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Involvement of multiple signaling pathways in PACAP-induced EM66 secretion from chromaffin cells

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1. Introduction

The secretory granules of adrenal medullary chromaffin cells contain a high concentration of soluble peptides and proteins, that are co-released with catecholamines into the circulation in response to splanchnic nerve stimulation. Among

the proteins stored in the chromaffin granules are the chromogranins (Cgs) which constitute a family encompassing chromogranin A (CgA), chromogranin B (CgB) and secretogranin II (SgII). Cgs are characterized by a high proportion of acidic amino acids and by the presence of multiple pairs of basic residues which represent potential cleavage sites for prohormone convertases (PC), suggesting that Cgs may serve as precursors to generate biologically active peptides. Indeed, several CgA- and CgB-derived peptides, including vasostatin, chromostatin, pancreastatin, parastatin, chromacin, catestatin and secretolytin have been shown to exert biological activities [1]. Concerning SgII, a 33-amino acid peptide named secretoneurin (SN) [2] has been shown to be implicated in

Abbreviations: Cgs, chromogranins; CgA/B, chromogranin A/B; SgII, secretogranin II; SN, secretoneurin; PACAP, pituitary adenylate cyclase-activating polypeptide; PC, prohormone convertase.

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angiogenesis, chemotaxis and modulation of the inflammatory response [3]. SgII is also the precursor of an evolutionarily conserved SN flanking-peptide termed EM66, in consideration of its N- and C-terminal glutamic acid and methionine residues [4]. It was shown that EM66 is generated early during the ontogenesis of the fetal human adrenal gland [5] and that the peptide occurs in the secretory granules of adrenergic cells in the rat adrenal gland, as well as in gonadotrophs and lactotrophs in the rat pituitary, suggesting that upon stimulation, EM66 may be released to act as an autocrine, paracrine or endocrine factor [6]. There is also evidence that EM66 is involved in energy homeostasis since food deprivation induces a significant increase in the number of EM66-containing neurons of the jerboa hypothalamus [7]. Finally, it has been recently shown that EM66 is expressed in tumoral chromaffin cells and represents a sensitive diagnostic and prognostic marker of pheochromocytoma [8,9].

Pituitary adenylate cyclase-activating polypeptide (PACAP), that is released from splanchnic nerve fibers innervating the adrenal medulla, acts as a regulator of chromaffin cell activity [10]. Indeed, PACAP has been shown to regulate, *in vivo* and *in vitro*, catecholamine and neuropeptide biosynthesis and release from chromaffin cells [11–17]. The effect of PACAP on chromaffin cells is mediated through the PACAP-preferring receptor, PAC1-R, via activation of the adenylate cyclase/PKA cascade and calcium (Ca^{2+}) influx (for review, [18]). In bovine adrenomedullary cells, nicotinic, histaminergic or PACAP stimulation increase SgII mRNA levels but not CgA expression [19–21]. PACAP also stimulates SN release through activation of the PKA and PKC pathways, and Ca^{2+} influx via L-type calcium channels [20]. Thus, PACAP activates multiple signaling pathways to regulate the synthesis and secretion of various neuropeptides, including SN, in primary chromaffin cells.

Although the function of EM66 is still unknown, several lines of evidence suggest that this peptide plays a role in the endocrine and neuroendocrine system. Determining the mechanisms that regulate the release of EM66 may contribute to the understanding of this role. In the present study, we have characterized EM66 and determined its localization in the bovine adrenal gland. We have also examined the signaling pathways activated by PACAP to regulate the secretion of EM66 from cultured bovine adrenochromaffin cells.

2. Materials and methods

2.1. Peptides

A fusion protein containing the human EM66 peptide was produced in *Escherichia coli* as previously described [5]. Briefly, the cDNA sequence encoding the EM66 region in human SgII was amplified by PCR, inserted downstream of the maltose-binding protein (MBP) region of the plasmid pMAL-c2 (New England Biolabs, Beverly, MA) and expressed in bacteria. The cleavage of the affinity-purified fusion protein with factor Xa released EM66 with the native amino acid sequence. The 38-amino acid form of PACAP was synthesized by solid-phase methodology as previously described [22].

2.2. Immunohistochemical procedure

Frozen bovine adrenal medulla and cortex were sliced into 10- μm sections with a cryomicrotome (Leica CM 3050, Heidelberg, Germany). Sections were thaw-mounted on gelatin-coated slides, dried in a desiccator overnight at 4 °C, and dipped for 10 min in Stephanini's fixative [4% paraformaldehyde and 0.2% picric acid in phosphate buffer (PB)]. Slides with cultured chromaffin cells were dipped for 30 min in 4% paraformaldehyde in PB. After several rinses in PB, fixed tissues and cultured cells were processed for indirect immunofluorescence. Tissue sections and cultured cells were incubated overnight at 4 °C with an EM66 antiserum directed against human EM66 (code no. 736-1806) [5] diluted 1:200 and the mouse monoclonal antibody directed against tyrosine hydroxylase (TH; Chemicon International, Temecula, CA) diluted 1:500 in PB containing 0.3% Triton X-100 and 1% bovine serum albumin (BSA, Roche Diagnostics, Mannheim, Germany). Then, they were rinsed in PB and incubated for 90 min at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit γ -globulins (Caltag Laboratories, San Francisco, CA) diluted 1:100 and Texas Red-conjugated sheep anti-mouse γ -globulins (Amersham Biosciences, Buckinghamshire, UK) diluted 1:50. Finally, tissues and cultured cells were rinsed in PB, mounted with buffer/glycerol (1:1), coverslipped, and examined using a confocal laser scanning microscope (CLSM TCS-SP2-AOBS, Leica) equipped with a fluorescence DMRX-A2 microscope and an argon (excitation wavelengths 458/476/488/514 nm) and two helium/neon (excitation wavelengths 543 and 633 nm, respectively) ion lasers. To verify the specificity of the immunoreaction, the following controls were performed: 1) substitution of the antiserum with PB; 2) incubation with nonimmune serum instead of the EM66 antiserum; 3) preincubation of the EM66 antiserum diluted (1:200) with purified recombinant EM66 (10^{-6} M).

2.3. Tissue extraction

For reversed-phase HPLC analysis, frozen tissue samples were boiled for 10 min in 0.5 M acetic acid, homogenized in a glass Potter, sonicated and centrifuged (3000 \times g, 4 °C, 30 min). The supernatants were collected and kept at 4 °C until pre-purification. The pellets were used for measurement of protein concentrations by the Bradford method. Adrenal medulla extracts were prepared from 87.5 mg wet tissue corresponding to 1.56 mg protein and adrenal cortex extracts were prepared from 100 mg wet tissue corresponding to 1.58 mg protein. For Western blot analysis, bovine adrenal medulla or cortex was homogenized in 50 mM Tris-HCl (pH 7.4), containing 1% Triton X-100, 10 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (21,000 \times g, 4 °C, 15 min), proteins contained in the supernatant were quantified using the Bradford method and then precipitated with 10% trichloroacetic acid.

2.4. Prepurification of tissue extracts

Each sample of adrenal tissue extract was loaded onto a Sep-Pak C₁₈ cartridge (Waters Corp., St-Quentin en Yvelines, France)

equilibrated with a solution of 0.1% trifluoroacetic acid (TFA) in water. Bound material was eluted from the cartridge with a solution of acetonitrile/water/TFA (59.9:40:0.1, vol/vol/vol), dried by vacuum centrifugation and kept at room temperature until chromatographic analysis.

2.5. HPLC analysis

Prepurified samples were reconstituted in 1 ml of 0.1% TFA in water, centrifuged (21,000 \times g, 4 °C, 10 min) and injected onto a 4.6 \times 250-mm Vydac 218TP54 (C₁₈) column equilibrated with a solution of acetonitrile/water/TFA (9.9:90:0.1, vol/vol/vol) at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 60% over 25 min using a linear gradient. HPLC standard consisted of 1 μ g purified recombinant EM66. Fractions of 0.5 ml were collected, evaporated and kept dry until RIA.

2.6. Radioimmunoassay

Purified recombinant EM66 was iodinated by the chloramine-T method and separated from free iodine on Sep-Pak C₁₈ cartridges using a gradient of acetonitrile (0–100%) in 0.1% TFA, as previously described [5]. The RIA was performed in veronal buffer (pH 7.4) supplemented with 0.4% BSA and 0.1% Triton X-100. The EM66 antiserum, used at a final dilution of 1:48,000, was incubated with 7000 cpm of tracer/tube in the presence of graded concentrations of standard (purified EM66), culture medium, tissue extracts or HPLC fractions. After a 2-day incubation at 4 °C, the antibody-bound

fraction was immunoprecipitated by addition of 200 μ l goat anti-rabbit γ -globulins (1:30), 200 μ l normal rabbit serum (1:150) and 1 ml of 5% polyethyleneglycol 8000. After a 2-hour incubation at room temperature, the mixture was centrifuged (5000 \times g, 4 °C, 30 min) and the pellet containing the bound fraction was counted on a gamma-counter (LKB, Wallack, Rockville, MD). The standard curve was set up with concentrations of EM66 ranging from 5 to 10,000 pg/tube. The inter- and intra-assay coefficients of variation were, respectively, 5.00–9.24% and 0.74–1.98% [8].

2.7. Western blot analysis

Proteins were electrophoresed on polyacrylamide gels under denaturing conditions (SDS-PAGE, 10% total acrylamide), electroblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech) and revealed with the various antisera using a chemiluminescence detection kit (Amersham Pharmacia Biotech). EM66, PC1 and PC2 antibodies were used at a dilution of 1:4000 and 1:1000, respectively. PC1 and PC2 antisera, raised in rabbit [23,24], were a generous gift of Dr. N. Seidah (Montreal, Canada).

2.8. Cell culture and treatments

Primary cultures of bovine adrenochromaffin cells were obtained after retrograde perfusion of bovine adrenal glands with 0.1% collagenase (Serlabo, Bonneuil-sur-Marne, France) and 30 U/ml DNase I (Sigma-Aldrich), followed by mechanical dissociation of the digested adrenal medulla. Isolated cells were

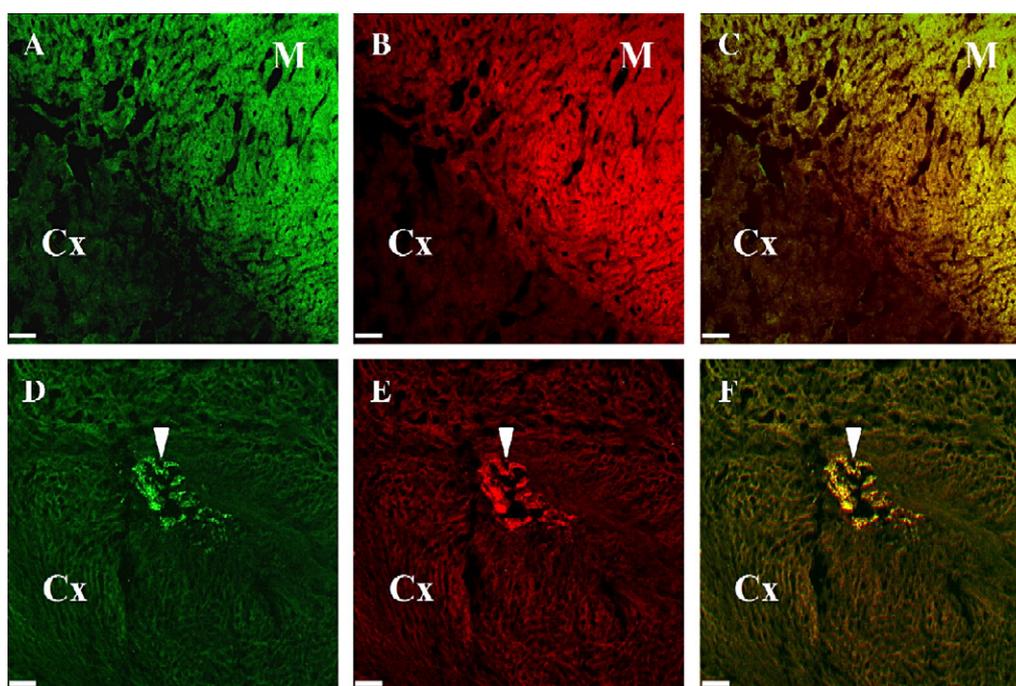


Fig. 1. Photomicrographs comparing the distribution of EM66 and TH immunoreactivity (IR) in the bovine adrenal gland and cultured bovine chromaffin cells. Intense immunofluorescence labeling of EM66 (A, D) and TH (B, E) is observed in the medulla (M) and in a cluster of chromaffin cells (arrowhead) located in the cortex (Cx). Note that cortical cells are devoid of staining. Combination of the images acquired in A and B, and in D and E, demonstrated the presence of EM66 in all chromaffin cells (C, F). Bars, 100 μ m.

cultured in DMEM (Sigma-Aldrich) supplemented with 5% calf serum (Biowhittaker Europe, Verviers, Belgium), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Life Technologies, Cergy-Pontoise, France). Chromaffin cells were purified by differential plating to remove adherent nonchromaffin cells as described previously [25], and then plated at a density of 10^6 cells/ml in poly-L-lysine-coated 24-well plates (Sigma-Aldrich, Saint-Quentin Fallavier, France). After a resting period of one day, chromaffin cells were treated for 24 h with PACAP in the presence or absence of the non-specific protein kinase inhibitor H7 (100 µM), the protein kinase A inhibitor H89 (20 µM), the protein kinase C inhibitor chelerythrine (5 µM), the nonselective Ca^{2+} channel blocker $NiCl_2$ (3 mM), the cytosolic Ca^{2+} chelator BAPTA-AM (50 µM), the L-type Ca^{2+} channel blocker nimodipine (10 µM), the p42/44 ERK 1/2 MAPK inhibitor U0126 (10 µM), the p38 MAPK inhibitor SB203580 (10 µM), and the protein synthesis inhibitor cycloheximide (0.5 µg/ml). The drugs were either dissolved in the culture medium (cycloheximide), in dimethyl sulfoxide (H7, H89, chelerythrine, BAPTA-AM, nimodipine, SB203580 and U0126) or in ethanol (forskolin and TPA), and added 30 min before the onset of secretagogue treatment. The final concentration of dimethyl sulfoxide or ethanol never exceeded 0.1% except for BAPTA-AM (0.2%),

and control cells were treated with the same amount of solvent. After the incubation period, aliquots of culture medium supernatants were taken and immediately frozen at $-20\text{ }^\circ\text{C}$ until RIA determination of EM66. For immunocytochemical experiments, cells were cultured on glass slides coated with poly-L-lysine.

2.9. Data analysis

Data are reported as median (25th–75th percentile), where the 25th–75th percentile represents the dispersion of the distribution. The nonparametric Mann–Whitney *U* test and Kruskal–Wallis test were used. Probability values less than 0.05 were considered significant. Data were analyzed with the Prism program (GraphPad Software, San Diego, CA).

3. Results

3.1. Immunohistochemical detection of EM66

In the adrenal gland, the EM66 antibody strongly labeled the medulla (Fig. 1A) and groups of cells located in the cortex (Fig. 1D). Double labeling experiments revealed that adrenomedullary and cortical cells that were immunolabeled with the EM66 antiserum were also stained with the TH antibodies (Fig. 1A and F), indicating that EM66-IR material is restricted to chromaffin cells. Preabsorption of the primary antiserum with 10^{-6} M EM66 totally abolished the immunoreaction (data not shown). When the antiserum was substituted with either nonimmune rabbit serum or PB, no immunostaining was observed.

3.2. Characterization of EM66-IR in bovine adrenal gland

HPLC analysis combined with RIA detection was performed to characterize EM66 in adrenal gland extracts and chromaffin cell culture medium (Fig. 2). The position of the main immunoreactive peak of recombinant EM66 varied between fractions 34 and 36 (44–46% acetonitrile), depending on the experiment (data not shown). In adrenal medulla and adrenal cortex extracts as in culture medium, a major immunoreactive peak was resolved that co-eluted with recombinant EM66 (Fig. 2A, B and C, respectively). More hydrophobic EM66-immunoreactive compounds eluting in fractions 39–45 (49–55% acetonitrile) were also resolved in the adrenal medulla extracts and culture medium (Fig. 2A and C, respectively).

As previously described [26], in bovine adrenal medulla, Western blot analysis using the EM66 antiserum revealed the presence of a SgII-immunoreactive band with an apparent molecular mass of 97-kDa (Fig. 3, lane 1M). The same band with less intensity was observed in adrenal cortex extracts (Fig. 3, lane 1Cx). Adrenal medulla and cortex extracts exhibited additional bands of lower mass but with a different pattern (Fig. 3, lane 1). Immunoblots with anti-PC1 and PC2 indicated that bovine adrenal medulla expresses both convertases as revealed by the 66-kDa bands (Fig. 3, lanes 2M–3M). In the adrenal cortex, a less intense PC1-immunoreactive band (Fig. 3, lane

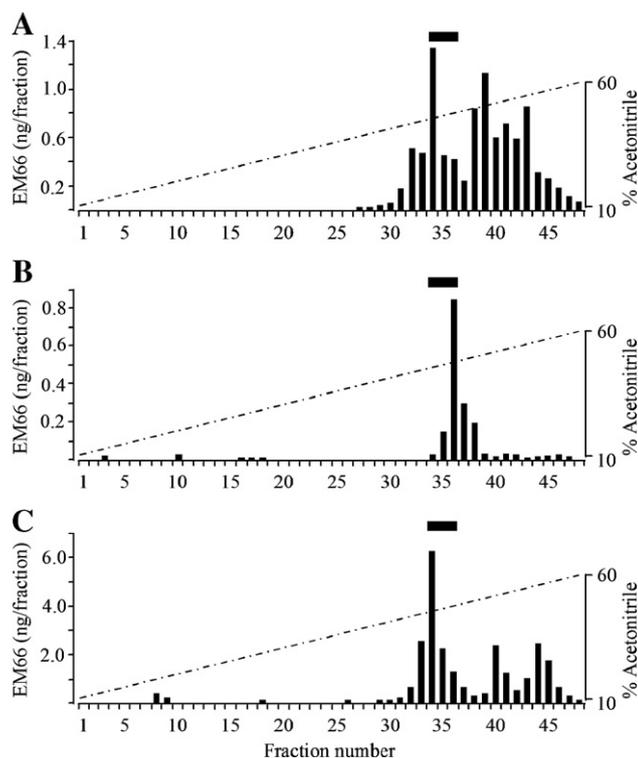


Fig. 2. Reversed-phase HPLC analysis of EM66-immunoreactivity in extracts of bovine adrenal medulla (A) and cortex (B), and in chromaffin cell culture medium (C). Samples were prepurified on Sep-Pak cartridges and chromatographed onto a Vydac C_{18} column. Fractions were collected (0.5 ml/tube), dried and radioimmunoassayed for EM66. The bars above the peaks indicate the elution position of recombinant EM66 chromatographed the same day as the extracts. The dashed lines show the concentration of acetonitrile in the eluting solvent.

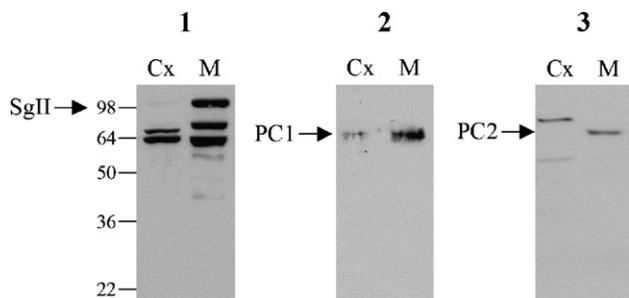


Fig. 3. Western blot analysis of SgII, PC1 and PC2 in bovine adrenal gland extracts. Adrenal cortex (Cx) and adrenal medulla (M) samples were homogenized and proteins (20 μ g) were analyzed by SDS-PAGE followed by immunoblotting using the EM66 antiserum diluted 1:4000 (lane 1), a PC1 antiserum diluted 1:1000 (lane 2) or a PC2 antiserum diluted 1:1000 (lane 3). Molecular weight markers (in kDa) and the SgII, PC1 and PC2 positions are indicated.

2Cx) and a band of 80-kDa corresponding to a non-processed form of PC2 (Fig. 3, lane 3Cx) were observed.

3.3. EM66 levels in the bovine adrenal gland

Serial dilutions of adrenal medulla extracts generated displacement curves that were parallel to those obtained with recombinant EM66 (Fig. 4A). Similar results were obtained with adrenal cortex extracts (Fig. 4A). EM66 concentrations determined from adrenal medulla extracts ($n=5$) ranged from 26.5 to 93.3 ng/mg protein with a median value of 35.9 ng/mg protein (Fig. 4B). In the cortex ($n=5$), EM66 levels were about 45-fold lower with values ranging from 0.6 to 1.8 ng/mg protein with a median of 0.8 ng/mg protein (Fig. 4B).

3.4. PACAP stimulates EM66 release from cultured chromaffin cells

After a 24-hour resting period in the absence of any secretagogue, bovine chromaffin cells released EM66 in the culture

medium at a rate of 6.9 (6.6–7.2) ng/ 10^5 cells per 24 h (Fig. 5A). Incubation of cells with graded concentrations of PACAP (0.1 nM–1 μ M) for 24 h induced a dose-dependent stimulation of EM66 release (up to 2.5-fold) with half-maximal (ED_{50}) effect at a concentration of 4.8 nM. Kinetic experiments showed that PACAP (50 nM) evoked EM66 release in the medium after 6 h of treatment (Fig. 5B). The PACAP effect gradually increased to reach a 2.6-fold stimulation after 48 h, remained elevated at 72 h and then declined at 96 h (Fig. 5B). Although the RIA measures mainly the EM66 peptide, other EM66-containing fragments are also included in this measurement as indicated by the HPLC analysis.

3.5. Contribution of calcium signaling to PACAP-induced EM66 release

The nonselective Ca^{2+} channel blocker $NiCl_2$ (3 mM), the cytosolic Ca^{2+} chelator BAPTA-AM (50 μ M) and the L-type Ca^{2+} channel blocker nimodipine (10 μ M) significantly inhibited by 78%, 65% and 57%, respectively, the stimulatory effect of PACAP on EM66 release. None of these drugs had any effect on EM66 secretion by its own (Fig. 6).

3.6. Contribution of protein kinases to PACAP-induced EM66 release

The nonselective protein kinase inhibitor H7 (100 μ M) markedly reduced (–90%) the effect of PACAP on EM66 release. The effect of PACAP was also significantly diminished by the PKA inhibitor H89 (20 μ M) and the PKC inhibitor chelerythrine (5 μ M) (–77% and –63%, respectively) (Fig. 7). Concomitant administration of H7 with BAPTA or with nimodipine reduced PACAP-induced EM66 release by 84% and 90%, respectively (Fig. 8). The association of H7 with the extracellular and intracellular Ca^{2+} chelators $NiCl_2$ and BAPTA totally abolished the stimulatory effect of PACAP on EM66 secretion. A similar blockage was observed when

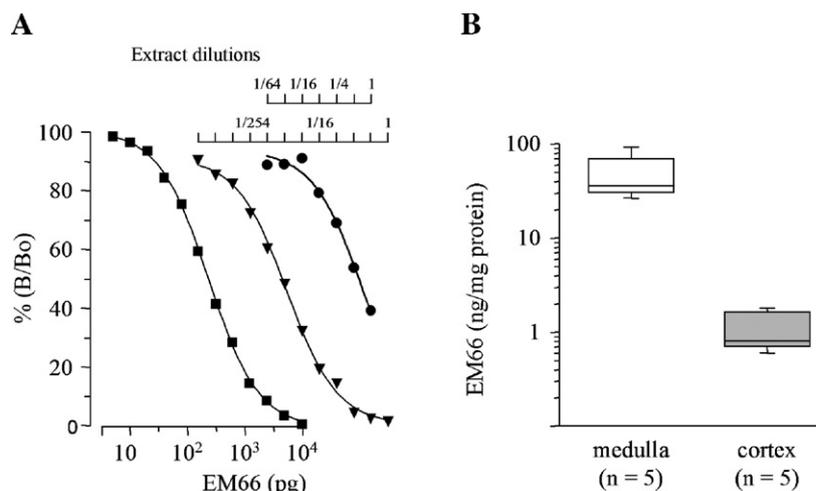


Fig. 4. (A) Semilogarithmic plots comparing competitive inhibition of antibody-bound ^{125}I -EM66 by recombinant EM66 (■) and serial dilutions of adrenal medulla (▼) or adrenal cortex (●) extracts. (B) Box-plots (with 25th–75th percentile and minimum–maximum values) showing EM66 concentrations in adrenal medulla and adrenal cortex extracts.

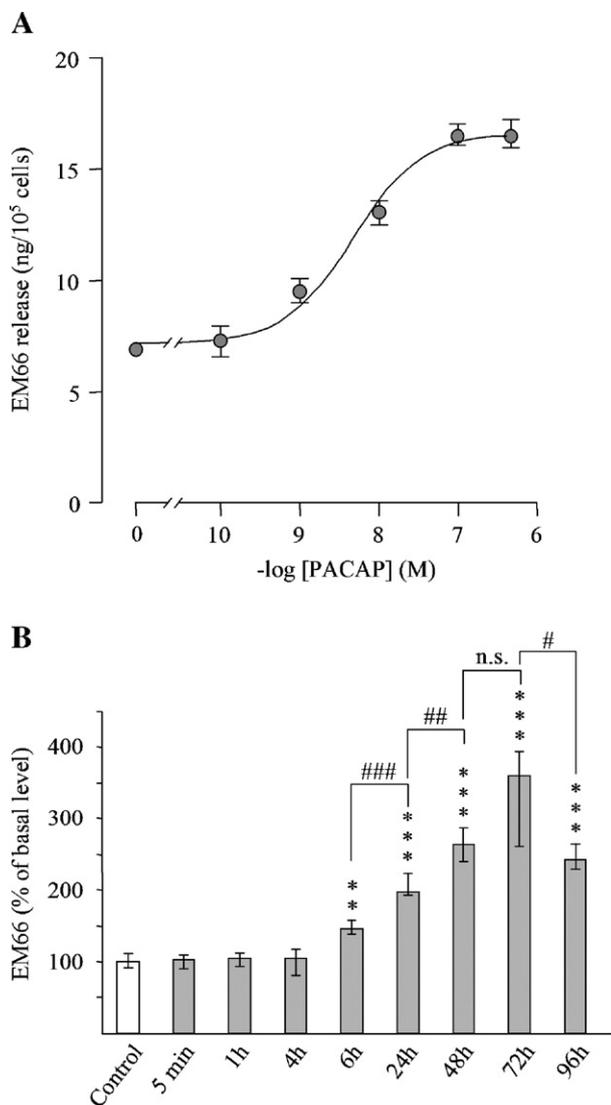


Fig. 5. (A) Dose-dependent effect of PACAP on EM66 release from cultured bovine chromaffin cells. Values are expressed as nanograms of EM66 secreted by 10^5 cells and represent the median and 25th–75th percentile. The EC_{50} value was 4.8 nM. (B) Time-course of PACAP-induced EM66 release. Chromaffin cells were incubated 24 h in the absence (control; open bar) or presence of 50 nM PACAP for various durations. Values represent the median and 25th–75th percentile of EM66 concentration in the culture medium, expressed as percentages of basal level. n.s., not significant; ** $p < 0.01$, *** $p < 0.001$ vs the corresponding control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

PACAP was co-incubated with chelerythrine, H89, $NiCl_2$ and BAPTA (Fig. 8).

3.7. Contribution of MAP kinases to PACAP-induced EM66 release

The p38 MAPK inhibitor SB203580 (10 μ M) and the p42/44 ERK1/2 MAPK inhibitor U0126 (10 μ M) decreased, respectively, by 72% and 63% the effect of PACAP on EM66 release (Fig. 9). Concomitant administration of both inhibitors suppressed by 93% PACAP-induced EM66 secretion (Fig. 9). The two inhibitors, alone or in combination, had no effect on peptide release.

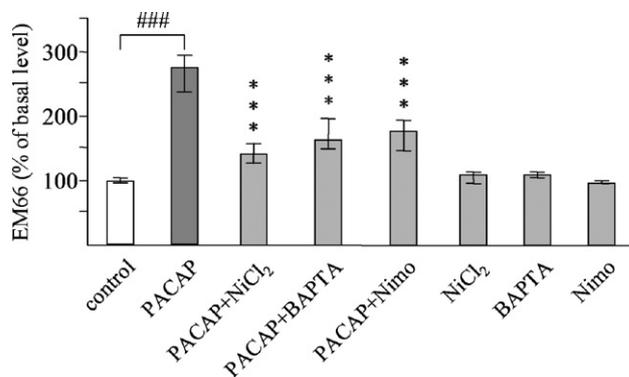


Fig. 6. Effect of Ca^{2+} mobilization inhibitors on PACAP-induced EM66 release. Chromaffin cells were incubated 24 h in control conditions or with 50 nM PACAP, in the absence or presence of 3 mM $NiCl_2$, 50 μ M BAPTA or 10 μ M nimodipine (Nimo). Values represent the median and 25th–75th percentile of EM66 concentration in the culture medium, expressed as percentages of basal level (corresponding controls). ### $p < 0.001$ PACAP vs control, *** $p < 0.001$ vs PACAP alone.

3.8. Contribution of protein synthesis to PACAP-induced EM66 release

The stimulatory effect of PACAP (50 nM) on EM66 release was mimicked by forskolin (25 μ M) and K^+ (25 mM), and to a lesser extent by TPA (100 nM) (Fig. 10). The protein synthesis inhibitor cycloheximide (0.5 μ g/ml), reduced by 80–90% the action of PACAP as well as that of forskolin and K^+ on EM66 release. In contrast, the effect of TPA was not affected by cycloheximide (Fig. 10).

4. Discussion

The results described in the present study have shown that bovine adrenochromaffin cells are able to produce and release the SgII-derived peptide EM66, and that PACAP is a strong regulator of this process through activation of diverse signaling components.

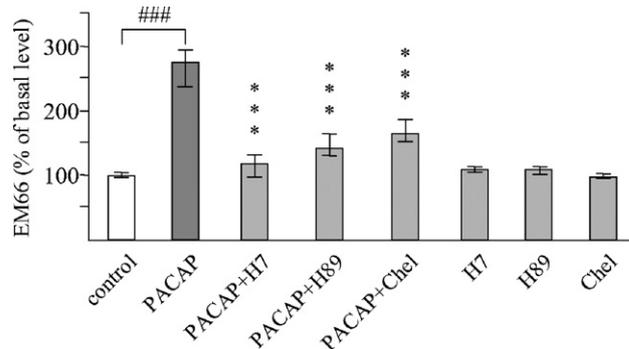


Fig. 7. Effect of protein kinase inhibitors on PACAP-induced EM66 release. Chromaffin cells were incubated 24 h in control conditions or with 50 nM PACAP, in the absence or presence of 100 μ M H7, 20 μ M H89 or 5 μ M chelerythrine (Chel). Values represent the median and 25th–75th percentile of EM66 concentration in the culture medium, expressed as percentages of basal level (corresponding controls). ### $p < 0.001$ PACAP vs control, *** $p < 0.001$ vs PACAP alone.

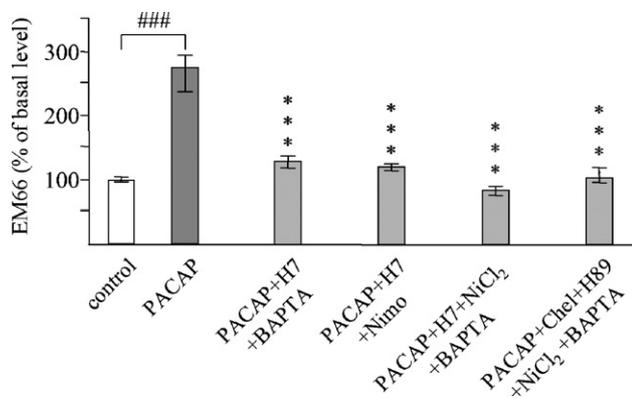


Fig. 8. Effect of concomitant administration of protein kinase inhibitors and Ca^{2+} mobilization inhibitors on PACAP-induced EM66 release. Chromaffin cells were incubated for 24 h in control conditions or with 50 nM PACAP, in the absence or presence of 100 μM H7, 50 nM BAPTA, 10 μM nimodipine (Nimo), 3 mM NiCl_2 , 5 μM chelerythrine (Chel) or 20 μM H89. Values represent the median and 25th–75th percentile of EM66 concentration in the culture medium, expressed as percentages of basal level (corresponding controls). ### $p < 0.001$ PACAP vs control, *** $p < 0.001$ vs PACAP alone.

Double labeling experiments revealed that EM66-IR is present in TH-producing cells *i.e.* chromaffin cells of the adrenal medulla, consistent with the data previously obtained in rat chromaffin cells [6]. As in the rat adrenal gland [6], islets of EM66-positive chromaffin cells were also observed within the adrenal cortex. Several studies have indicated the existence of rays and islets of extramedullary chromaffin cells in the adrenal gland [27–29]. The occurrence of EM66 in these cells suggests that the peptide could play a role as a paracrine factor to regulate steroidogenic cells in the adrenal gland [30,31].

HPLC analysis combined with RIA detection of adrenal medulla and adrenal cortex extracts, resolved a major immunoreactive peak that co-eluted with purified recombinant EM66, indicating that this peptide is produced in the adrenal gland. In fact, our previous observations indicated that SgII is actually processed to form EM66 in the human and rat adrenal gland [5,6,9], in the rat pituitary [6] and in the jerboa hypothalamus

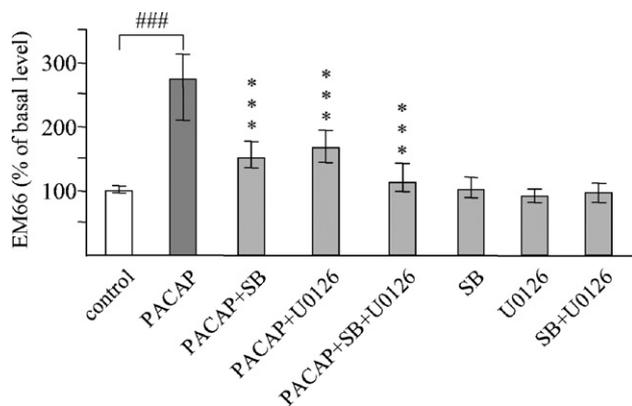


Fig. 9. Effect of MAPK inhibitors on PACAP-induced EM66 release. Chromaffin cells were incubated for 24 h in control conditions or with 50 nM PACAP, in the absence or presence of 10 μM SB203580 (SB), 10 μM U0126 or a combination of both drugs. Values represent the median and 25th–75th percentile of EM66 concentration in the culture medium, expressed as percentages of basal level (corresponding controls). ### $p < 0.001$ PACAP vs control, *** $p < 0.001$ vs PACAP alone.

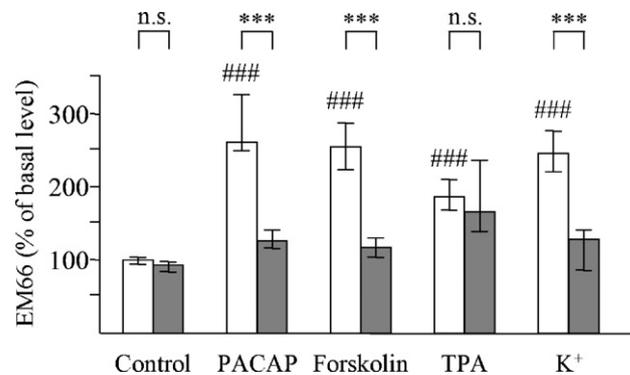


Fig. 10. Effect of protein synthesis inhibition on EM66 release stimulated by PACAP, forskolin, TPA or K^+ . Chromaffin cells were incubated for 24 h in control conditions or with 50 nM PACAP, 25 μM forskolin, 100 nM TPA or 25 mM K^+ in the absence (open bars) or presence (gray bars) of 0.5 $\mu\text{g}/\text{ml}$ cycloheximide. Values represent the median and 25th–75th percentile of EM66 concentration in the culture medium, expressed as percentages of basal level (corresponding controls). ### $p < 0.001$ vs control, *** $p < 0.001$ cycloheximide-treated cells vs cycloheximide untreated cells. n.s., not significant.

[7]. Moreover, EM66 is generated early during the ontogenesis of the fetal human adrenal gland [5], suggesting that its formation is necessary throughout life. The sequence of SgII contains nine pairs of basic amino acids which represent potential cleavage sites by the endopeptidases PC1 and PC2 [32]. It has been previously shown that, in the rat PC12 chromaffin cell line, SgII can be cleaved by PC1 and PC2 to generate several fragments including SN [33,34]. The fact that both PC1 and PC2 are expressed in the bovine adrenal gland strongly suggests that these prohormone convertases are involved in the processing of SgII to generate mature EM66. Interestingly, Western blot analysis revealed a differential pattern of EM66-immunoreactive bands between adrenal medulla and adrenal cortex extracts. This result indicates the existence of different processing mechanisms of SgII in medullary vs extramedullary adrenal chromaffin cells, which can be due to differential regulation of PCs activities probably related to the expression of PC inhibitors [35] or the accumulation of catecholamines which inhibit PCs [36].

It is well established that, in chromaffin cells of the adrenal medulla, PACAP acting through PAC1-R, exerts a stimulatory effect on catecholamine secretion that persists for several hours [11]. PACAP also stimulates the expression of genes encoding various neuropeptides such as galanin [37] and VIP [38], and the release of enkephalins and brain natriuretic peptides [11,12]. Concerning chromogranins, which are co-stored with catecholamines within secretory granules of chromaffin cells, Turquier et al. [20] previously showed that PACAP increases SgII gene transcription and SN release. In contrast, CgA mRNA levels were not affected by PACAP treatment, indicating that the neuropeptide exerts a gene-specific effect on members of the Cg family in bovine chromaffin cells. In addition to SN, PACAP also stimulated the secretion of EM66 from chromaffin cells which have the ability to release appreciable amounts of EM66 in the culture medium. Together with the immunohistochemical experiments indicating the occurrence of EM66-IR in cultured chromaffin cells and HPLC analysis showing the presence of a

major EM66-immunoreactive peak co-eluting with the recombinant peptide in culture medium extracts, these data demonstrate that EM66 is produced by chromaffin cells and is released from these cells upon stimulation.

The ED_{50} (≈ 5 nM) of PACAP-stimulated EM66 secretion was similar to that observed for PACAP-induced SN secretion ($ED_{50} \approx 3$ nM, [20]), in agreement with the high-affinity binding of PACAP to the PAC1-R expressed in bovine chromaffin cells. Kinetic experiments indicated that PACAP-evoked EM66 secretion occurred after 6 h of treatment, reached a plateau phase at 48–72 h and lasted up to 96 h. These data confirm those reported previously showing that PACAP exerts long-lasting effects on the secretory activity of chromaffin cells [11]. In contrast to SN release which occurred within 5 min [20], EM66 secretion could be detected only after 6 h of exposure to the neuropeptide. Since it has been shown that PACAP stimulated SgII gene expression after 1 h, these observations suggest that, in bovine chromaffin cells, PACAP induces the release of pre-existing pools of SN whereas the effect on EM66 secretion could be directly dependent on SgII biosynthesis and processing. In accordance, we found that PACAP, high potassium concentration and forskolin-induced EM66 secretion were almost completely blocked ($\approx 90\%$) by the protein synthesis inhibitor cycloheximide. Alternatively, since the basal level of EM66 is 5-fold higher than that of SN, and since PACAP induces a lower maximal fold increase of EM66 (2.6-fold) compared to SN (5-fold), one would predict that PACAP causes modest EM66 variation that remains undetectable until 6 h of treatment.

The PAC1-R has been shown to activate several second messengers including cAMP, IP_3 and Ca^{2+} in bovine chromaffin cells [39–41]. Using extra- and intracellular Ca^{2+} blockers, we found that the effect of PACAP on EM66 release is associated with activation of L-type Ca^{2+} channels and elevation of cytosolic Ca^{2+} concentrations. However, since nimodipine partially blocked the PACAP effect, other types of voltage-operated Ca^{2+} channels may be involved in EM66 secretion. Similar Ca^{2+} signaling routes were shown to be involved in PACAP-induced SN secretion [20]. However, the effects of Ca^{2+} inhibitors were much lower on SN than EM66 release, indicating a predominance of this second messenger pathway in EM66 secretion.

The general protein kinase inhibitor H7, almost completely blocked the PACAP-induced stimulation of EM66 secretion. In fact, we observed that PKA, PKC, p38 and ERK1/2 blockers were, with equivalent potency, very effective in reducing the PACAP effect. Moreover, concomitant administration of H7 and Ca^{2+} blockers totally suppressed PACAP-evoked EM66 release. Altogether these results indicate that multiple signaling pathways involving Ca^{2+} , PKA, PKC and MAPK (Fig. 11) are collectively activated by PACAP to induce maximal stimulation of EM66 secretion. Further studies will be necessary to elucidate the crosstalks between these transduction pathways and the sequence of their activation by PACAP to trigger EM66 release. It has been shown in PC12 cells, that PACAP induces GTP-loading of Rap1 and co-activation of PKA, PKC or Ras to efficiently stimulate ERK1/2 [42] and p38 through activation of a phospholipase C, mobilization of intracellular Ca^{2+} stores and Ca^{2+} influx via voltage-dependent Ca^{2+} channels [43]. Because

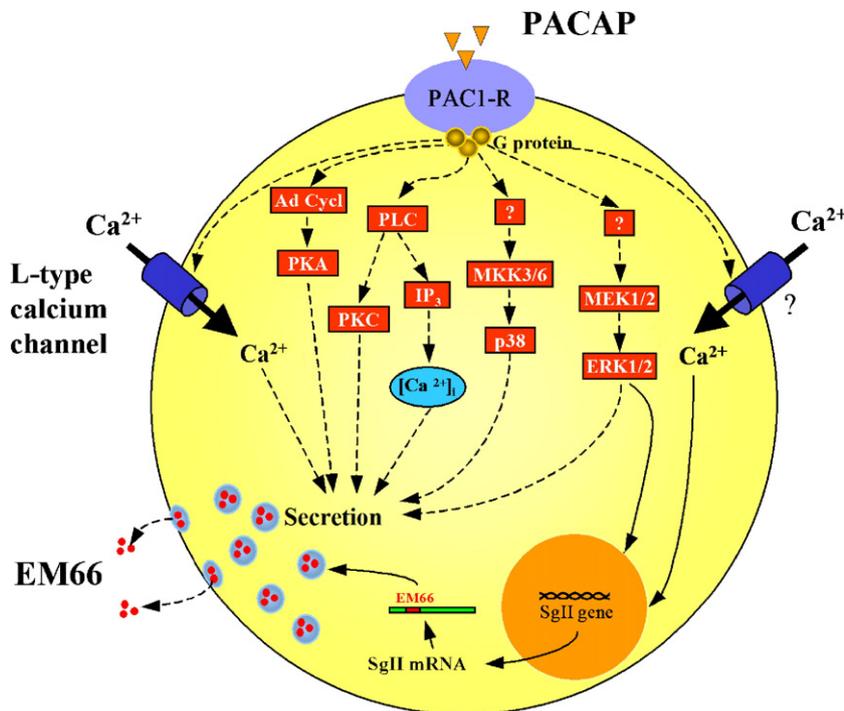


Fig. 11. Model depicting the transduction pathways involved in the effect of PACAP on EM66 release in chromaffin cells. The dashed line arrows indicate the activated transduction pathways leading to EM66 secretion and the solid line arrows indicate the pathways involved in SgII gene expression under PACAP stimulation as described previously [20]. Ad Cycl, adenylyl cyclase; $[Ca^{2+}]_i$, intracellular calcium pool; ERK1/2, p42/44 extracellular signal regulated kinase; IP_3 , inositol trisphosphate; MEK, MAPK/ERK kinase; MKK, MAP kinase kinase; PAC1-R, type 1 PACAP receptor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.

PACAP stimulation of EM66 involved several of these transduction components, it could be interesting to determine whether a similar sequential activation of these mechanisms also occurs in primary chromaffin cells.

Taken together, as summarized in Fig. 11, our results show that, in bovine chromaffin cells, PACAP, probably through PAC1-R, activates multiple signaling pathways including intra- and extracellular Ca²⁺ mobilization, PKA, PKC, p38 and ERK 1/2 MAPK to induce EM66 secretion. Although the release of EM66 exhibited several similarities with that of SN, the former seems to rely primarily on pools of newly synthesized peptide whereas the latter probably involves a pre-existing pool of peptide. Moreover, p38 and ERK1/2 MAPK transduction routes are implicated to the same extent in EM66 release while mainly ERK1/2 is involved in SN secretion. Thus, the fine tuning of the release of SgII-derived peptides by a neurocrine factor suggests that SN and EM66 may act differently after release from neuroendocrine cells.

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