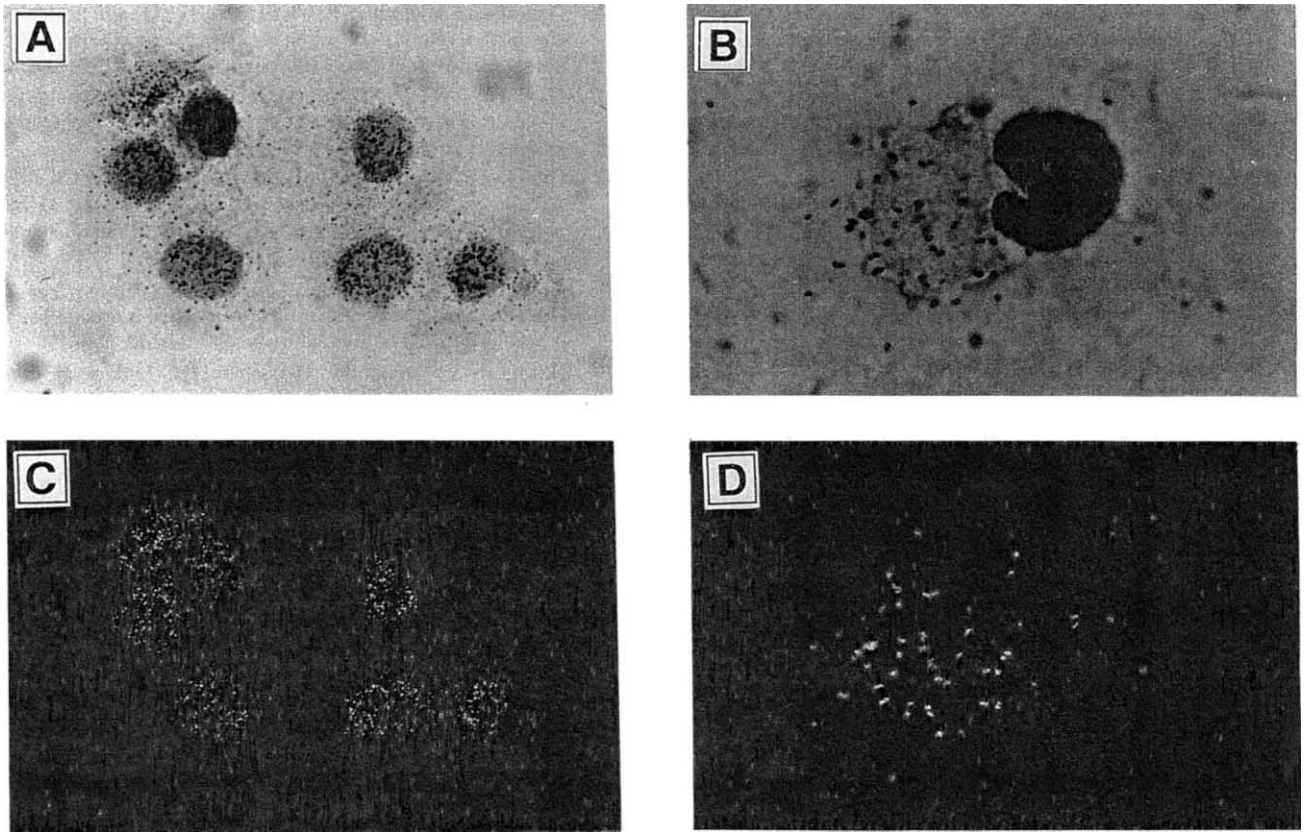

FIG. 6. Visualization by cytoautoradiography of [^{125}I]PACAP27 binding sites on cultured adrenal cells. A and B, Photomicrographs of a cluster of adrenocortical cells (A) and a chromaffin cell (B) stained with toluidine blue, showing a dense accumulation of silver grains over the cells (magnification, ×850 and ×1500, respectively). C and D, Darkfield illumination micrographs of the cells shown in A and B, respectively. Reproduced from [100] with permission from the Endocrine Society.

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