Annals of the New York Academy of Sciences 971:474-490 (2002)

Role of Protein Kinases in Neuropeptide Gene Regulation by PACAP in Chromaffin Cells

A Pharmacological and Bioinformatic Analysis

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Ann. N.Y. Acad. Sci. 971: 474-490 (2002).

ABSTRACT

Pituitary adenylate cyclase—activating polypeptide (PACAP) is an adrenomedullary cotransmitter that along with acetylcholine is responsible for driving catecholamine and neuropeptide biosynthesis and secretion from chromaffin cells in response to stimulation of the splanchnic nerve. Two neuropeptides whose biosynthesis is regulated by PACAP include enkephalin and vasoactive intestinal polypeptide (VIP). Occupancy of PAC1 PACAP receptors on chromaffin cells can result in elevation of cyclic AMP, inositol phosphates, and intracellular calcium. The proenkephalin A and VIP genes are transcriptionally responsive to signals generated within all three pathways, and potentially by combinatorial activation of these pathways as well. The characteristics of PACAP regulation of enkephalin and VIP biosynthesis were examined pharmacologically for evidence of involvement of several serine/threonine protein kinases activated by cAMP, IP3, and/or calcium, including calmodulin kinase II, protein kinase A, and protein kinase C.

Evidence is presented for the differential involvement of these protein kinases in regulation of enkephalin and VIP biosynthesis in chromaffin cells, and for a prominent role of the mixed-function (tyrosine and serine/threonine) MAP kinase family in mediating transcriptional activation of neuropeptide genes by PACAP.

Key Words: calcium/calmodulin kinase • cyclic AMP • mitogen-activated protein kinase • pituitary adenylate cyclase-activating polypeptide • phorbol myristate acetate • proenkephalin • protein kinase A • protein kinase C • vasoactive intestinal polypeptide

INTRODUCTION

Several converging lines of evidence suggest that PACAP is a physiologically important transmitter at the adrenomedullary synapse. PACAP administered *in vivo* causes enhanced catecholamine secretion.[1] PACAP elicits catecholamine release from PC12 cells, and rat, porcine, and bovine chromaffin cells in culture at nanomolar concentrations.[2-5] PACAP stimulates the synthesis of both catecholamines and neuropeptides in bovine chromaffin cells.[6-9] PACAP has been proposed as a neuropeptide transmitter important in maintaining the link between catecholamine secretion and synthesis during prolonged splanchnic nerve stimulation based on studies in the perfused rat adrenal gland,[10] and we have recently reported that mice deficient in PACAP also fail to maintain adequate adrenomedullary catecholamine synthesis and secretion following prolonged hypoglycemia induced by insulin.[11]

Regulation of neuropeptide biosynthesis in the adrenal medulla is a second transmitter role of potential physiological importance played by PACAP. VIP, an adrenomedullary neuropeptide stimulated by PACAP, may be involved in adrenal vasodilation,[12] assisting in dissemination of the effects of catecholamines cosecreted from the adrenal medulla. Enkephalins released from the adrenal medulla and regulated by PACAP may play a role in stress-related analgesia, cardiovascular function, and, in an autocrine fashion, on catecholamine secretion itself, *in vivo*.[13] Elucidating the signaling pathways that PACAP uses to couple control of hormone secretion and biosynthesis is of significant interest in revealing how PACAP and other neuropeptide neurotransmitters work together with classical neurotransmitters to exert short-term and long-term control over synaptic transmission important in homeostatic regulation by the autonomic nervous system and the adrenal medulla.

PACAP stimulates several signaling pathways in chromaffin cells by enhancement of calcium influx, mobilization of sequestered intracellular calcium to the cytosol, and elevation of cyclic AMP (cAMP) levels. Calcium influx is required for PACAP-induced secretion of catecholamines[2,14] and neuropeptides.[8] Elevation of cAMP is important in regulation of catecholamine-synthesizing enzymes.[15] The second messengers responsible for PACAP stimulation of neuroendocrine-specific gene

transcription have only recently been investigated. Combined elevation of cyclic AMP and calcium would be predicted to activate at least four types of protein kinases in chromaffin cells—protein kinase C, protein kinase A, mitogen-activated protein kinases (MAP kinases), and calmodulin-activated kinases (CaM kinases).[16-18]

Several neuropeptide genes are known to be targets of *trans*-synaptic activation in chromaffin cells, including the VIP and proenkephalin genes. These genes have *cis*-acting promoter-proximal sequences responsive to the *trans*-activators AP-1 and CRE[19] and are both transcriptionally activated by the adrenomedullary cotransmitter PACAP,[6-8] which in turn is able to signal to both AP-1- and CREB-responsive reporter genes in heterologous systems.[20-22] This regulation presumably occurs because PACAP is able to couple to both adenylate cyclase, which elevates cAMP and can activate protein kinase A (a major upstream regulator of CREB), and to phospholipase C, which can elevate diacylglycerol and cause an IP3-dependent increase in intracellular calcium, which together can lead to activation of protein kinase C, a major regulator of signaling pathways leading to activation of AP-1 through MAP kinase activation. It is not necessarily so, however, that all activators of cAMP cause gene activation through PKA/CREB, and that all activators of PKC cause gene activation solely through *trans*-activation at a TPA/PMA-responsive element (TRE).[19] Here, we examine the intermediate signaling components that link upregulation of second messengers by PACAP to control of the VIP and proenkephalin genes in chromaffin cells. Control of enkephalin and VIP gene transcription by PACAP appears to be exerted by different signaling pathways, or combinations of signaling pathways, with differential convergence on protein kinase C, protein kinase A, and calmodulin kinase/calcineurin.

REGULATION OF NEUROPEPTIDE GENE TRANSCRIPTION/BIOSYNTHESIS BY PMA IN CHROMAFFIN CELLS: ROLE OF CULTURE CONDITIONS

PMA has been reported to inhibit PEnk mRNA induction in chromaffin cell cultures under some conditions[23,24] and to stimulate PEnk mRNA induction under others.[25,26] The mechanism of action of phorbol esters in inhibition of calcium influx is thought to be through PKC-mediated phosphorylation of L-type voltage-sensitive calcium channels.[23,27] In order to compare neuropeptide gene regulation by protein kinase C in chromaffin cells to that induced by PACAP as well as stimulators of other protein kinases such as PKA, we cultured differentially plated bovine chromaffin cells for two days in DMEM with 5% heat-inactivated fetal bovine serum before addition of inducing agents including PMA, and for seven days under the same conditions before addition of potential inducers of neuropeptide biosynthesis, using otherwise identical culture conditions as adapted from Fenwick *et al.* and described previously.[7,28,29] Proenkephalin mRNA was upregulated by 2.5-3-fold by 40 mM KCl, 25 µM forskolin, or 100 nM PMA in short-term cultures (Fig. 1).

Induction by KCl, forskolin, and PMA was not influenced by cotreatment with 25 μM dexamethasone or by differential plating to remove adherent nonchromaffin cells before plating and treatment (data not shown). With time in culture, however, the induction of proenkephalin by PMA was markedly diminished (Met-enkephalin peptide levels 212 ± 8.3% of control 72 hours after exposure to 100 nM PMA when treatment was begun 48 hours after cell isolation, versus 80 ± 7.0% of control 72 hours after exposure to 100 nM PMA when treatment was begun 168 hours after cell isolation) (Fig. 1). Significantly, the ability of PMA to inhibit upregulation of enkephalin biosynthesis by elevated potassium[23,24,26] persisted throughout the entire culture period: only the ability of PMA to increase enkephalin peptide levels on its own was lost with increased time in culture (Fig. 1). Induction of VIP peptide by PMA, on the other hand, was not significantly affected by time in culture (Fig. 1). We have observed that upregulation of galanin peptide and its mRNA is likewise unaffected by duration of culture of chromaffin cells (data not shown). These results suggest that multiple isoforms of PKC, some diminishing in expression as a function of time in culture and others not, may differentially mediate the regulation of transcription of enkephalin, VIP, and other neuropeptides by PMA and perhaps by first messengers such as PACAP, histamine, and angiotensin II that activate "conventional" or "novel" protein kinase C isoforms via coupling to phospholipase C and elevation of intracellular calcium and diacylglycerol. Consistent with this, the presence of multiple PKC isoforms has been described in bovine chromaffin cells, conventional PKC-α, novel PKC-ϵ, and atypical PKC-ι and PKC-ζ.[30-35] Conventional isoforms are sensitive to both calcium and diacylglycerol/phorbol ester; whereas novel isoforms are sensitive to only diacylglycerol/phorbol ester, and atypical isoforms are sensitive to neither. Both PKC-α and PKC-ϵ are rapidly translocated from cytosol to membrane following short-term PMA exposure, and PKC-ι and -ζ are not. In particular, antagonism of depolarization-induced L-type calcium channel opening, stimulation of VIP biosynthesis, and stimulation of galanin biosynthesis by 100 nM PMA appear to require a protein kinase C isoform that is present in both short- and long-term chromaffin cell cultures, whereas stimulation of enkephalin biosynthesis requires a protein kinase C isoform whose expression is downregulated in long-term chromaffin cells cultures, at least under the conditions described here. Differential involvement of PKC isoforms in calcium channel regulation has been described.[32,33] The present results suggest that there are indeed differential signaling pathways to gene transcription that rely on utilization of PKC isoforms in chromaffin cells, although whether such modulation occurs *in vivo* as well as in culture remains to be investigated.

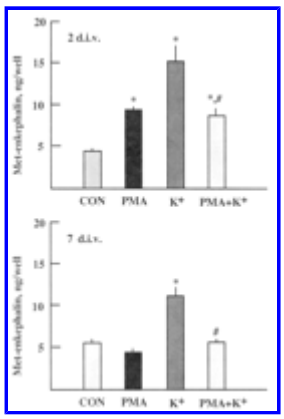

FIGURE 1. Stimulation of proenkephalin A biosynthesis by PMA is dependent on time of culture of bovine chromaffin cells. Cells were prepared as described in Materials and Methods as nondifferentially plated cells. After 2 or 7 days *in vitro* (48 or 168 h after cell isolation), medium was changed to complete medium containing PMA, 40 mM K^+, or both. Medium on long-term cultures was also changed on day 5. Similar results were obtained with cells that were first differentially plated on day 1. *Indicates significantly greater than control values of Met-enkephalin at $P < 0.05$; # indicates significantly less than 40 mM K^+ values at $P < 0.05$, using Student's *t* test. Values represent the mean ± SEM of four determinations (wells) from a single chromaffin cell preparation. Experiment was repeated once with similar results.

View larger version (27K): [in this window] [in a new window]	Increased biosynthesis of VIP in response to 100 nM PMA was similar when PMA was added either at 2 or 7 d.i.v. (control, 50 ± 2 pg/well; 0.1 µM PMA 3428 ± 415 pg/well at 2 d.i.v., control 52 ± 2; 0.1 µM PMA 4567 ± 371 pg/well at 7 d.i.v.; total immunoreactive VIP per well ± SEM, *n* = 4; 72 hours of exposure to PMA initiated at d.i.v. indicated). Induction of galanin mRNA by PMA likewise occurred at both 2 and 7 d.i.v. (data not shown).

PACAP REGULATION OF NEUROPEPTIDE BIOSYNTHESIS BY PROTEIN KINASE C

In all subsequent experiments described in this report, bovine chromaffin cells (BCCs) were cultured as previously reported.[28,29] In brief, 500,000 cells per well were differentially plated and treated with PACAP-27 (100 nM) or PMA (100 nM). Parallel cultures were also treated with the calcineurin inhibitor ascomycin (0.1 µM) or the protein synthesis inhibitor cycloheximide (0.5 µg/mL) in addition to either PACAP or PMA. Treatments commenced at 2 days *in vitro* (2 d.i.v.; acute culture) so that the effects of pharmacological treatments on enkephalin and VIP induction following stimulation by PACAP or PMA could be directly compared. Cells were harvested for neuropeptide radioimmunoassay (RIA) 72 hours after treatment[28] or extracted 18-24 h after drug additions into sodium dodecylsulfate-EDTA-Tris buffer containing 100 mg/mL of proteinase K for mRNA.[7] Northern blot analysis was performed with a nicktranslated human cDNA or AccI cut 386-basepair fragment of bovine VIP cDNA. Drugs were initially dissolved in ethanol, water, or complete medium such that the final concentration of vehicle in incubation medium was 0.1% (vol/vol).

Both 100 nM PACAP-27 and 100 nM PMA elevate VIP peptide levels more than 30-fold, and VIP mRNA levels greater than 10-fold by densitometric scanning following Northern blot hybridization (Fig. 2A). Elevation of VIP mRNA or peptide by PACAP was completely blocked by coapplication of ascomycin, as reported previously,[8] but not by cycloheximide (CHX, 0.5 µg/mL). In contrast, the effects of PMA on VIP biosynthesis were not inhibited by ascomycin, but were completely dependent on new protein synthesis, that is, blocked by CHX (Fig. 2B). These differential pharmacological profiles of VIP induction by PMA and PACAP-27 suggest that PACAP stimulation of VIP biosynthesis in chromaffin cells is independent of protein kinase C, but is dependent on elevation of intracellular calcium, leading to activation of the calcium-calmodulin-dependent protein phosphatase calcineurin (CnA). Intracellular calcium elevation driving VIP induction by PACAP is supplied by calcium entering the cell through either L- or non-L-type calcium channels (Fig. 3). PACAP-induced elevation of enkephalin peptide levels is also blocked by ascomycin (S.-H. Hahm, C. Hamelink, C.-M. Hsu, and L. E. Eiden, unpublished observations). Dependence on calcineurin points toward a requirement for elevation of intracellular calcium in PACAP signaling to the Penk gene, yet blockade of calcium entry by either L- or

non-L-type voltage-sensitive calcium channels, or both, fails to block enkephalin upregulation by PACAP (Fig. 3). Blockade of PACAP signaling through PKC is suggested by chelerythrine inhibition of PACAP-induced elevation of Metenkephalin levels (Table 1). We hypothesize that PACAP signaling to the enkephalin gene is mediated through the theta isoform of protein kinase C or another novel (n)-type PKC, since PKC-theta is reported to activate CREB independently of either PKA or MAPK in T lymphocytes,[36] and activates AP-1 via several pathways in a variety of cells.[37] Both AP-1 and CREB are reported to enhance enkephalin gene transcription via binding to the ENKCRE2 *cis*-regulatory element.[38-40] Thus, either of these *trans*-activating proteins may be involved in enkephalin gene transcription via binding to the ENKCRE2, depending on the first messenger and subsequent signaling pathways employed.

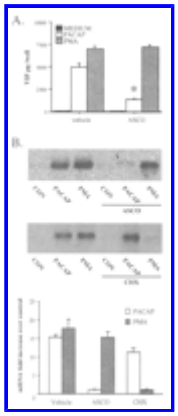

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FIGURE 2. PACAP and PMA stimulation of VIP biosynthesis are distinguished by different requirements for calcineurin and *de novo* protein synthesis. (**A**) VIP peptide induction by PACAP and PMA are differentially sensitive to ascomycin. Experiments were carried out as described in Materials and Methods. Values represent the mean ± SEM of quadruplicate wells from a single cell dispersion, repeated once with similar results. (**B**) VIP mRNA induction by PACAP and PMA are differentially sensitive to ascomycin and to cycloheximide. RNA was harvested, and Northern blot hybridization carried out with a ^{32}P-labeled bVIP cDNA probe. Typical results of Northern hybridization are illustrated. Bovine VIP mRNA levels were quantified in triplicate samples for each condition by densitometric scanning of autoradiograms made by exposing hybridized Northern blots for 2 days.

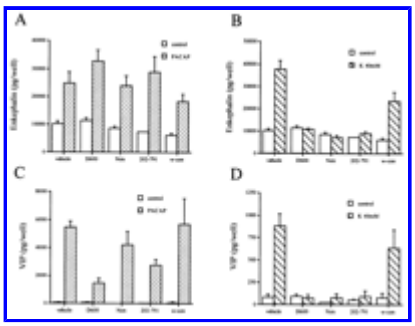

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FIGURE 3. PACAP and KCl stimulation of Met-enkephalin and VIP biosynthesis are distinguished by different requirements for calcium influx. (**A**) Enkephalin peptide levels after treatment with 10 nM PACAP-27 in the presence of calcium channel blockers. D600 was used at 30 µM, nimodipine (Nim) at 10 µM, (-)-202-791 at 1 µM, and w-conotoxin MVIIC (w-con) at 1 µM. Inhibitors were added 30 min before addition of PACAP-27 at 10 nM, and cells and media harvested for radioimmunoassay for VIP and Met-enkephalin 72 hours later. (**B**) Met-enkephalin levels following treatment with KCl (40 mM) in the presence and absence of channel blockers. Experimental design and conditions as described for (A) above. Met-enkephalin peptide data (3A and 3B) represent the mean ± SEM of three to four determinations (wells) from a single experiment. (**C**) VIP peptide levels after treatment with 10 nM PACAP-27 in the presence of calcium channel blockers. Experimental

	design and conditions as described for (A) above. VIP peptide data (3C and 3D) are pooled from two separate experiments with three to four determinations (wells) from a single experiment. (**D**) VIP peptide levels following KCl (40 mM) in the presence and absence of calcium channel blockers. Experimental design and conditions as described for (A) above.

View this table: [in this window] [in a new window]	**TABLE 1. Met-enkephalin peptide induction by PACAP-27: effects of inhibition of MAPK, PKA, and PKC**

PACAP REGULATION OF NEUROPEPTIDE GENE TRANSCRIPTION AND PROTEIN KINASE A

PACAP is reported to elevate cAMP levels via PAC1 receptor coupling to adenylate cyclase through Gs[41] and in fact does elevate intracellular cAMP in bovine chromaffin cells at nanomolar concentrations.[42,67] Thus, the possibility that PACAP acts through activation of protein kinase A was also examined in bovine chromaffin cells. The protein kinase A inhibitor H89 at 10 μM failed to block PACAP-induced upregulation of either enkephalin or VIP peptide levels (Table 1). This concentration of H89 is inhibitory for forksolin-induced elevation of VIP mRNA in NBFL neuroblastoma cells (Hamelink, unpublished observations).

THE CASE FOR COMBINATORIAL REGULATION OF NEUROPEPTIDE BIOSYNTHESIS BY PACAP IN BOVINE CHROMAFFIN CELLS

PACAP-27 at nanomolar concentrations increases levels of the neuropeptides Met-enkephalin and VIP in bovine chromaffin cells, via elevation of the mRNAs, and thus presumably the transcription of the genes encoding these neuropeptides. What are the protein kinase signaling pathways that mediate this regulation? First, enkephalin is an already-abundant mRNA and peptide species in chromaffin cells whose abundance is increased several-fold (3-5-fold) by PACAP, while VIP is present at relatively low levels and is upregulated many-fold (>100-fold by

quantitative measurements of VIP mRNA[67]). Enkephalin is regulated by calcium entry (depolarization), by cyclic AMP elevation, by phorbol ester, and by PACAP to a similar extent. Because the effects of cyclic AMP elevation, protein kinase C stimulation, and calcium influx through depolarization are not synergistic as for VIP, and perhaps not even additive, we hypothesize that while PACAP activates multiple signaling pathways to the enkephalin gene, some of these may be essentially redundant. We further hypothesize that although both PACAP- and KCl-induced elevation of Penk mRNA are sensitive to inhibition by CnA (S. H. Hahm, C. Hamelink, and L. E. Eiden, in preparation), PACAP regulation of enkephalin biosynthesis reflects stimulation of an alternative pathway to that initiated by calcium influx, because it is unaffected by blockade of calcium channels by D600 (see Fig. 3). VIP biosynthesis is regulated by calcium entry and by cyclic AMP elevation to the same extent as enkephalin transcription (3-5-fold) but is much more strongly upregulated by PACAP (several hundred fold stimulation of VIP mRNA when measured by Q-RT-PCR). This, we hypothesize, is because the VIP gene responds to calcium and cAMP combinatorially, because of the presence on the VIP gene of *cis*-active elements that respond separately to calcium and cAMP, and synergistically to both.

The nature of the signaling pathways mediating the effects of PACAP on enkephalin and VIP biosynthesis are not yet known. The simplest assumption is that two pathways are involved, one initiated by cAMP and one by calcium, for regulation of both genes and that (see above) this combinatorial regulation is obligate but not synergistic for the enkephalin gene and synergistic for the VIP gene. We have used static network analysis (see L. Lok, this volume) with approximately 60 known signaling dyads (see CClist, below) that are components of pathways linking cyclic AMP and calcium to genes containing TRE- and CRE-responsive transcriptional elements and the static network analysis tool pathFinder (http://pathfinder.nimh.nih.gov) to delimit the possible pathways linking PAC1 receptor occupancy to transcriptional activation of the enkephalin and VIP genes. The following assumptions are included in the analysis, based on the literature and current pharmacological data presented here, about the regulation of these genes in bovine chromaffin cells[7,8,19,26,28,39,43-51] and the characteristics of the signaling response elements within each gene obtained from transient expression/reporter gene studies in a variety of cell systems.[8,38,40,47,52-64] We assume here that (i) the enkephalin gene can be regulated at a proximal *cis*-active transcriptional element acting as a compound CnARE/CaRE (calcineurin/calcium-response element) and CRE (cAMP-response element), whereas the VIP gene is regulated via simultaneous activation of two spatially distinct (C. Hamelink and L. Eiden, in preparation) *cis*-active elements, one a promoter-distal CnARE/CaRE and the other a promoter-proximal CRE; (ii) regulation of the VIP gene does not require activation of PKC, while enkephalin gene activation by PACAP may require activation of an as-yet uncharacterized isoform of PKC, designated here as the (candidate) PKC-theta; (iii) activation of the VIP gene but not the enkephalin gene requires calcium influx; (iv) activation of the VIP and enkephalin genes is occurring in the same cell, that is, that the same signaling components are available to both genes; and (v) calmodulin kinase II, but not calmodulin kinase IV, is present in bovine chromaffin cells; and (vi) protein kinase A is not involved in the signaling to either the enkephalin or VIP genes. Pathways were eliminated that did not require a step involving both cyclic AMP and calcium for VIP, and a step involving cyclic AMP but not calcium for enkephalin. The number of pathways obtained when no exclusions are made is very large (greater than 200). The results are as follows.

For VIP, the following potential pathways were obtained after exclusion of PLC activation (10-100 nM PACAP induction of VIP peptide and mRNA is not blocked by 10 μM U773122, a concentration that inhibits enkephalin peptide induction by 10-100 nM PACAP—C. Hamelink, C.-M. Hsu, and L. Eiden, unpublished observations); exclusion of PKA activation (lack of blockade of PACAP-induced elevation of VIP peptide and mRNA by 10 μM H89—Table 1); and exclusion of PKC activation (lack of involvement of PKC in PACAP induction of VIP—see Figs. 1 and 2 above).

Activated: PACAP Exclude: CaMKIV PKA PKC PKC-theta PLC Target: VIPgeneholo

Path 1-
VIPgeneholo VIPCnARE/CaRE NFAT CnA CaM Ca^{2+}in PACAP
VIPgeneholo VIPCRE CREB RSK2 ERK MEK Raf Rap1 cAMP-GEFII cAMP AC
Ca^{2+}in PACAP

Path 2-
VIPgeneholo VIPCnARE/CaRE NFAT CnA CaM Ca^{2+}in PACAP
VIPgeneholo VIPCRE CREB RSK2 ERK MEK Raf Rap1 cAMP-GEFII cAMP AC
PACAP

Path 3-
VIPgeneholo VIPCnARE/CaRE NFAT CnA CaM Ca^{2+}in PACAP
VIPgeneholo VIPCRE CREB RSK2 ERK MEK Raf Ras RasGRF Ca^{2+}in PACAP

Path 4-
VIPgeneholo VIPCnARE/CaRE NFAT CnA CaM Ca^{2+}in PACAP
VIPgeneholo VIPCRE CREB RSK2 ERK Ras RasGRF Ca^{2+}in PACAP

Synergistic activation of the VIP gene via its CRE and CnARE/CaRE elements can occur via simultaneous activation of cAMP and calcium pathways (Paths 1 and 2), or activation of two separate calcium-initiated pathways (Paths 3 and 4). Work in progess will determine whether cAMP is indeed required for PACAP signaling to the VIP holo gene, as strongly suggested by the dramatic synergism in VIP gene regulation observed in the presence of agents that independently elevate cAMP, and increase intracellular calcium (C. Hamelink, H.-Y. Lee, M. Grimaldi, and L. Eiden, in preparation). D600 blocks PACAP stimulation of VIP bioysnthesis not to basal levels, but to the levels seen in the presence of forskolin or 8-Br-cAMP alone.[67] This implies that calcium-influx activation of adenylate cyclase is probably not occurring in chromaffin cells, or at least is not required for signaling to the VIP gene. It also implies that at least one component of combinatorial regulation of VIP gene transcription by PACAP is independent of calcium influx. With these considerations in mind, Path 2 is the most likely candidate pathway for VIP gene regulation by PACAP in bovine chromaffin cells, given the current information available.

The following pathways were obtained for enkephalin after exclusion of Ca^{2+} influx (i.e., the activation step PACAP→Ca^{2+}in is excluded, whereas PACAP→PLC→IP3→Ca^{2+}mob is preserved) because

PACAP signaling to the enkephaline gene is not blocked by D600 (Fig. 3); exclusion of CaMKIV (not found in chromaffin cells); exclusion of ERK (PACAP induction of enkephalin peptide is not blocked by 10 μM U0126, see Table 1); and exclusion of PKA (PACAP signaling to the enkephalin gene is not blocked by H89, see Table 1).

Activated: PACAP Exclude: Ca^{2+} in CaMKIV ERK PKA Target: PEnkgeneholo

Path 1-
PEnkgeneholo ENKCRE AP-1 Fos CREB PKC-theta DAG PLC PACAP
PEnkgeneholo ENKCRE AP-1 Jun CREB PKC-theta DAG PLC PACAP
PEnkgeneholo ENKCnARE/CaRE NFAT CnA CaM Ca^{2+}mob IP3 PLC PACAP

Path 2-
PEnkgeneholo ENKCRE CREB PKC-theta DAG PLC PACAP
PEnkgeneholo ENKCnARE/CaRE NFAT CnA CaM Ca^{2+}mob IP3 PLC PACAP

These two pathways can potentially be distinguished by treatment with the protein synthesis inhibitior cycloheximide, since immediate early gene regulation (Fos/Jun) by *de novo* protein synthesis would be abrogated, while proenkephalin mRNA elevation by pre-existing CREB would not be sensitive to protein synthesis blockade. If additional pathways are involved besides those shown, they are either novel (as yet undiscovered) or not yet incorporated into the dyad listing for pathFinder (reproduced as CC list, below).

CCLIST

Most abbreviations are standard, except for VIPgeneholo (indicates CRE and CaRE of VIP gene) and Penkgeneholo (indicates both CRE-like and TRE-like responsiveness of proenkephalin A gene contained within the CRE2 element within the proximal promoter of the proenkephalin gene). VIPgeneholo indicates coactivation of VIP gene transcription at a calcium-response element (CnARE/CaRE) activatable by NFAT and CREB, and a TRE (activatable by AP-1) and ENKgeneholo indicates activation of enkephalin gene transcription at the ENKCRE2 acting either as a nominal CRE or TRE.

Results obtained and described above used the 2001.09 version of CClist shown here, which will be modified (expanded) continuously beginning in early 2002. First item in each column represents target protein, factor, or process activated by items to the right. Presence of two or more items to the right indicates requirement for coactivation. Activation of both cAMP and calcium (cAMP/Ca^{2+}) is a device that allows prediction of pathways activated when agents (e.g., elevated potassium chloride and forskolin) that allow simultaneous elevation of cAMP and influx of calcium are applied to chromaffin

cells.

The protein kinases included in CClist are PKA, PKC, CaMKII, CaMKIV, and the three MAPKs (mitogen activated protein kinases, aka microtubule associated protein kinases) ERK, p38, and JNK (Jun NH_2-terminal kinase).[18] "Big" MAPK and other MAPKs including individual ERKs, isoforms of PKC, and CaMKI are not yet incorporated into CClist because their presence and roles in chromaffin cells have not yet been addressed. CaMKIV is included, although absent from chromaffin cells[65] because its signaling pathways are prominent in signal transduction in some neurons; and contrast with these pathways, and their possible recruitment in chromaffin cells expressing exogenous CaMKIV, is useful. Ras is included as both directly activating ERK1/2, or activating ERK1/2 via Raf, to indicate that Raf-independent activation of ERK by Ras is not ruled out in signaling by neuroendocrine cells. The target genes discussed here, VIP and Penk (proenkephalin A) are notated as VIPgeneholo and Penkgeneholo to indicate full transcriptional activation of each gene by bipartite regulation via multiple *cis*-active elements defined biochemically and pharmacologically as ENKCRE, VIPCRE, ENKCnARE/CaRE, and VIPCnARE/CaRE. CRE elements are those responsive to CREB and AP-1 (i.e., canonical or classical cyclic AMP-response element; called CRE for VIP gene and ENKCRE2 for Penk gene) and CnARE/CaRE elements are those responsive to calcium signaling through calcineurin, as defined by blockade of response with FK506, ascomycin, or cyclosporin A, but not rapamycin.

AC Ca^{2+}in
AC Ca^{2+}mob
AC FOR
AC PACAP
AP-1 Fos Jun
ATF1 CaMKII
ATF1 PKA
ATF2 JNK
Ca^{2+}in ACh
Ca^{2+}in cAMP/Ca^{2+}in
Ca**2**+mob IP3
Ca^{2+}in KCl
Ca^{2+}in PACAP
CaM Ca^{2+}mob
CaM Ca^{2+}in
CaMKII CaM
CaMKIV CaM
cAMP cAMP/Ca^{2+}in
cAMP AC
cAMP-GEFII cAMP
CnA CaM
CRE CREB
CREB CaMKIV

CREB MAPKAPK2/3
CREB PKA
CREB PKC-theta
CREB RSK2
DAG PLC
Elk ERK
ENKCRE AP-1
ENKCRE CREB
ENKCnARE/CaRE NFAT
ERK MEK
ERK Ras
Fos CREB
IP3 PLC
Jun CREB
MAPK Rap1
MAPKAPK2/3 p38
MEK Raf
neurites ERK
NFAT CnA
PEnkgeneholo ENKCRE ENKCnARE/CaRE
PKA cAMP
p38 JNKK
p38 MKK6
JNK JNKK
JNKK MEKK
Jun JNK
PKC Ca^{2+}in DAG
PKC Ca^{2+}mob DAG
PLC PACAP
PKC-theta DAG
Raf PKC
Raf Rap1
Raf Ras
Rap1 cAMP-GEFII
Ras RasGRF
RasGRF Ca^{2+}in
RasGRF Ca^{2+}mob
RSK2 ERK
TRE AP-1
VIPCnARE/CaRE NFAT
VIPCRE CREB
VIPgeneCRE VIPCRE

VIPgeneCnARE/CaRE VIPCnARE/CaRE
VIPgeneholo VIPCnARE/CaRE VIPCRE

It is extremely important for us to note that the use of pathFinder as a static network analysis tool provides at this point nothing more than a bookkeeping mechanism for rapidly and reliably checking the biochemical and cell biological assumptions built into a proposed pathway analysis. Its authority derives only from the understanding of the authors about the likelihood of the pathway components shown, and its usefulness will be tested only by (i) how interestingly pathFinder pathways will lead to explicit data contradictions resolvable by further experimentation and (ii) a steady accretion of pathway authority through inputs from other chromaffin cell biologists. Importantly, proposed pathways might well require significant alteration to include new pharmacological as well as biochemical information. Thus, the role of CaMKII in PACAP signaling is not included in the pathways proposed here because information about blockade of PACAP signaling by CaMKII inhibitors remains equivocal: inhibition of signaling to the VIP gene, for example, depends on calcium influx, which can be affected nonspecifically by agents such as KN-93 and KN-62. The ability to introduce dominant negative and constitutively active signaling components into bovine chromaffin cells with an efficiency high enough to measurably affect endogenous single-copy gene transcription will be a major step forward in unraveling the complete PACAP signal transduction pathway to any one of the genes regulated *trans*-synaptically by this critical adrenomedullary neurotransmitter.

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- TOP
- ABSTRACT
- INTRODUCTION
- REGULATION OF NEUROPEPTIDE GENE...
- PACAP REGULATION OF NEUROPEPTIDE...
- PACAP REGULATION OF NEUROPEPTIDE...
- THE CASE FOR COMBINATORIAL...
- CCLIST
- REFERENCES

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Mol. Pharmacol., December 1, 2003; 64(6): 1503 - 1511.
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