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Differential Activation of Enkephalin, Galanin, Somatostatin, NPY, and VIP Neuropeptide Production by Stimulators of Protein Kinases A and C in Neuroendocrine Chromaffin Cells

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Abstract

Neuropeptides function as peptide neurotransmitters and hormones to mediate cell-cell communication. The goal of this study was to understand how different neuropeptides may be similarly or differentially regulated by protein kinase A (PKA) and protein kinase C (PKC) intracellular signaling mechanisms. Therefore, this study compared the differential effects of treating neuroendocrine chromaffin cells with stimulators of PKA and PKC on the production of the neuropeptides (Met)enkephalin, galanin, somatostatin, NPY, and VIP. Significantly, selective increases in production of these neuropeptides was observed by forskolin or PMA (phorbol myristate acetate) which stimulate PKA and PKC mechanisms, respectively. (Met)enkephalin production was stimulated by up to 2-fold by forskolin treatment, but not by PMA. In contrast, PMA treatment (but not forskolin) resulted in a 2-fold increase in production of galanin and somatostatin, and a 3-fold increase in NPY production. Notably, VIP production was highly stimulated by forskolin and PMA, with increases of 3-fold and 10–15-fold, respectively. Differences in elevated neuropeptides occurred in cell extracts compared to secretion media, which consisted of (i) increased NPY primarily in cell extracts, (ii) increased (Met)enkephalin and somatostatin in secretion media (not cell extracts), and (iii) increased galanin and VIP in both cell extracts and secretion media. Involvement of PKA or PKC for forskolin or PMA regulation of neuropeptide biosynthesis, respectively, was confirmed with direct inhibitors of PKA and PKC. The selective activation of neuropeptide production by forskolin and PMA demonstrates that PKA and PKC pathways are involved in the differential regulation of neuropeptide production.

Keywords

neuropeptide biosynthesis; enkephalin; galanin; somatostatin; NPY; VIP; forskolin; PMA; protein kinase; regulation; chromaffin cells

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Introduction

Peptide neurotransmitters and hormones, collectively known as neuropeptides, function in the nervous and endocrine systems to mediate cell-cell communication that is required for biological control. Neuropeptides mediate activity-dependent neurotransmission of information among neuronal circuits, and function as endocrine regulators of target cellular and organ systems (Krieger et al., 1983). Neuropeptides possess diverse physiological actions defined by their unique primary sequences, which includes the enkephalin opioid peptide that regulates analgesia (Gustein and Akil, 2006; Law et al., 2000) and immune functions (Hedner and Cassuto, 1987; Owens and Smith, 1987), galanin which regulates cognition (Steiner et al., 2001; Robinson, 2004), neuropeptide Y (NPY) which participates in regulating feeding behavior and blood pressure (Ramos et al., 2005; Waeber et al., 1990), somatostatin which mediates growth regulation (Norris, 1997), and vasoactive intestinal peptide (VIP) that regulates immune functions (Gomariz et al., 2001). Knowledge of the regulatory mechanisms that control the biosynthesis of neuropeptides is important for understanding biological control mechanisms that influences the physiological functions of neuropeptides.

Neuropeptides are produced and stored within secretory vesicles that undergo regulated secretion upon stimulation of neuroendocrine cells by receptor-mediated mechanisms. Intracellular second messenger signaling mechanisms mediate the regulation of neuropeptide production to replace intracellular stores of these biologically active peptides as they are secreted. Notably, the protein kinase A (PKA) and protein kinase C (PKC) pathways are known to regulate neuropeptide secretion (Sirianni et al., 1999; Giraud et al., 1991; Rokaeus et al., 1990; Magni and Barnea, 1992; Vlotides et al., 2004). Because neuropeptide secretion is linked to cellular production of neuropeptides, it is likely that PKA and PKC pathways may regulate neuropeptide production. Furthermore, comparison of PKA and PKC mechanisms in regulating neuropeptides is important for assessing whether similar or distinct PKA or PKC pathways regulate neuropeptide biosynthesis.

Therefore, the goal of this study was to evaluate the differential regulation of several neuropeptides during activation of PKA and PKC in neuroendocrine chromaffin cells in primary culture (prepared from bovine adrenal medulla). Cellular levels of the neuropeptides (Met)enkephalin, galanin, somatostatin, NPY, and VIP were assessed after treating chromaffin cells with forskolin or PMA (phorbol myristate acetate) that result in cellular activation of PKA and PKC, respectively. Activation of PKA results from forskolin stimulation of adenylate cyclase (Seamon et al., 1983) that catalyzes the production of cAMP, which stimulates cAMP-dependent PKA (Meinkoth et al., 1993; Lodish et al., 2000). Cellular PKC is activated by PMA (phorbol myristate acetate) that is known to directly stimulate PKC (Goel et al., 2007).

Results from this study illustrated three distinct, selective modes for PKA and PKC regulation of neuropeptide production. Total (Met)enkephalin production was stimulated primarily by forskolin, rather than by PMA. However, production of total cellular galanin, somatostatin, and NPY was preferentially stimulated by PMA, compared to forskolin. VIP production was extensively stimulated by both forskolin and PMA. Differences were observed in the location of increased neuropeptide with respect to changes in neuropeptide levels in cell extracts or secretion media. These novel findings demonstrate the selective regulation of neuropeptide production by PKA compared to PKC pathways in neuroendocrine chromaffin cells.

Experimental Procedures

Treatment of chromaffin cells in primary culture with forskolin or PMA, combined with inhibitors of PKA or PKC

Primary cultures of bovine chromaffin cells were prepared from fresh adrenal medulla as described previously (O'Connor et al., 2007), plated at 4×10^5 cells/well to 2×10^6 cells/well (6-well plate). Experiments were conducted after 3–5 days in culture. Cell cultures at this time showed consistent numbers of cells/well, assessed by protein determination.

Experiments were conducted by treating cells with forskolin (50 μ M) or PMA (phorbol myristate acetate, 100 nM) for 48 and 72 hours in complete culture media (O'Connor et al., 2007); controls included incubation with vehicle control (0.001% DMSO). In addition, forskolin-treated cells were incubated with or without the protein kinase A inhibitor, KT-5720 (10 μ M) (Hidaka and Kobayashi, 1992; Haddad et al., 2005), and PMA-treated cells were incubated with or without the protein kinase C inhibitor, bisindolylmaleimide I (Bis I) (Hidaka and Kobayashi, 1992; Langford et al., 2005), for 72 hours.

At the end of each treatment period, cells were harvested and prepared for neuropeptide analyses, achieved by lysing cells in 0.1 N acetic acid that was prepared as an acid extract, as we have described previously (Miller et al., 2003). In addition, culture media was collected for measurement of neuropeptides secreted during the treatment period. The cell culture media included bacitracin (10 μ M) as protease inhibitor. Total neuropeptide production during the treatment period was measured as the combined sum of neuropeptides in cell extracts and culture media.

Each experiment utilized replicate cell samples (triplicate), and experiments were conducted at last three times. Results are expressed as the amount of total neuropeptide measured from cell extracts and media per well, and expressed as the mean $\bar{x} \pm$ s.e.m., with evaluation of statistical significance (student's t-test, with $p < 0.05$ for significance).

It was noted that there was some variability among primary chromaffin cell preparations on the amounts of neuropeptides measured. Such variation is quite common for commercial sources of *in vivo* tissues. This may be due to different groups of animals, specific conditions for dissection of the fresh tissue by the commercial vendor and transportation.

Measurement of (Met)enkephalin, galanin, somatostatin, NPY, and VIP in chromaffin cell samples

Specific radioimmunoassays (RIAs) were utilized to measure levels of (Met)enkephalin, galanin, somatostatin-28, neuropeptide Y (NPY1-36), and VIP (vasoactive intestinal polypeptide) in chromaffin cell extracts and secretion media. The RIA procedure for (Met)enkephalin was performed as described previously (Yasothornsrikul et al., 2003). RIAs for NPY, somatostatin-28, and galanin utilized RIA kits and protocols from Peninsula Laboratories (San Carlos, CA). The RIA for VIP utilized a kit from Phoenix Pharmaceuticals, Inc. (Burlingame, CA). These RIA assays were highly sensitive with detection limits of 1–2 pg for each of these neuropeptides. In addition, control assays showed that the secretion media (with serum) had no effect on the standard curves for these neuropeptides.

Results

Selective elevation of total (Met)enkephalin production by forskolin: comparison in cell extracts and secretion media

Treatment of chromaffin cells with forskolin, which activates cAMP production by adenylate cyclase and activates cAMP-dependent protein kinase A (PKA), resulted in elevation of (Met)enkephalin production. Total (Met)enkephalin (sum of enkephalin content in cell extracts and secretion media) was increased by approximately 2-fold after 72 hours treatment with forskolin, and was increased by 50% after 48 hours forskolin treatment (fig. 1). However, PMA, a stimulator of protein kinase C (PKC) had little effect on (Met)enkephalin levels (fig. 1). These results demonstrate that (Met)enkephalin production in chromaffin cells is selectively increased by forskolin, compared to PMA.

Analyses of the effects of forskolin and PMA in cell extracts compared to secretion media showed that PMA increased (Met)enkephalin in the secretion media (Table 1). After forskolin treatment, (Met)enkephalin in the secretion media was elevated 2–3 fold, and a modest increase of (Met)enkephalin in the cell extract was observed. PMA had minimal effects on (Met)enkephalin. These results demonstrate that forskolin-induced stimulation of (Met)enkephalin production was detected primarily in the secretion media.

Selective elevation of total galanin, somatostatin, and NPY production by PMA: comparison in cell extracts and secretion media

In contrast to the selective elevation of (Met)enkephalin by forskolin (and not by PMA), total amounts of the neuropeptides galanin, somatostatin, and NPY were selectively increased by PMA. Total galanin production was increased after treatment of cells with PMA, resulting in a 2-fold increase in cellular galanin levels after 48 hr or 72 hr PMA treatment (fig. 2). However, forskolin treatment had little effect on cellular galanin levels (fig. 2). PMA also increased cellular levels of somatostatin by 2-fold after 48 hr PMA treatment; after 72 hr PMA treatment, somatostatin levels were also increased (fig. 3). These findings demonstrate significant increases in galanin and somatostatin that were induced by PMA, but not by forskolin.

While PMA increased both total galanin and somatostatin by approximately two-fold, differences in increased neuropeptides in either cell extracts or secretion media were found. PMA increased galanin in both the cell extract and secretion media (Table 2). However, the increased somatostatin induced by PMA was observed in the secretion media, but not in the cell extract (Table 3). These results indicate differences in PMA-stimulated production of galanin compared to somatostatin with respect to their increases in cell extracts compared to secretion media.

Total NPY levels in chromaffin cells were increased by approximately 3-fold after 72 hr treatment with PMA (fig. 4); at 48 hours PMA treatment NPY was not yet altered. There was a modest elevation in NPY levels after 72 hours forskolin treatment (fig. 4). Analyses of cell extracts and secretion media showed that PMA and forskolin increased NPY in the secretion media (Table 4).

Evaluation of PMA-stimulation of the production of galanin, somatostatin, and NPY illustrated differences in the neuropeptide increases residing in cell extracts or secretion media. Three different stimulated conditions were observed consisting of PMA stimulation of (1) increased galanin in both cell extracts and secretion media, (2) increased somatostatin and NPY primarily in the secretion media. Apparently, each neuropeptide system may be uniquely regulated by PMA.

VIP production is substantially stimulated by PMA, as well as by forskolin

Among the neuropeptides examined in this study, VIP production was substantially increased by both forskolin and PMA (fig. 5). VIP production was stimulated by approximately 3-fold after forskolin treatment of cells for 48 hr or 72 hr (fig. 5). Notably, VIP production was highly increased by 10- to 15-fold by PMA treatment for 48 hr or 72 hr (fig. 5). PMA stimulation of VIP occurred in both cell extracts and secretion media (Table 5). These dramatic increases in VIP demonstrate that its production is notably stimulated by PMA.

Inhibitors demonstrate involvement of PKA and PKC in the regulation of neuropeptide production

Stimulation of (Met)enkephalin production by forskolin was predicted to involve protein kinase A (PKA), since forskolin is known to activate adenylate cyclase that results in increased production of cAMP that stimulates cAMP-dependent protein kinase A (PKA). To explore the role of PKA in forskolin stimulation of (Met)enkephalin, the effect of the PKA inhibitor KT-5720 was tested. Treatment with KT-5720 resulted in blockade of forskolin-induced increases in (Met)enkephalin production (fig. 6). These results support a role for PKA in mediating forskolin stimulation of (Met)enkephalin production.

PMA stimulation of neuropeptide production suggests involvement of protein kinase C (PKC) activation by PMA. Therefore, direct inhibition of PKC by bisindolylmaleimide (Bis I) (Hidaka and Kobayashi, 1992; Davies et al., 2000) was tested for its effects on PMA stimulation of neuropeptide levels in chromaffin cells. Treatment of cells with Bis I resulted in nearly complete inhibition of PMA-stimulation of galanin, NPY, and VIP production (fig. 7). These data provide support for the role of PKC in mediating PMA stimulation of NPY, galanin, and VIP neuropeptide production in chromaffin cells.

Discussion

Differential stimulation of neuropeptide production in neuroendocrine chromaffin cells occurred with treatment with forskolin compared to PMA (phorbol myristate acetate), which stimulate protein kinase A (PKA) and protein kinase C (PKC), respectively. Total (Met)enkephalin production was stimulated by up to 2-fold by forskolin, but PMA had no effect. In contrast, production of total galanin, somatostatin, and NPY was stimulated by PMA, but forskolin had little effect. Moreover, total VIP production was dramatically increased by 3-fold and 10–15-fold by treatment of cells with forskolin and PMA, respectively. Differences in elevated neuropeptides occurred in cell extracts compared to secretion media, which consisted of (i) increased NPY primarily in cell extracts, (ii) increased (Met)enkephalin and somatostatin in only secretion media (not cell extracts), and (iii) increased galanin and VIP in both cell extracts and secretion media. Involvement of PKA or PKC for forskolin or PMA regulation of neuropeptide biosynthesis, respectively, was confirmed with direct inhibitors of PKA and PKC. These novel findings demonstrate the differential regulation of neuropeptide production by PKA compared to PKC pathways in neuroendocrine chromaffin cells.

The release of neuropeptides from neuroendocrine cells occurs via receptor-stimulated secretion during requirements for peptide neurotransmission in the nervous system, and peptide hormone actions in the endocrine system. For example, PACAP (pituitary adenylate cyclase-activating polypeptide) regulates enkephalin and VIP in adrenal medulla (Hamelink et al., 2002). Receptor-mediated stimulation of neuropeptide secretion and production involves activation of protein kinase A (PKA) and protein kinase C (PKC) signal transduction mechanisms in many neuroendocrine cell types. It was, therefore, of interest in this study to assess similar or differential PKA and PKC mechanisms for mediating increased production of several different neuropeptides in chromaffin cells.

Chromaffin cells have provided an excellent model for elucidating mechanisms involved in neuropeptide production and secretion (Hook et al., 1994; Hook et al., 2007). Short-term stimulation of neuropeptide secretion by KCl depolarization or nicotine activation of the nicotinic cholinergic receptor results in regulated secretion of neuropeptides, representing a small fraction of cellular neuropeptides. However, long-term stimulation of chromaffin cells with forskolin or PMA in this study (48–72 hrs) results in substantial increases in neuropeptide production, with a large portion of secreted neuropeptides. The relative amounts of secreted neuropeptides depends on the stimulating secretagogues and their time of incubation with chromaffin cells. Importantly, results from this study indicate differential regulation of neuropeptide production through PKA, PKC, or both PKA and PKC pathways.

Neuropeptides are synthesized by proteolytic processing of inactive proneuropeptide precursors. Therefore, it is possible that forskolin and PMA, via PKA and PKC, may regulate protease pathways that participate in the biosynthesis of active neuropeptides. Two distinct protease pathways have, thus far, been demonstrated for proneuropeptide processing. These pathways consist of the subtilisin-like prohormone convertases combined with carboxypeptidase E, and the more recently identified cysteine protease pathway consisting of secretory vesicle cathepsin L and aminopeptidase B (Hook et al., 2007; Hook et al., 2004). Earlier studies found that forskolin stimulates the cathepsin L cysteine protease complex, known as ‘prohormone thiol protease’ (PTP), during increased production of (Met)enkephalin in chromaffin cell (Tezapsidis et al., 1995). Thus, it is known that PKA may be involved in stimulating proteolytic processing of a proneuropeptide to enhance production of the smaller, active neuropeptide.

Cellular production of neuropeptides also requires expression of the proneuropeptide genes, whose primary mRNA transcripts undergo protein translation to generate proneuropeptide precursors. PKA and PKC are known to regulate gene expression through nuclear transcriptional mechanisms. It is, therefore, possible that PKA and PKC regulation of neuropeptide production may involve control of proneuropeptide gene expression. Indeed, the neuropeptides examined in this study – (Met)enkephalin, galanin, somatostatin, NPY, and VIP – utilize PKA- or PKC-mediated transcriptional regulators to control proneuropeptide gene expression (Tezapsidis et al., 1995; Suh et al., 1995; Rokaeus et al., 1990; Montminy et al., 1986; Magni et al., 1998; Williams et al., 1998; Hahm and Eiden, 1999). It will be fruitful in future studies to investigate the coordinate regulation of proneuropeptide gene expression and proneuropeptide processing to gain knowledge of how PKA and PKC may influence transcriptional and post-translational regulatory mechanisms in the control of neuropeptide biosynthesis.

Regulation of neuropeptide production in chromaffin cells may have profound physiological effects. Adrenal medullary neuropeptides are known to regulate catecholamine secretion from adrenal medulla. VIP stimulates the secretion of catecholamines from the adrenal gland (Malhotra and Wakade, 1987; Wakade et al., 1991). Somatostatin potentiates acetylcholine-induced catecholamine release from adrenal medullary cells (Ribeiro et al., 2006). NPY regulates catecholamine release evoked by interleukin-1 β in chromaffin cells, and regulates catecholamine secretion under physiological conditions (Rosmaninho-Salgado et al., 2007; Spinazzi et al., 2005). Furthermore, adrenomedullary galanin regulates glucocorticoid secretion from adrenal cortex (Andreis et al., 2007). Enkephalins released from adrenal medulla regulate immunological functions (Hedner and Cassuot, 1987; Owens and Smith, 1987). These physiological functions of adrenal medullary neuropeptides implicate their possible *in vivo* regulation by protein kinase A and C mechanisms.

These data demonstrate that the chromaffin cells differentially regulate the amount of each neuropeptide produced which may allow selective regulation of the amount of neuropeptides

produced and secreted for their physiological effects. The different amounts of each neuropeptide synthesized by chromaffin cells leads to secretion of galanin, somatostatin, NPY, and VIP into plasma for selective regulation of their respective physiological functions. These physiological functions may consist of galanin regulation of glucocorticoid secretion from adrenal cortex, somatostatin control of acetylcholine-induced catecholamine release from adrenal medulla, NPY and VIP regulation of catecholamine release, and enkephalin regulation of the immune system.

In summary, the production of diverse neuropeptides may be differentially regulated by PKA and PKC mechanisms, as demonstrated in this study. Notably, the production of specific neuropeptides may be regulated the PKA or PKC pathways, or by both of these kinase pathways. It will be of interest in future studies to examine the components and targets of the PKA and PKC pathways that participate in the control of neuropeptide production that is involved in physiological peptidergic functions.

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Figure 1. Selective stimulation of (Met)enkephalin production induced by forskolin, but not by PMA

Chromaffin cells in primary culture (4×10^5 cells/well) were treated with forskolin or PMA for 48 hr or 72 hr. Total cellular (Met)enkephalin was then measured by RIA as the sum of enkephalin in cell extracts and media. Data are shown as (Met)enkephalin in control (C), forskolin-treated (F), and PMA-treated (P) cells, indicated as the mean $\pm$ s.e.m from triplicate wells (this experiment was repeated three times). Statistical significance is indicated by * $p < 0.05$ (by student's t-test). Results show that forskolin, but not PMA, increased (Met)enkephalin production.

Figure 2. Selective elevation of galanin production induced by PMA, but not by forskolin
 Galanin was measured (by RIA) in chromaffin cells treated with forskolin or PMA for 48 hr and 72 hr (4×10^5 cells/well). Total cellular galanin was calculated as the sum of galanin content in cell extracts and media. Results are shown as galanin levels in control (C), forskolin-treated (F), and PMA-treated (P) cells, as the mean $\pm$ s.e.m. from triplicate wells (the experiment was repeated three times). Statistical significance is indicated by * $p < 0.005$ (by students' t-test) of treated cells compared to untreated controls. Results indicated elevation of galanin by PMA, but not by forskolin.

Figure 3. Elevated somatostatin production induced by PMA, but not by forskolin

Somatostatin was measured after treatment of chromaffin cells with forskolin or PMA for 48 hr and 72 hr (4×10^5 cells/well). Total cellular somatostatin was measured as the sum of its content in cell extracts and media. Results indicate somatostatin in control (C), forskolin-treated (F), and PMA-treated (P) cells, shown as the mean $\pm$ s.e.m. from triplicate wells (the experiment was repeated three times). Statistical significance is indicated by * $p < 0.05$ (students t-test). Results show increased somatostatin production after PMA treatment.

Figure 4. Elevated NPY production induced by PMA

Total NPY was measured in chromaffin cells after treatment of cells with forskolin or PMA for 48 hr and 72 hr (4×10^5 cells/well). Results are shown as total cellular NPY (cell extract plus media) in control (C), forskolin-treated (F), and PMA-treated (P) cells, expressed as the mean $\pm$ s.e.m. from triplicate wells (the experiment was repeated three times). Results show significant increases in NPY with $*p < 0.05$ (by student's t-test). NPY production was increased by PMA, with lesser effects by forskolin.

Figure 5. Stimulation of VIP production by both forskolin and PMA

VIP was measured in chromaffin cells after treatment of cells for 48 hr and 72 hr with forskolin or PMA (4×10^5 cells/well). Results are shown as total cellular VIP (sum of content in cell extract and media) in control (C), forskolin-treated (F), and PMA-treated (P) cells. Data are indicated as the mean $\pm$ s.e.m. from triplicate wells (the experiment was repeated three times). Results show significant elevation of cellular VIP by forskolin and PMA with $*p < 0.005$ (by student's t-test).

Figure 6. The PKA inhibitor, KT-5720, blocks forskolin stimulation of (Met)enkephalin production Chromaffin cells were treated with KT-5720 (10 μ M), an inhibitor of protein kinase A, in the presence or absence of forskolin for 72 hrs, and total cellular (Met)enkephalin was measured (as described in the methods) (2×10^6 cells/well). Results are shown as total (Met)enkephalin in control (C) and forskolin-treated (F) cells, with or without KT-5720. Significant results show that KT-5720 blocked forskolin-induced elevation of cellular (Met)enkephalin, with * $p < 0.05$ (by student's t-test).

Figure 7. The PKC inhibitor, bisindolylmaleimide (Bis), blocks PMA stimulated production of galanin, NPY, and VIP

Chromaffin cells were treated with bisindolylmaleimide (Bis) (10 μM) in the presence or absence of PMA for 72 hrs (2×10^6 cells/well), and total cellular galanin, NPY, and VIP were measured by RIA assays as described in the methods. Results are shown for each neuropeptide in control (C) and PMA-treated (P) cells, with or without Bis inhibitor. Significant results show that Bis blocked PMA-induced increases in galanin, NPY, and VIP, with $*p < 0.05$ (by student's t-test).

Table 1
(Met)enkephalin in Chromaffin Cell Extracts and Secretion Media During Treatment with Forskolin and PMA

Cell Condition	(Met)enkephalin (pg/μg protein)		
	Cell Extract	Secretion Media	Total in Cells and Media
48 hrs treatment:			
Control	10.3±0.7	19.4±1.6	29.7±2.4
Forskolin	12.8±1.6	32.6±0.2*	45.4±1.9*
PMA	12.7±1.3	19.3±0.7	32.7±2.0
72 hrs treatment:			
Control	8.8±1.3	19.1±1.8	27.9±3.1
Forskolin	10.4±1.2	50.9±0.7*	61.2±8.8*
PMA	8.6±0.4	26.2±1.7*	34.9±2.1

Chromaffin cells in primary culture were treated with forskolin or PMA (phorbol myristate acetate) for 48 hr or 72 hr (from figure 1). Cells were harvested by collecting the secretion media, and cell extracts were prepared as described in the methods. The content of (Met)enkephalin in samples was measured by radioimmunoassay, shown as the mean + s.e.m. Statistical significance was calculated for the 'total' (Met)enkephalin represented as the sum in cell extracts and secretion media, and significance is indicated by * $p < 0.05$ (by student's t-test).

Table 2
Galanin in Chromaffin Cell Extracts and Secretion Media During Treatment with Forskolin and PMA

Cell Condition	Galanin (pg/μg protein)		
	Cell Extract	Secretion Media	Total in Cells and Media
48 hrs treatment:			
Control	30.2±0	23.0±2.1	53.2±2.1
Forskolin	28.9±1.1	34.0±1.7	63.0±2.8
PMA	44.3±1.3*	61.7±5.7*	106.0±7.1*
72 hrs treatment:			
Control	27.5±0	24.1±3.7	51.6±3.7
Forskolin	26.1±2.9	26.2±3.4	52.4±6.3
PMA	42.4±0.8*	67.2±2.1*	110.0±3.0*

Chromaffin cells in primary culture were treated with forskolin or PMA (phorbol myristate acetate) for 48 hr or 72 hr (from figure 2). Cells were harvested by collecting the secretion media, and cell extracts were prepared as described in the methods. The content of galanin in samples was measured by radioimmunoassay, shown as the mean ± s.e.m. Statistical significance was calculated for the 'total' galanin represented as the sum in cell extracts and secretion media, and significance is indicated by *p < 0.05 (by student's t-test).

Table 3
Somatostatin in Chromaffin Cell Extracts and Secretion Media During Treatment with Forskolin and PMA

Cell Condition	Somatostatin (pg/μg protein)		
	Cell Extract	Secretion Media	Total in Cells and Media
48 hrs treatment:			
Control	0.021±0.001	0.40±0.06	0.42±0.07
Forskolin	0.019±0.360	0.36±0.01	0.38±0.01
PMA	0.022±0.001	0.83±0.11*	0.85±0.12*
72 hrs treatment:			
Control	0.022±0.002	0.52±0.02	0.54±0.03
Forskolin	0.021±0.003	0.35±0.03	0.37±0.03
PMA	0.018±0.003	0.83±0.08*	0.85±0.08*

Chromaffin cells in primary culture were treated with forskolin or PMA (phorbol myristate acetate) for 48 hr or 72 hr (from figure 3). Cells were harvested by collecting the secretion media, and cell extracts were prepared as described in the methods. The content of somatostatin in samples was measured by radioimmunoassay, shown as the mean ± s.e.m. Statistical significance was calculated for the 'total' somatostatin represented as the sum in cell extracts and secretion media, and significance is indicated by * $p < 0.05$ (by student's t-test).

Table 4
Neuropeptide Y (NPY) in Chromaffin Cells Extracts and Secretion Media During Treatment with Forskolin and PMA

Cell Condition	NPY (pg/ μ g protein)		
	Cell Extract	Secretion Media	Total in Cells and Media
48 hrs treatment:			
Control	5.8 $\pm$ 0.7	26.7 $\pm$ 1.0	32.6 $\pm$ 1.7
Forskolin	3.6 $\pm$ 0.6	25.7 $\pm$ 2.3	29.3 $\pm$ 2.9
PMA	17.2 $\pm$ 0.3*	26.4 $\pm$ 1.0	43.6 $\pm$ 1.3*
72 hrs treatment:			
Control	17.9 $\pm$ 2.4	42.2 $\pm$ 3.3	60.1 $\pm$ 1.1
Forskolin	16.3 $\pm$ 0.3	90.2 $\pm$ 4.8*	106 $\pm$ 4.9*
PMA	9.9 $\pm$ 1.7	151.0 $\pm$ 7.4*	161 $\pm$ 7.8*

Chromaffin cells in primary culture were treated with forskolin or PMA (phorbol myristate acetate) for 48 hr or 72 hr (from figure 4). Cells were harvested by collecting the secretion media, and cell extracts were prepared as described in the methods. The content of NPY in samples was measured by radioimmunoassay, shown as the mean $\pm$ s.e.m. Statistical significance was calculated for the 'total' NPY represented as the sum in cell extracts and secretion media, and significance is indicated by * $p < 0.05$ (by student's t-test).

Table 5
 Vasoactive Intestinal Polypeptide (VIP) in Chromaffin Cells Extracts and Secretion Media During Treatment with Forskolin and PMA

Cell Condition	VIP (pg/ μ g protein)		
	Cell Extract	Secretion Media	Total in Cells and Media
48 hrs treatment:			
Control	1.9 $\pm$ 0.4	1.8 $\pm$ 0.1	3.7 $\pm$ 0.1
Forskolin	6.7 $\pm$ 0.6*	5.0 $\pm$ 0.3*	11.7 $\pm$ 0.9*
PMA	27.3 $\pm$ 1.1*	12.2 $\pm$ 2.3*	39.6 $\pm$ 3.5*
72 hrs treatment:			
Control	2.3 $\pm$ 0.3	2.5 $\pm$ 0.1	4.8 $\pm$ 0.1
Forskolin	6.3 $\pm$ 0.6*	6.2 $\pm$ 0.3*	12.6 $\pm$ 0.9*
PMA	33.0 $\pm$ 2.6*	16.2 $\pm$ 3.8*	49.2 $\pm$ 6.4*

Chromaffin cells in primary culture were treated with forskolin or PMA (phorbol myristate acetate) for 48 hr or 72 hr (from figure 5). Cells were harvested by collecting the secretion media, and cell extracts were prepared as described in the methods. The content of VIP in samples was measured by radioimmunoassay, shown as the mean $\pm$ s.e.m. Statistical significance was calculated for the 'total' VIP represented as the sum in cell extracts and secretion media, and significance is indicated by *p < 0.05 (by student's t-test).