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Characterization of an Endooligopeptidase A-Like Protein in PC12 Cells: Activity Modulation by cAMP but Not by Basic Fibroblast Growth Factor

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Abstract Endooligopeptidase A is a putative neuropeptide-metabolizing enzyme. It converts small enkephalin-containing peptides into the corresponding enkephalins and inactivates biopeptides such as bradykinin and neurotensin in vitro. We investigated the presence of endooligopeptidase A in PC12 cells. This cell line was derived from a rat pheochromocytoma tumor and resembles fetal chromaffin cell. Depending on the supplements added to the cell culture, this cell line can be differentiated into mature chromaffin cell or sympathetic neuron-like cell. Endooligopeptidase A activity was measured in soluble cellular extracts using a specific fluorogenic substrate QF-ERP7. The PC12 endooligopeptidase A-like activity shared similar but not identical biochemical properties with rabbit brain endooligopeptidase A. Similarly to rabbit brain endooligopeptidase A, the PC12 endooligopeptidase A-like activity was enhanced by DTT, totally inhibited by DTNB and 1-10 Phenanthroline, partially inhibited by cFP-AAF-pAb, and not affected by PMSF. Furthermore, the PC12 endooligopeptidase A-like activity displayed identical elution profile as rabbit brain endooligopeptidase A in gel filtration and anion-exchange chromatography. In addition, an antiserum raised against rabbit brain endooligopeptidase A cross-reacted with a 71 kDa component from PC12 cell extracts in Western blotting and was also able to partially neutralize the PC12 endooligopeptidase A-like activity. Treatment of PC12 cells with basic fibroblast growth factor (bFGF), a neurotrophic factor for this cell line, did not modify the specific activity of this enzyme. However, cAMP analogs decreased the specific activity of the enzyme. These results indicate the presence of an endooligopeptidase A-like activity in PC12 cells which is modulated by cAMP but not by bFGF.

Key words: endooligopeptidase A, PC12 cells, basic fibroblast growth factor, cyclic AMP, opioid peptides, EC 3.4.22.15

PC12 cell is a clonal cell line derived from catecholamine-secreting tumor (pheochromocytoma) of rat adrenal medulla [Greene and Tischler, 1976]. When cultured under normal conditions, these cells resemble fetal adrenal chromaffin cells in morphology and biochemistry, which can be induced to differentiate into two different pathways: (1) to mature chromaffin-like cell by glucocorticoids [Greene and Tischler, 1982; Guroff, 1985], sodium butyrate [Naranjo et al., 1986; Byrd et al., 1987], or coculture with adrenal medullary endothelial cells [Mizrachi et al., 1990]; and (2) to sympathetic neuron-like cell by neurotrophic factors like nerve growth factor (NGF) [Greene and Tischler, 1982; Guroff, 1985] or fibroblast growth factors [Togari et al., 1983; Burgess and Maciag, 1989]. Furthermore, these cells express several bioactive peptides like enkephalins, dynorphin, and neurotensin [Tischler et al., 1982; Naranjo et al., 1986; Byrd et al., 1987;

Abbreviations used: bFGF, basic fibroblast growth factor; cFP-AAF-pAB, N-[1-(R,S)-carboxyl-2-phenylethyl]-Ala-Ala-Phe-p-amino-benzoate; dbcAMP, dibutyryl adenosine 3′:5′ cyclic monophosphate; DTNB, 5′5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EOPA, endooligopeptidase A; LH-RH, luteinizing hormone-releasing hormone; MEP, metallo-endopeptidase; NGF, nerve growth factor; PMSF, phenylmethylsulphonyl-fluoride; QF-ERP7, ortoaminobenzoyl-Gly-Gly-Phe-Leu-Arg-Arg-Val-N-(2,4 dinitrophenyl)-ethylenediamine; 1,10 Phenan, 1,10 Phenanthroline; 8brcAMP, 8-bromoadenosine 3′:5′ cyclic monophosphate.

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Margioris et al., 1992], whose expression is modulated by the chromaffinic and/or neurotrophic factors. These properties make this cell line a potentially interesting model to study the expression of enzymes thought to be involved in bioactive peptides metabolism.

Endooligopeptidase A (EOPA, formerly EC 3.4.22.19) is a putative bioactive metabolizing enzyme first described in rabbit brain as a cysteine-endopeptidase [Camargo et al., 1973; Shaw, 1990]. A similar enzyme was also described in rat brain and classified as a metalloendopeptidase (MEP 24.15, EC 3.4.24.15) [Orlowski et al., 1983]. The identity of these two enzymes is still controversial and awaiting confirmation [Camargo, 1991; Barret, 1991; Gomes et al., 1993], once both enzymes are able to hydrolyse and inactivate bioactive peptides such as bradykinin, neurotensin, and luteinizing hormone releasing hormone (LHRH) [Oliveira et al., 1976; Carvalho and Camargo, 1981; Camargo et al., 1982, 1984; Chu and Orlowski, 1985; Orlowski et al., 1989]. In addition, they convert by single cleavage small enkephalin-containing peptides (8–13 amino acidis residues) into [Leu5]-enkephalin or [Met5]-enkephalin [Camargo et al., 1985, 1987; Chu and Orlowski, 1985].

Using PC12 cells as a promising in vitro cellular model to study bioactive peptide metabolism, we described here the preliminary characterization of an EOPA-like activity in PC12 cells and we also showed evidence that cyclic AMP analogs but not FGF modulate this activity.

MATERIALS AND METHODS

Materials

Endooligopeptidase A (EOPA) from cytosol of rabbit brain purified to apparent homogeinity and monospecific mouse antibodies raised against the enzyme were obtained essentially as described [Carvalho and Camargo, 1981]. Metorphamide (Y1-G2-G3-F4-M5-R6-R7-V8-NH_2) was purchased from Cambridge Research Biochemicals (Cambridge, England). Dibutyryl adenosine 3′:5′ cyclic monophosphate (dbcAMP) and 8-bromoadenosine 3′:5′ cyclic monophosphate (8brcAMP), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), phenylmethylsulphonylfluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), 1-10 Phenanthroline (1-10 Phenan) were purchased from Sigma Chemical Co. (St. Louis, MO). The quenched fluorogenic substrate ortoaminobenzoyl-Gly-Gly-Phe-Leu-Arg-Arg-Val-N-(2,4 dinitrophenyl)-ethylenediamine (QF-ERP7) was synthesized by solid phase method and purified by preparative HPLC as described [Chagas et al., 1991]. bFGF was purified from human placenta [Costa et al., 1993] and N-[1-(R,S)-carboxyl-2-phenylethyl]-Ala-Ala-Phe-p-aminobenzoate (cFP-AAF-pAb) was a generous gift of Dr. M. Orlowski (Mount Sinai School of Medicine of the City University of New York, NY). All other reagents were of analytical grade.

Cell Culture

PC12 cells were a generous gift of Dr. A. Howllet (Saint Louis University, St. Louis, MO). Cells were grown in Dulbecco's Modified Eagle's Medium (DME) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2. For enzyme and Western-blotting assays, cells were grown on a 6–12 well microplate (Corning, Corning, NY) with the presence of the desired factors (5 ng/ml of bFGF and/or 1 mM dbcAMP or 8brcAMP or 10 μM forskolin). The cells were carefully washed with PBS without detaching them from the plates where they were further released by flushing with 0.5 ml of PBS. The cells were collected by centrifugation and resuspended in a small volume (100–500 μl) of 20 mM Tris-HCl, pH 7.5, and 0.15 M NaCl and broken by freezing/thawing 5 times. The homogenate was clarified by centrifugation at maximum speed for 25 min in a microcentrifuge at 4°C. The supernatant was used as the PC12 soluble cellular extract for enzyme assay and Western blotting.

Enzyme Assay

The assays using the quenched fluorogenic substrate QF-ERP7 were performed as described [Juliano et al., 1990] on a Shimadzu model RF-540 spectrofluorimeter set at the wavelengths of 319 nm (excitation) and 418 nm (emission). Prior to the addition of 10 μl of 3 mM QF-ERP7 in 30% dimethylsulphoxide to start the reaction, the samples were preincubated for 2 min in a thermostated cuvette in a final volume of 3.0 ml of Tris-HCl, pH 7.5, containing 0.5 mM DTT at 37°C. One mU of EOPA activity is defined as the amount of enzyme that is able to cleave 1 nmol of QF-ERP7/min/ml at 37°C at pH 7.5. For immunoneutralization assay, 0.02 mU of rabbit brain EOPA or PC12 EOPA-like protein in 50 μl were preincubated for 30 min with the amount of the anti-rabbit brain EOPA antiserum as indicated in Figure 4. Then, the volume was increased to 3.0

ml with Tris-HCl, pH 7.5, containing 0.5 mM DTT and the assay proceeded as described above.

Alternatively, EOPA activity was followed by RP-HPLC using metorphamide as substrate and the detection of the cleavage product [Met5]-enkephalin was done essentially as described [Camargo et al., 1987]. Briefly, the products of peptide hydrolysis were analysed over a Bondex 10/C18 column (300 × 2.9 mm; Phenomenex) using a 12 min linear gradient of acetonitrile (5–35%) in 0.1% H_3PO_4 (vol/vol), pH 2.7, at a flow rate of 2.0 ml/min. The retention time of the products was confirmed by the corresponding synthetic peptide used as standards. The enzymatic reaction was stopped by the addition of 10 µl of H_3PO_4. Quantification of [Met5]-enkephalin generation was calculated measuring the peak height or area. Enzyme unit was defined as the amount of [Met5]-enkephalin released from metorphamide hydrolysis/min/ml in 25 mM Tris-HCl containing 0.15 M NaCl and 0.5 mM DTT at 37°C. Protein determination was according to Lowry et al. [1951], using BSA as standard.

Gel Filtration and Anion-Exchange Chromatography

The Mr of PC12 cell EOPA-like protein was determined by gel filtration on a Sephacryl S-300 (97 × 1.5 cm) equilibrated in 25 mM Tris-HCl, pH 7.5, and 0.15 M NaCl. The column was previously calibrated with Mr standards (Pharmacia, Uppsala, Sweden). The samples were applied and eluted at a constant flow of 0.5 ml/min in equilibration buffer. Fractions of 2 ml were collected for enzymatic activity determination. Anion-exchange chromatography was carried on a TSK DEAE-5PW column (10 × 0.8 cm) equilibrated in 25 mM Tris-Gly buffer, pH 8.2, at a constant flow rate of 0.5 ml/min. The adsorbed proteins were eluted in a 1-h linear gradient from 0 to 0.3 M NaCl in 25 mM Tris-HCl, pH 7.5. Protein elution was followed spectrophotometrically at 280 nm. Fractions of 0.5 ml were collected.

Western Blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was according to Laemmli [1970]. The gel was 10% acrylamide and 2.6% cross-linking and the samples were dissolved in the SDS sample buffer containing 10% 2-mercaptoethanol. After running, the proteins were semi-dry transferred to a nitrocellulose sheet by a Multiphor 2117 Pharmacia-LKB (Pharmacia) system, according to the manufacturer's instructions. The total protein after transfer was visualized and photographed after staining with 0.1% (w/v) Ponceau S in 10% acetic acid (v/v). Mouse anti-rabbit brain EOPA was used at 1:1,000 dilution in a 1-h incubation time. Immunoreactive components were revealed by immuno-alkaline phosphatase conjugates and NBT/BCIP essentially as described [Harlow and Lane, 1988] and quantified by densitometry (Laser Densitometer Shimadzu model CS-9000).

RESULTS

PC12 EOPA-Like Activity Characterization

The presence of EOPA-like activity in the soluble fractions of PC12 cells was first indicated by using a very specific assay for EOPA, based on the cleavage of the quenched fluorogenic substrate QF-ERP7 [Juliano et al., 1990]. In order to further characterize this activity, we undertook biochemical, chromatographic, and immunological studies.

Table I shows the effects of several compounds on PC12 EOPA-like activity. Similarly to rabbit brain EOPA, PC12 EOPA-like activity was enhanced by DTT and inhibited by DTNB, a thiol reactive compound [Soper et al., 1979]. In addition, both rabbit brain EOPA and PC12 EOPA-like activities were inhibited by the metal chelator 1-10 Phenan but not affected by PMSF. cFP-AAF-pAB which is a specific competitive inhibitor for MEP [Orlowski et al., 1988] was able to partially inhibit rabbit brain EOPA and PC12 EOPA-like activity. However, PC12 EOPA-like activity was partially inhibited by EDTA, whereas rabbit brain EOPA was not affected in this condition. These results indicate that, similarly to rabbit brain EOPA, the PC12 EOPA-like activity displays both cysteinyl and metalloendopeptidase features but not the properties of a serine-protease.

Gel filtration chromatography of PC12 soluble cellular extracts on Sephacryl S300 showed an EOPA-like activity peak with an apparent Mr of 70–72 kDa (Fig. 1). DEAE-TSK chromatography of PC12 soluble cellular extracts also showed an EOPA-like activity being eluted with similar if not identical molar salt concentration as rabbit brain EOPA (Fig. 2). Moreover, using an antiserum raised against rabbit brain EOPA, an immunoreactive component of 71 kDa from PC12 cellular extracts was detected (Fig. 3).

TABLE I. Effects of Several Compounds on PC12 EOPA-Like Activity and Purified Rabbit Brain EOPA*

	PC12 EOPA (%)	Rabbit brain EOPA (%)
Control	100	100
0.5 mM DTT	200	200
1 mM DTNB	3	1
1 mM 1,10 Phenan	2	2
50 nM cFP-AAF-pAB	50	53
1 mM EDTA	51	100
1 mM PMSF	100	100

*Activity was assayed using the quenched fluorogenic substrate QF-ERP7.

However, this antiserum was only able to partially inhibit PC12 EOPA-like activity (Fig. 4). The maximum inhibition value was around 50%. All these data together suggest that PC12 EOPA-like protein is very similar but not identical to rabbit brain EOPA.

Negative Modulation of PC12 EOPA-Like Activity by cAMP Analogs but Not by BFGF

Until now, PC12 cells has been one of the most studied in vitro models of neuronal differentiation [Shafer and Atchison, 1991]. In the presence of NGF or FGFs, this cell line differentiates into sympathetic neuron-like cell [Greene and Tischler, 1976; Togari et al., 1983]. It was shown that cAMP analogs potentiate the neurotrophic response of PC12 cells to both NGF and bFGF [Gunning et al., 1981; Richter-Landsberg and Jastorff, 1986; Ho and Raw, 1992]. For this reason, we undertook studies in order to verify possible differences in EOPA-like activity in PC12 cells after induction to a neuronal phenotype switched by bFGF and/or cAMP analogs treatment. The results in Figure 5 showed that bFGF, which is a potent neurotrophic factor, did not affect the PC12 EOPA-like activity. However, soluble cellular extracts from cells treated with cAMP analogs, dbcAMP or 8brcAMP, showed a reduction of about 40% of the EOPA-like activity compared to control or bFGF-treated PC12 cells. Western-blotting studies (Fig. 3) did not clarify if this decreasing activity was due to a decrease of EOPA-like protein content. Statistical analysis from three independent blots did not show a significant difference among the densitometric values obtained from the bands (results not shown). The results also showed that the effect of cAMP analogs on PC12 EOPA-like activity was dominant over bFGF treatment (Fig. 5).

This negative modulation was further analysed by RP-HPLC using metorphamide as an

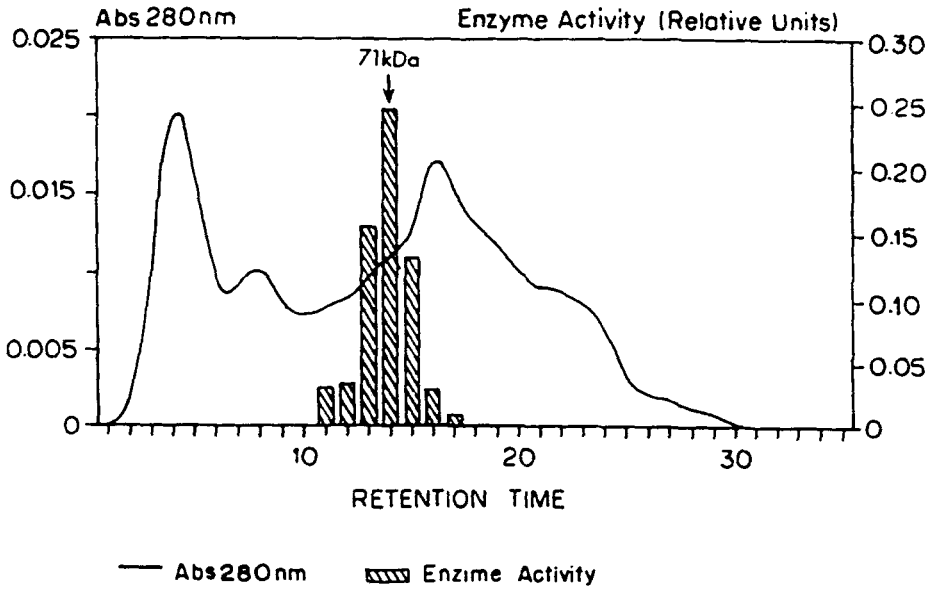

Fig. 1. Gel filtration of PC12 EOPA-like activity on Sephacryl S-300. PC12 EOPA-like activity (2 mU) was loaded on a Sephacryl S-300 column (97 × 1.5 cm) equilibrated in 25 mM tris-HCl, pH 7.5, and 0.15 M NaCl at a constant flow rate of 0.5 ml/min. Fractions of 2.0 ml were collected for activity determination using the quenched fluorogenic substrate QF-ERP7.

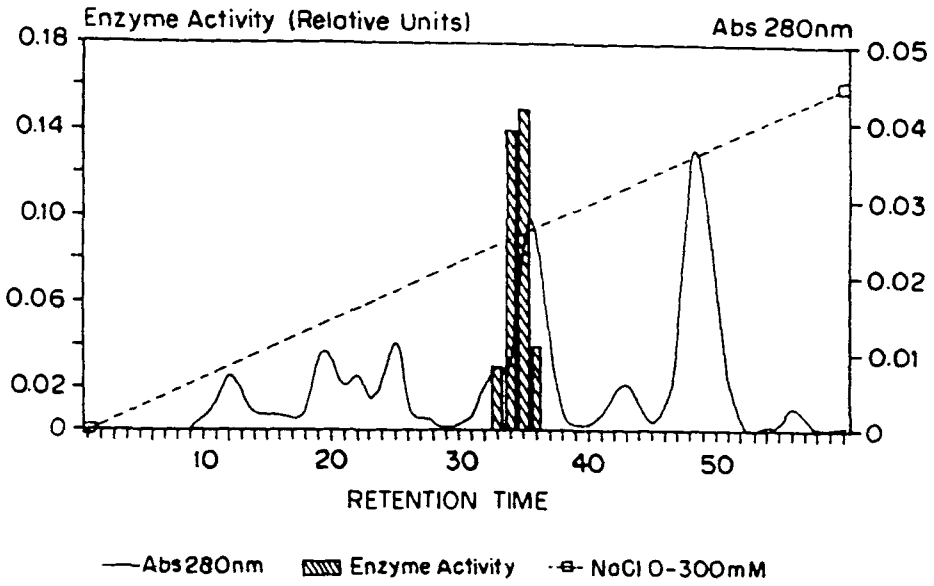

Fig. 2. Anion-exchange chromatography of PC12 EOPA-like activity on TSK DEAE-5PW. PC12 EOPA-like activity (0.5 mU) was loaded on a TSK DEAE-5PW column (10 × 0.8 cm) equilibrated in 25 mM Tris-Gly buffer, pH 8.2, at a constant flow rate of 0.5 ml/min. The adsorbed proteins were eluted in a 1-h linear gradient from 0 to 0.3 M NaCl in 25 mM Tris-HCl, pH 7.5. Fractions of 0.5 ml were collected for activity determination using the quenched fluorogenic substrate QF-ERP7.

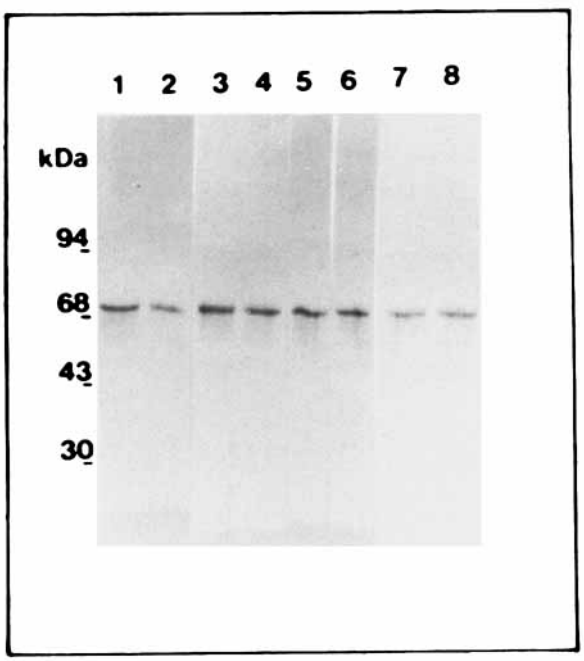

Fig. 3. Western blotting of PC12 soluble cellular extracts using mouse anti-rabbit brain EOPA antiserum. The cells were previously treated for 5 days with (1) none; (2) 1 mM dbcAMP; (3) 1 mM 8brcAMP; (4) 10 μM forskolin; (5) 5 ng/ml bFGF; (6) 1 mM dbcAMP/5 ng/ml bFGF; (7) 1 mM 8brcAMP/5 ng/ml bFGF; (8) 10 μM forskolin/5 ng/ml bFGF (See Materials and Methods for details). Quantifications of immunoreactive components from three independent blots were done by densitometry and did not show statistical difference among them.

EOPA substrate. In the presence of EOPA, metorphamide (Y1-G2-G3-F4-M5-R6-R7-V8-NH2) is cleaved, generating [Met5]-enkephalin. The results in Figure 6 confirm those shown in Figure 5. The generation of [Met5]-enkephalin (peak 1) is less when PC12 cells are either treated with dbcAMP alone or together with bFGF, presenting an inhibition around 25% in both treatments (Fig. 6c,d). Furthermore, there is a concomitant appearance of another substrate cleavage product (peak 2). This peak 2 is not present after incubation of metorphamide with soluble extracts of undifferentiated PC12 cells or PC12 cells treated with only bFGF. As a control, we also incubated extracts of PC12 cells treated with dbcAMP in the absence of metorphamide. In this condition, we did not detect the presence of peak 2 (not shown), indicating that peak 2 resulted from the cleavage of metorphamide. These data suggest the activation or induction of unknown protease(s) that cleaves metorphamide in a site different than that cleaved by EOPA in PC12 cells treated with cAMP analogs.

DISCUSSION

EOPA is a monomeric 70 kDa thiol-dependent endopeptidase [Carvalho and Camargo, 1981] able to metabolize several bioactive peptides comprising 8–13 amino acids in length [Camargo et

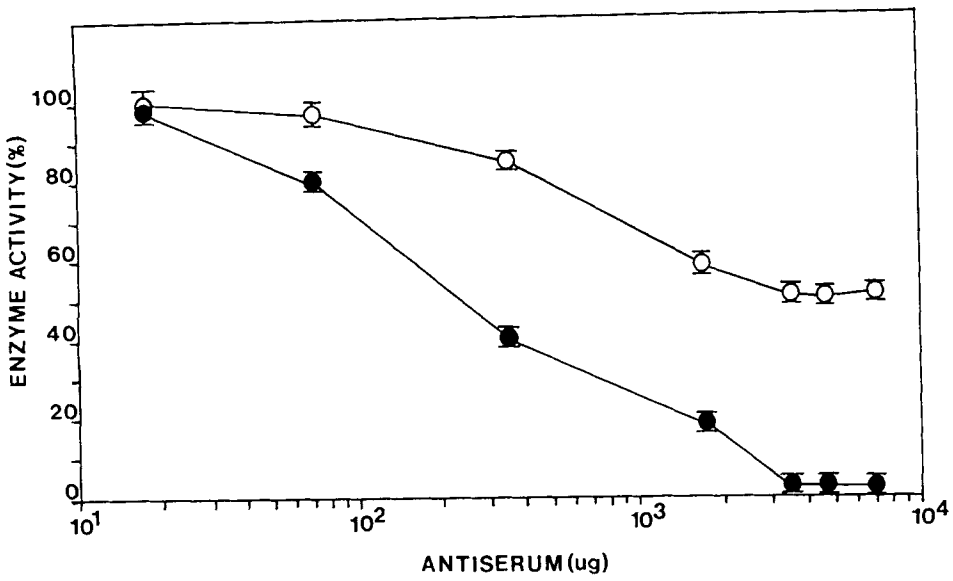

Fig. 4. Inhibition of PC12 EOPA-like activity by mouse anti-rabbit brain EOPA antiserum. Enzyme activity was assayed using the quenched fluorogenic substrate QF-ERP7. Indicated amount of anti-rabbit brain EOPA antiserum was incubated for 30 min at 37°C with 0.02 mU of rabbit brain EOPA (●) or PC12 EOPA-like activity (○) prior to the measurement of the enzymatic activity. The values expressed in % are relative to rabbit brain EOPA or PC12 EOPA-like activity preincubated with preimmune mouse balb-c antiserum, considered as 100%. Each point is the mean ± SE of three independent experiments.

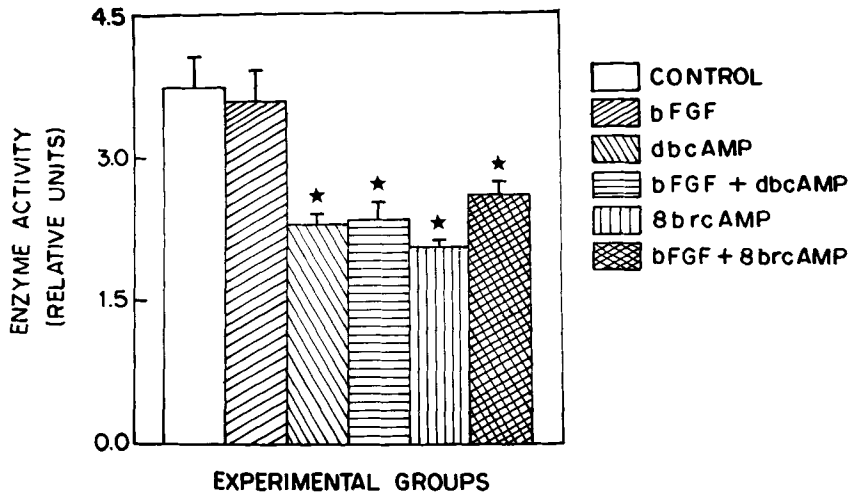

Fig. 5. Negative modulation of PC12 EOPA-like activity by cAMP analogs. EOPA activity was determined using the quenched fluorogenic substrate QF-ERP7. The cells were previously treated with none (control), 5 ng/ml bFGF and/or 1 mM dbcAMP or 1 mM 8brcAMP for 5 days. Each point is the mean ± SE of five independent experiments (*$P < 0.01$, two sample t-test).

al., 1994]. These include bradykinin, neurotensin, and opioid peptides. The main biochemical feature of EOPA is to biotransform and/or to inactivate these peptides. In the case of 8–13 amino acids opioid peptides, this enzyme is able to generate by single cleavage the smallest bioactive opioid peptides [Leu5]-enkephalin or [Met5]-enkephalin [for review, see Tisljar, 1993]. Although intensive biochemical characterization of this enzyme has been done since its first description [Camargo and Graeff, 1969; Camargo et al., 1973], little is known about its

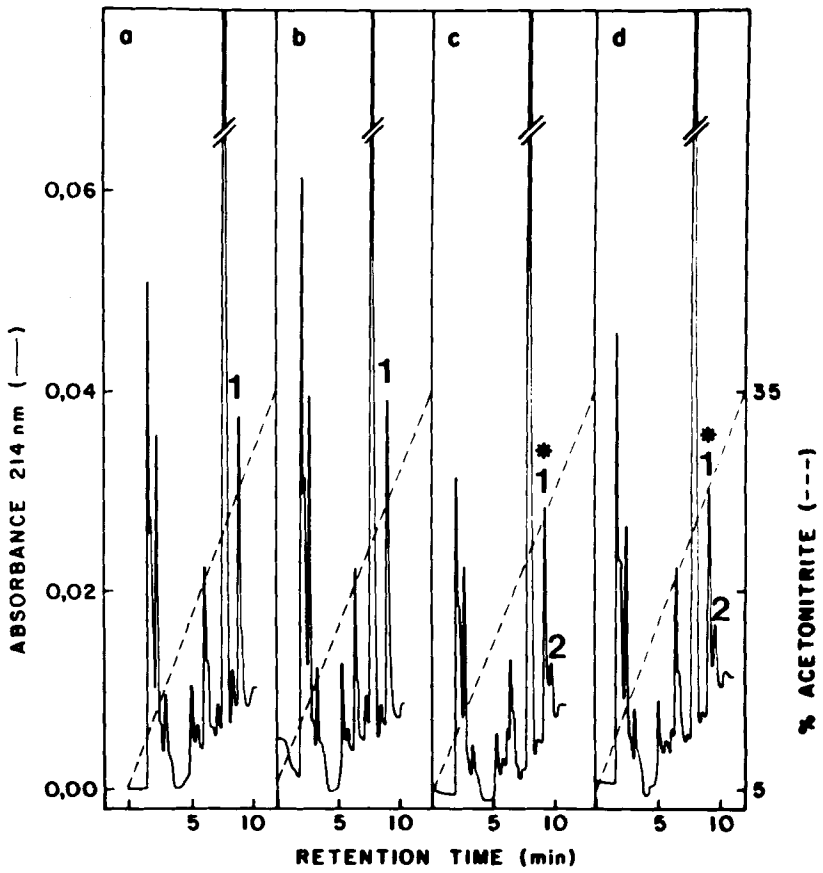

Fig. 6. Measurement of [Met5]-enkephalin generated by incubation of PC12 soluble cellular extracts on metorphamide by C18 RP-HPLC. PC12 cells were previously treated with (a) none; (b) 5 ng/ml bFGF; (c) 1 mM dbcAMP; (d) 5 ng/ml bFGF/1 mM dbcAMP. Peak (1), [Met5]-enkephalin generated by metorphamide cleavage; Peak (2), undetermined product generated from metorphamide cleavage. The chromatographic profiles shown in this figure are representative of 4 independent experiments (*$P < 0.01$, two sample t-test).

physiological and biological role. Based on colocalization of opioid peptides and EOPA in brain and retina neurons [Oliveira et al., 1990; Ferro et al., 1991], it was suggested that EOPA plays a role in opioid peptide generation. Using MEP 24.15 inhibitor, cFP-AAF-pAB, an EOPA-like enzyme characterized by Orlowski et al. [1983], a blockage of dynorphin degradation, a reduction of LH-RH degradation, and a decrease in the blood pressure in normotensive rats attributed to the inhibition of bradykinin degradation were shown [Molineaux and Ayala, 1990; Genden and Molineaux, 1991; Lasdun et al., 1989; Lasdun and Orlowski, 1990]. All these data together suggested a function of EOPA or EOPA-like proteins as bioactive peptide metabolizing enzymes.

PC12 cells are derived from a rat pheochromocytoma tumor able to differentiate into mature chromaffinic cells or into sympathetic neuron-like cells, depending on the external stimuli [Greene and Tischler, 1982; Guroff, 1985]. Besides this property, this cell line is rich in bioactive peptides such as opioid peptides, neurotensin, and dynorphin, whose level are modulated during differentiation to one or another phenotype [Tischler et al., 1982; Byrd et al., 1987; Mizrachi et al., 1990; Margioris et al., 1992]. We hypothesized that if EOPA acts as a bioactive peptide metabolizing enzyme: (1) we would be able to detect its presence in PC12 cells; (2) since the level of bioactive peptides changes according to PC12 differentiation, we inferred that EOPA activity would also be modulated during differentiation to one or another pathway.

Using a selective assay for EOPA based on the cleavage of the quenched fluorogenic substrate QF-ERP7, a 71 kDa protein was detected with

physical, biochemical, and immunological properties similar to purified rabbit brain EOPA. PC12 EOPA-like activity displayed identical behavior on gel filtration and anion exchange chromatography as purified rabbit brain EOPA. It also presented biochemical similarities with rabbit brain EOPA since it is activated by DTT and inhibited by the thiol reacting compound DTNB. The inhibition by the metal chelator 1,10 Phenan and inhibition by the MEP active site competitive inhibitor cPF-AAF-pAB [Orlowski et al., 1988] seem to argue that this enzyme shares the properties of the cysteine EOPA and those of the MEP as the thimet-oligopeptidase suggested by Barret [1991]. The similarity between rabbit brain EOPA and PC12 EOPA-like protein is also suggested by Western blotting which detected a 71 kDa band in PC12 cytosol extracts, which is indistinguishable from rabbit brain EOPA. On the other hand, there was a difference between PC12 EOPA-like and rabbit brain EOPA enzyme activity when assayed in the presence of EDTA. This difference may not be of importance since we have shown that a simple dilution of enzyme-chelating compound mixture was able to revert the inhibition [Camargo et al., 1987]. However, this result could reflect structural differences between these two enzymes (e.g., hydrofobicity of the catalytic pocket) [Hrycyna and Clarke, 1993]. This possibility is further supported by immunoneutralization assays using anti-rabbit brain EOPA antiserum. In contrast with the purified brain EOPA, which is fully inhibited by the antiserum, the PC12 EOPA-like activity was only partially inhibited. This result could be due to the presence of two immunologically distinct forms of EOPA-like activities or to structural differences between rabbit brain EOPA and PC12 EOPA-like proteins.

We also showed that the EOPA-like activity present in the PC12 cells soluble extracts is modulated by cAMP analogs but not by bFGF. Although cAMP analogs are poor neurotrophic factors for PC12 cells [Richter-Landsberg and Jastorff, 1986; Ho and Raw, 1992] compared to NGF or bFGF, they are potent neurotrophic agents for rat sympathetic sensory neurons [Rydel and Greene, 1988] and are able to potentiate NGF- or bFGF-induced neurite outgrowth in PC12 cells [Gunning et al., 1981; Richter-Landsberg and Jastorff, 1986; Ho and Raw, 1992]. Using both the quenched fluorogenic assay [Juliano et al., 1990] or detection of [Met5]-enkephalin formation by RP-HPLC after hydrolysis of metorphamide as EOPA substrate, a decrease was observed in the EOPA-like activity of extracts from cells treated with cAMP analogs. The influence of cAMP on opioid peptides metabolism is well known. Previous work has shown that cholinergic stimulus is connected to the adenylate cyclase system in rat adrenal medulla. Surgical denervation of adrenal gland leads to a decrease in cAMP [Guidotti and Costa, 1973] and the transection of the splanchinic nerves inervating the rat adrenal medulla results in a 145% increase in [Leu5]-enkephalin content. The same result was mimicked by nicotinic receptor antagonist, supporting the idea that cholinergic nicotinic stimulation decreases [Leu5]-enkephalin in the rat adrenal medulla, at least, in part, through cAMP decrease [La-Gamma et al., 1984]. If the PC12 EOPA-like protein is the putative converting enzyme for the 8–13 amino acids enkephalin containing peptides, these data support our results, showing a decrease of PC12 EOPA-like activity when the cells are treated with cAMP analogs. How cAMP analogs have this effect is unknown. The Western blotting showed in Figure 3 suggests that is not a consequence of a decrease in the content of the EOPA-like protein. One possibility is that a decrease in EOPA-like activity may be due to the difference of EOPA-like protein phosphorylation by protein kinase A (PKA) activated by cAMP analogs or through other kinases activated or attenuated by PKA [Graves et al., 1993; Sevetson et al., 1993]. This question has to be addressed in future experiments.

In conclusion, it was shown that PC12 cells possess an EOPA-like activity and that such activity can be modulated by cAMP analogs but not by bFGF. These results also show that PC12 cells can be a very interesting in vitro cellular model to study the expression of EOPA-like proteins and their role in metabolizing bioactive peptides.

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