ELSEVIER

European Journal of Pharmacology 364 (1999) 255–262

ejp

κ_1-Opioid binding sites are the dominant opioid binding sites in surgical specimens of human pheochromocytomas and in a human pheochromocytoma (KAT45) cell line

Marilenna Kampa [a], Andrew N. Margioris [b], Anastassia Hatzoglou [a], Irene Dermitzaki [b], Anne Denizot [d], Jean-Francois Henry [d], Charles Oliver [e], Achille Gravanis [c], Elias Castanas [a,*]

[a] *Department of Experimental Endocrinology, Medical School, University of Crete, Heraklion GR-71110, Crete, Greece*
[b] *Department of Clinical Chemistry, Medical School, University of Crete, Heraklion GR-71110, Crete, Greece*
[c] *Department of Pharmacology, Medical School, University of Crete, Heraklion GR-71110, Crete, Greece*
[d] *Department of Endocrine Surgery, Hopital Timone, Marseille, France*
[e] *Department of Endocrinology, Hopital Nord, Marseille, France*

Received 25 June 1998; revised 6 November 1998; accepted 10 November 1998

Abstract

The adrenal medulla produces opioids which exert paracrine effects on adrenal cortical and chromaffin cells and on adrenal splanchnic nerves, via specific binding sites. The opioid binding sites in the adrenals are detectable mainly in the medullary part of it and differ in type between species. Thus, the bovine adrenal medulla contains mostly κ-opioid binding sites and fewer δ- and μ-opioid binding sites while primate adrenals contain mainly δ sites and few κ-opioid binding sites. Most chromaffin cell tumors, the pheochromocytomas, produce opioids which suppress catecholamine production by the tumor. The aim of the present work was to identify the types of opioid binding sites in human pheochromocytomas. For this purpose, we characterized the opioid binding sites on crude membrane fractions prepared from 14 surgically excised pheohromocytomas and on whole KAT45 cells, a recently characterized human pheochromocytoma cell line. Our data showed that human pheohromocytomas are heterogeneous, as expected, with regard to the production of catecholamines and the distribution and profile of their opioid binding sites. Indeed, only one out of the 14 pheochromocytomas expressed exclusively δ and μ opioid sites, while in the remaining 13 tumors κ-type binding sites were dominant. The KAT45 cell line possessed a significant number of κ_1 binding sites, fewer κ_2-opioid binding sites and κ_3-opioid binding sites, and minimal binding capacity for δ- and μ-opioid receptor agonists sites. More specifically, the κ_1 sites/cell were approximately 18,000, the κ_2 4500/cell and the κ_3 sites 2000/cell. Our findings for the surgical specimens and the cell line combined with previously published pharmacological data obtained from KAT45 cells suggest that κ sites appear to be the most prevalent opioid binding sites in pheochromocytomas. Finally, in normal bovine adrenals the profile of opioid binding sites differs in adrenaline and noradrenaline producing chromaffin cells. To test the hypothesis that the type of catecholamine produced by a pheochromocytoma depends on its cell of origin, we compared our binding data with the catecholamine content of each pheochromocytoma examined. We found no correlation between the type of the predominant catecholamine produced and the opioid binding profile of each tumor suggesting that this hypothesis may not be valid.

Keywords: κ-opioid binding site; Pheochromocytoma, human; KAT45 cell line, human

1. Introduction

In addition to catecholamines, adrenal chromaffin cells synthesize and secrete several neuropeptides, including opioids (Margioris, 1993; Karl et al., 1996). It is now well established that opioids from the adrenal medulla exert mostly local (paracrine and/or autocrine) effects either on chromaffin cells (Venihaki et al., 1996a,b), adrenal splanchnic nerve terminals (Hirokami et al., 1994), or on adrenal cortical cells (Fallo et al., 1988). The biological effects of opioids on the adrenals are mediated by specific

* Corresponding author. Tel.: +30-81-394580; Fax: +30-81-394581; E-mail: castanas@med.uch.gr

0014-2999/99/$ - see front matter
PII: S0014-2999(98)00834-6

binding sites which are almost exclusively confined to the medullary part of the gland (Mansour et al., 1986; Bunn et al., 1988).

The types of opioid binding sites in the adrenal medulla are species-specific. Thus, in the most commonly used model for the in vitro study of adrenal medulla, the bovine adrenal, the predominant sites are κ and there are much fewer δ and μ sites (Castanas et al., 1984, 1985a,b). In monkeys, δ sites predominate while κ sites constitute only a minor component (Mansour et al., 1986). Furthermore, the κ sites in bovine adrenal medulla are heterogeneous and are categorized in three subtypes. The κ_1 sites bind mainly dynorphin A-(1–13) and other archetypal κ-opioid receptor agonists; the κ_2 sites bind mainly ß-endorphin; and the κ_3 sites bind preferentially [Met^5]enkephalyl-Arg^6-Phe^7 (Castanas et al., 1985a,b; Pesce et al., 1990; Bourhim et al., 1993). In addition, the distribution of opioid binding sites exhibits a cell-specific pattern. Thus, the κ sites are mainly concentrated on the peripheral, adrenaline-containing medullary chromaffin cells, and at a much lower density on the central noradrenaline-containing chromaffin cells and the medullary nerve tracts. The δ opioid binding sites are mainly localized on the central, noradrenaline-containing cells while the μ opioid sites, although low in number, are equally distributed throughout the bovine adrenal medulla (Bunn et al., 1988; Bunn, 1991; Bunn and Dunkley, 1991).

Most chromaffin cell tumors, the pheochromocytomas, produce catecholamines (mainly noradrenaline) and opioids (Margioris, 1993) which, as in their normal counterparts, play important local micro-regulatory roles, one of which is suppression of catecholamine secretion (Castanas et al., 1983). Indeed, in the well-known model for the in vitro study of these tumors, the PC12 rat pheochromocytoma cell line, opioids exert multiple paracrine tonic inhibitory effects including suppression of catecholamine secretion (Venihaki et al., 1996a,b) and inhibition of cell proliferation (Venihaki et al., 1996a,b). The aim of the present work was to identify the type(s) of opioid binding sites in human pheochromocytomas and their relationship to the catecholamine type produced. For this purpose, we analyzed the opioid receptor profile in the cell membranes of 14 surgically excised human pheohromocytomas, and in whole cell preparations of KAT45 cells, a recently characterized human pheochromocytoma cell line (Venihaki et al., 1998). This cell line responds to opioids in an opioid type-specific and dose-dependent manner.

In normal adrenals, the profile of the opioid binding sites differs in adrenaline and noradrenaline-producing chromaffin cells. Because of this, we compared our binding data to the catecholamine content of each pheochromocytoma, to examine whether the type of the predominant catecholamine produced correlates with the opioid binding profile, i.e., whether the opioid binding profile can give us a clue about the possible origin of the neoplastic chromaffin cell.

2. Materials and methods

2.1. Surgical specimens of human pheochromocytomas. Preparation of cell membranes and conditions of binding

The surgical specimens were obtained from 14 patients with established pheochromocytoma, operated at the University Hospital of Marseilles, France. Each specimen was collected in the operation room, washed briefly with normal saline, and immediately frozen in liquid nitrogen and stored at −80°C. Subsequently, the frozen tissues were pulverized in 10 volumes of Tris–HCl buffer, in a Teflon pestle tissue homogeniser after a brief disruption in liquid nitrogen by the use of a dismembrator (type MM2, Retsch, Germany). The membrane fraction was prepared by differential centrifugation between 1500 and 20,000 × *g*, at 4°C, for 30 min. The membrane pellet was resuspended in Tris–HCl buffer, at a protein concentration of 2 mg/ml, as assayed by the Coomasie brilliant blue dye technique of Bradford. Membranes were preincubated for 30 min at 37°C in order to dissociate any endogenously bound opioid, washed twice with cold Tris–HCl buffer, and resuspended, at the same concentration (2 mg/ml), in the same buffer. Ligand binding was performed in a Tris buffer for 30 min at 37°C, in a total volume of 1.0 ml, containing 0.5 ml membrane suspension, 0.1 ml radioligand (20 nM), and effectors (see below). Nonspecific binding (which varied from 12% to 28% of total binding) was assayed by the use of 10^{-6} M of the same unlabelled opioid. Separation of free and bound radioactivity was performed by rapid filtration through Whatman GF/B filters (Castanas et al., 1985a,b). Filter disks were washed twice with 5 ml cold Tris–HCl buffer, dried at 50°C, added to vials containing 5 ml scintillation cocktail (SigmaFluor, Sigma, St. Louis, MI), and counted in a Tricarb series scintillation counter (Packard), with 60% efficiency for tritium.

2.2. The KAT45 human pheochromocytoma cell line. Whole cell preparations and binding conditions

The KAT45 cell line grew spontaneously from continuous primary culture of a human pheochromocytoma for more than 9 months, as described before (Venihaki et al., 1998). Cells were cultured in flasks containing antibiotic-free RPMI 1640 medium with 10% (v/v) fetal bovine serum (all from Gibco BRL, Gaithersburg, MD) in humidified 5% CO_2 at 37°C (Venihaki et al., 1998).

Ligand binding assays in whole KAT45 cells were performed as described by Hatzoglou et al. (1995a,b, 1996a,b). For saturation binding experiments with whole cells, about 10^6 cells/well were used. Before binding, cells were subjected to brief acidification in glycine buffer, in order to remove endogenous opioids from opioid binding sites (Hatzoglou et al., 1994, 1995a,b). Thereafter, they were washed twice with 2 ml of phosphate-buffered saline. Binding was performed in the same buffer, in a total

volume of 0.4 ml, containing radioactive opioid, without (total binding) or with (nonspecific binding) a thousand-fold molar excess of the same unlabelled opioid and effectors (see below). At least 8–10 different concentrations of radiolabelled opioid (varying from 1 to 16 nM for ethylketocyclazocine, and 1–5 nM for diprenorphine) were used in duplicate for saturation binding experiments. The cells were incubated for 2 h at room temperature (18°–22°C). At the end of the incubation period, the unbound radioactivity was eliminated by washing the cells twice with 2 ml cold buffer. Cells were removed from plates with 0.4 ml 2 N NaOH and mixed with 4 ml scintillation cocktail (SigmaFluor, Sigma). The bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000, Packard), with a 60% efficiency for tritium. Binding experiments were repeated at least two times (in triplicate). Nonspecific binding, under the described conditions, ranged from 20% to 36%. The results were analyzed with the Origin (MicroCal) V 4.1 package, using equations described by Munson and Rodbard (1980).

2.3. Specific conditions for the differential detection of the type of opioid binding sites

The conditions used for the differential detection of various opioid sites have been described in previous studies from our group (Castanas et al., 1985a,b; Hatzoglou et al., 1995a,b, 1996a,b). Briefly, δ opioid sites were detected by using tritiated [D-Pen^2, D-Pen^5]enkephalin (DPDPE). μ opioid sites were detected by using selective ligand [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (DAGO). The interaction of tritiated ethylketocyclazocine with κ_1 opioid binding sites was assayed by measuring binding in the presence of 5 μM [D-Ala^2, D-Leu^5]enkephalin (DADLE), which masks δ, μ, and κ_2-opioid sites to which ethylketocyclazocine could bind with high affinity (Attali et al., 1982; Castanas et al., 1985b). Finally, diprenorphine binding (reacting mainly with δ, μ, κ_2 and κ_3 opioid sites) was measured under the same conditions as for ethylketocyclazocine. The interaction of the ligand with the κ_3 opioid site was estimated in the presence of 5 μM DADLE, as described previously (Castanas et al., 1985b). The exact conditions for the opioid ligand binding experiments are summarized in Table 1.

2.4. Measurement of catecholamines

The separation and measurement of catecholamines was performed as previously described (Venihaki et al., 1998). Samples were acidified with 0.1 N HCl to ensure the stability of the catecholamines and extracted by acetone precipitation. The extracts were then evaporated and reconstituted in 200 μl of the mobile phase of the high performance liquid chromatography (HPLC). Samples of 25 μl (i.e., 1/8 of the initially extracted material) were injected into the reverse phase (RP) HPLC (HP-1090, Hewlett-Packard, Germany) equipped with an electrochemical S100A detector (ESA, Coulochem, USA). The detector was set at +0.45 mV (with respect to an H_2/H^+ couple reference electrode) while the guard cell was set at +0.5 mV. The guard and main columns were RP-18, 5-μm particle size (Hewlett-Packard); the main column was 200 mm × 4.6 mm. The mobile phase was composed of 25 mM $NaH_2PO_4 \cdot H_2O$, 3.2 mM 1-heptane sulfonic acid sodium salt, 0.5 mM EDTA, and 7.5% methanol. The signals were recorded in an analogue digital converter (HP-35900 C, Hewlett-Packard) connected to an HP-1040 computer.

2.5. Radiochemicals and chemicals

[^{3}H]ethylketocyclazocine (S.A. 18 Ci/mmol) and [^{3}H]DPDPE (S.A. 37 Ci/mmol) were bought from New England Nuclear. [^{3}H]diprenorphine (S.A. 29 Ci/mmol) and [^{3}H]DAGO (S.A. 60 Ci/mmol) were from Amersham (UK). Unlabelled DAGO, [D-Ser^2, Leu^5]enkephalin-Thr^6 (DSLET) and DADLE were from Sigma. Ethylketocyclazocine was a gift from Sterling–Winthrop. Diprenorphine and etorphine were from Reckit and Coleman. All other chemicals were either from Merck (Darmstad, Germany) or from Sigma (St. Louis, MA).

2.6. Statistical analysis

Statistical analysis was performed by classical parametric and nonparametric tests, using the Statgraphics (V 6.0) microcomputer program (Manugistics, USA).

Table 1
Characterization of the opioid binding sites on KAT45 cells

Ligand	Effector	K_D (nM)	Sites/cell	Detected sites
[^{3}H]DPDPE		ND	0	δ
[^{3}H]DAGO		ND	0	μ
[^{3}H]EKC		5.26 ± 0.11	23020 ± 921	δ, μ, κ_1, κ_2
	DADLE (5 μM)	3.60 ± 0.83	18286 ± 1203	κ_1
[^{3}H]diprenorphine		3.02 ± 0.60	6485 ± 234	δ, μ, κ_2, κ_3
	DADLE (5 μM)	5.04 ± 0.72	1938 ± 174	κ_3

3. Results

3.1. Characterization of opioid binding sites in whole cell preparations from the KAT45 cell line

We have previously shown that the simultaneous use of several opioid ligands with variable selectivity for each type of opioid binding site is a useful tool in the characterization of opioid binding sites, both in membrane preparations and whole cells (Castanas et al., 1985a,b; Hatzoglou et al., 1995b, 1996b). Table 1 depicts the results. Further analysis of these data showed the following.

[^{3}H][D-Pen^2, D-Pen^5]enkephalin (DPDPE, a ligand which binds mainly to δ opioid sites), and [^{3}H][D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (DAGO), a specific μ ligand), did not exhibit any specific binding for KAT45 cells.

[^{3}H]ethylketocyclazocine, a ligand of δ, μ, κ_1, and κ_2 opioid sites, demonstrated a monophasic binding curve (Fig. 1 upper panel) with an affinity of 5.26 ± 0.11 nM, and a binding capacity of 23020 ± 921 sites/cell. In view of the absence of δ and μ opioid sites in our cell population, this binding corresponds to the interaction of ethylketocyclazocine with κ_1 and κ_2 opioid sites. The addition of micromolar concentrations of [D-Ala^2, D-Leu^5]enkephalin (DADLE) to the binding assay masked all sites with the exception of κ_1 sites (Castanas et al., 1985b). Therefore, when saturation binding was performed in the presence of [D-Ala^2, D-Leu^5]enkephalin (5 μM) the ligand interacted only with κ_1 sites. The results of this binding assay are presented in Fig. 1 lower panel and in Table 1. Under these conditions, we found a monophasic curve with an apparent dissociation constant of 3.60 ± 0.60 nM and a binding capacity of 18286 sites/cell, which might represent the number and the affinity of κ_1 opioid sites.

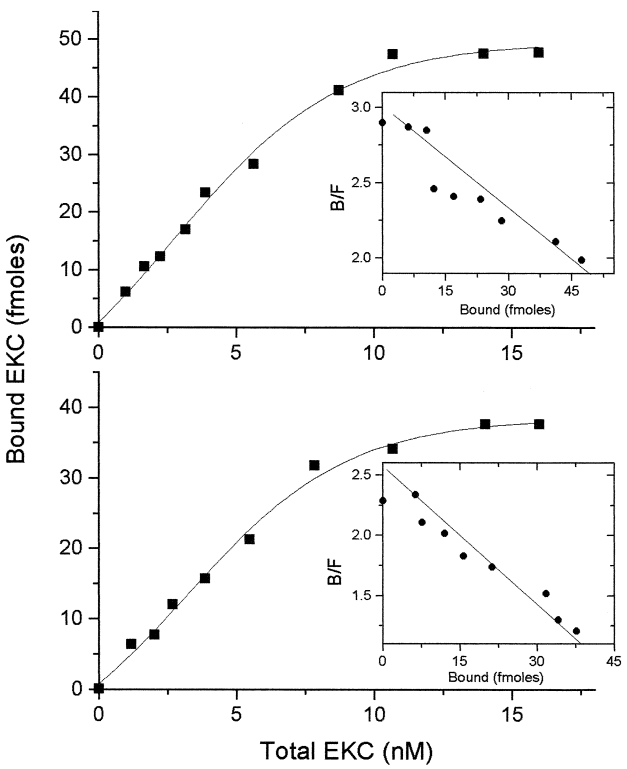

Fig. 1. Saturation binding of [^{3}H]ethylketocyclazocine to KAT45 cells. Cells (10^6) were incubated for 2 h, at room temperature, with nine concentrations of [^{3}H]ethylketocyclazocine, varying from 1 to 16 nM, in phosphate-buffered saline, pH 7.4. Upper panel presents the binding of ethylketocyclazocine in the absence of an effector. Lower panel shows the same binding, in the presence of 5 μM [D-Ala^2, D-Leu^5]enkephalin, which masks the binding to δ, μ, and κ_2 opioid sites, to which ethylketocyclazocine could bind with high affinity. In each panel, the insert presents the analysis of data in Scatchard coordinates. See Sections 2 and 3 for further details. The figure presents the results of a typical experiment, in triplicate.

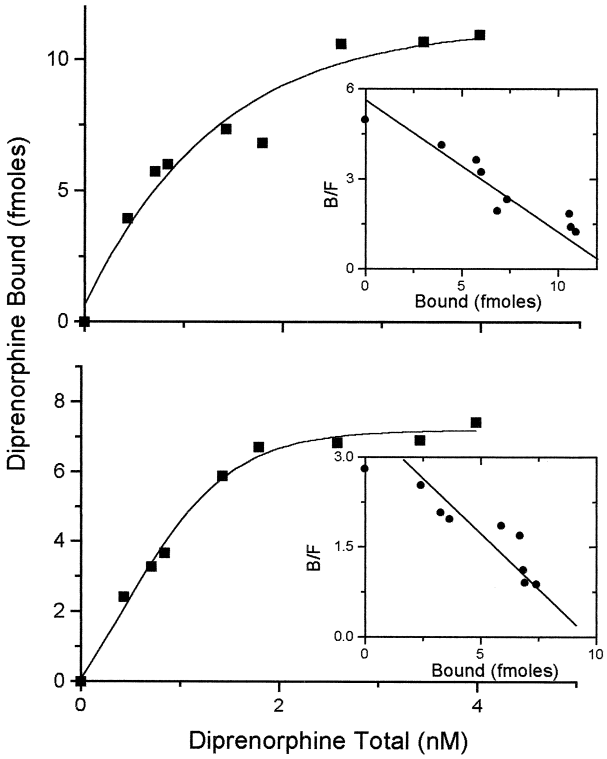

Fig. 2. Saturation binding of [^{3}H]diprenorphine to KAT45 cells. About 10^6 cells were incubated for 2 h at room temperature with nine concentrations of [^{3}H]diprenorphine, varying from 1 to 5 nM, in phosphate-buffered saline, pH 7.4. Upper panel presents the binding of diprenorphine in the absence of any effector. Lower panel shows the same binding, in the presence of 5 μM [D-Ala^2, D-Leu^5]enkephalin, which masks the binding to δ, μ, and κ_2 opioid sites. In each panel, the insert presents the analysis of data in Scatchard coordinates. See Sections 2 and 3 for further details. The figure presents the results of a typical experiment, in triplicate.

[^{3}H]diprenorphine (an almost universal ligand of opioid sites with a selectivity for δ, μ, κ_2 and κ_3 opioid sites) interacted with KAT45 cells. In the absence of any effector (Fig. 2 upper panel), this ligand exhibited a monophasic curve, with an apparent K_D of 3.02 ± 0.60 nM and a binding capacity of 6485 ± 234 sites/cell. Under these conditions, and in view of the absence of δ and μ sites, [^{3}H]diprenorphine binding was considered to reflect an interaction with κ_2 and κ_3 opioid sites. The addition of

5 μM DADLE (masking κ_2 opioid sites) during [^{3}H]diprenorphine binding permitted assessment of the interaction of the ligand with the κ_3 sites (Fig. 2, lower panel). Under these conditions, we observed one apparent site with a K_D of 5.04 nM and a binding capacity of 1938 ± 174 sites/cell.

Finally, using a simulation program, we calculated the number and affinity of κ_2 opioid binding sites, taking into consideration the absence of δ and μ opioid sites and the results of the interaction of ethylketocyclazocine and diprenorphine with opioid sites, in the absence or presence of blocking concentrations of DADLE. Our results showed that the κ_2 opioid binding sites on KAT45 cells had a concentration of 4547 sites/cell, and affinity of 2.58 nM for diprenorphine and 11.52 nM for ethylketocyclazocine.

3.2. Catecholamine content and opioid binding sites in cell membranes from the surgical specimens of human pheochromocytomas

Table 2 depicts the catecholamine content of the 14 human pheochromocytomas examined. The content of epinephrine varied, ranging from 5 to 8277 ng/mg tissue. The content of norepinephrine also varied ranging from 10 to 38442 ng/mg tissue, while that of dopamine ranged from 0 to 254 ng/mg tissue. The medians were 219, 762, and 29 ng/mg tissue, respectively. The majority of pheochromocytomas (10/14) exhibited a high content of either epinephrine or norepinephrine (total catecholamine content > 100 ng/mg tissue). Two cases (no. 5 and 12) exhibited an epinephrine to norepinephrine ratio greater than one (i.e., mostly epinephrine secreting pheos), while in all other specimens the ratio varied from 0.0076 (no. 13, almost exclusively norepinephrine producing tumor) to 1.1 (no. 10, a tumor producing the two catecholamines in an almost equimolar proportion). The content of all three catecholamines per tissue were correlated between them in a significant manner ($P < 0.01$ at least), as the Spearman nonparametric correlation coefficient showed.

Table 2
Surgical specimens of human pheochromocytomas

	Epinephrine	Norepinephrine	Dopamine
1	17	76	2
2	3008	38.442	145
3	5912	7949	254
4	313	602	31
5	8277	3.400	216
6	124	3.104	101
7	32	59	
8	97	400	6
9	616	2.648	38
10	21	19	
11	5	10	
12	1355	190	14
13	7	921	60
14	442	1188	27

Content of catecholamines in nanogram per milligram of tissue wet weight.

Table 3
Surgical specimens of human pheochromocytomas

	δ	μ	κ_1	κ_2	κ_3
1	6	nd	52	58	nd
2	7	12	22	79	nd
3	35	nd	60	nd	nd
4	2	nd	40	nd	31
5	nd	nd	80	nd	nd
6	11	5	31	22	22
7	8	8	21	nd	13
8	nd	6	95	nd	nd
9	5	3	85	nd	nd
10	nd	nd	121	76	nd
11	103	157	nd	nd	nd
12	57	28	148	17	23
13	nd	nd	177	nd	nd
14	6	9	45	15	nd

Content of opioid binding sites in femtomole per milligram of protein (nd = non detectable).

Table 3 depicts the profile of opioid binding sites on each of the pheochromocytomas examined. Compared to the sites for catecholamines, the total number of opioid sites per tissue specimen varied less from 50 to 273 fmol/mg protein (median 98, mean 129, SE 18 fmol/mg protein). Regarding the type of the binding sites the data are as follows: the δ opioid binding sites were non-detectable in 4/14 cases and predominant in only one specimen (no. 11), ranging form 0 to 103 fmol/mg protein (median 6 fmol/mg protein). Similarly, the μ opioid sites were non-detectable in 6/14 cases. It is interesting to note that cases 5, 10, and 13 did not exhibit any δ or μ opioid binding site. In two other cases (no. 1 and 4), there were few δ sites, while in the sixth case (no. 3) there were many δ sites. The concentration of μ sites varied from 0 to 157 fmol/mg protein (median 4 fmol/mg protein). Case no. 11 is interesting in that the pheochromocytoma had a low catecholamine content, but had high concentrations of both δ and μ opioid sites, and a total absence of κ sites.

The κ opioid binding sites were the most abundant sites in our human pheochromocytoma specimens. Indeed, they were present in 13/14 cases, varying from 34 to 197 fmol/mg protein (median 83, mean 95, SE 15 fmol/mg protein). Regarding the subgrouping of the κ opioid sites, κ_1 sites were the most common. They were present in all 13 cases (median 56, mean 70, SE 14 fmol/mg protein), followed by the κ_2 sites, which were present in six cases and the κ_3 sites, which were found in only four cases. In two cases (1 and 2) the κ_2 sites were more abundant than the κ_1 sites in a ratio 1.1 and 3.6, respectively. The ratio of κ_3/κ_1 was always less than one, indicating that the κ_1 type of opioid binding site was the predominant form. However, its concentration was highly variable in a few specimens (about 0.7 in three cases—no. 4, 6 and 7—, and about 0.15 in one other case—no. 12).

Comparison between catecholamine content and opioid binding profile in the 14 pheochromocytomas studied revealed that there was no relationship between the type of catecholamines produced and the opioid binding profile. For instance, case no. 3 (which exhibited a high epinephrine and norepinephrine content) had an exclusively δ and κ_1 opioid binding profile; case no. 5 (which exhibited a high epinephrine content) had an exclusively κ_1 opioid binding profile; case no. 12 (which exhibited a high epinephrine content) had all five types of opioid binding sites, with the δ and κ_1 sites present in high concentrations; and finally case no. 13 (which exhibited a high content of norepinephrine) had an exclusively κ_1 opioid binding profile.

4. Discussion

Our data show that specific opioid binding sites are detectable on cell membranes from surgically removed human pheochromocytomas as well as on whole cell preparations from the KAT45 human pheochromocytoma cell line. The profile of opioid binding sites in the majority of cell membrane preparations from the surgical specimens and in the whole cell preparations from the cell line were directly comparable. Indeed, in both types of preparations the binding capacity for δ and μ opioid agonists was low while that for κ_1 agonists was high. More specifically, the κ_1 binding sites in the surgically resected human pheochromocytoma specimens varied from 21 to 177 fmol/mg protein while in the KAT45 were approximately 18,000/cell. The κ_2 sites were about 4500/cell and the κ_3 sites about 2000/cell. The affinity of all three κ opioid binding sites was in the low nanomolar range. The predominance of κ_1 opioid binding sites appears to be an almost constant characteristic of human pheochromocytomas. Although the opioid binding profile of normal human adrenal chromaffin cells is not known, we can postulate that the δ binding sites predominate since, as has been shown in primate adrenals, the δ opioid binding sites predominate, while κ sites are only present as a minor component (Mansour et al., 1986) and since human adrenals produce δ and κ opioid agonists. However, it should be noted that κ opioid binding sites predominate over δ and μ sites in crude bovine adrenal medullary membranes (Castanas et al., 1985a,b). If the type of opioid binding site in human chromaffin cells resembles that of primates, then the predominance of κ_1 opioid binding sites in pheochromocytomas should be related to the neoplastic transformation per se. However, it is also possible that human pheochromocytomas may retain the profile of opioid receptors of the originating cell. Indeed, as we have mentioned above, adrenal chromaffin cells can be subdivided in two major categories: cells in the periphery of the adrenal medulla producing mainly adrenaline and those in the center of the gland producing noradrenaline. In normal bovine adrenals, κ opioid binding sites are present in a high density in the peripheral (adrenaline-containing) medullary chromaffin cells and in low density in the central (noradrenaline-containing) chromaffin cells and the medullary nerve tracts (Bunn et al., 1988; Bunn, 1991; Bunn and Dunkley, 1991). δ opioid binding sites are mainly localized in the central, noradrenaline-containing chromaffin cells while μ opioid sites, low in number, are equally distributed throughout the adrenal medulla (Bunn et al., 1988). It is thus possible that those pheochromocytomas originating in the periphery of the adrenal medulla retain their richness in κ opioid receptors and the production of adrenaline, while the noradrenaline-producing pheochromocytomas may originate from the center of the medulla and retain their δ opioid binding profile. However, only a minority of pheochromocytomas produce adrenaline while the κ opioid binding profile seems to be universal in pheochromocytomas. Our results suggest that there is no association between the catecholamine profile of pheochromocytomas and the type of opioid binding sites they possess. For instance, the three tumors containing mostly epinephrine in our study (no. 5, 10 and 12) had a variable profile for opioid binding sites; one of them (no. 5) had exclusively κ_1 opioid binding sites, while no. 12 possessed all five opioid sites on its membranes (i.e., δ, μ, and all three subtypes of the κ site), although κ_1 appeared to predominate. The third specimen (no. 10), which contained almost equimolar amounts of epinephrine and norepinephrine, expressed κ_1 and κ_2 sites. The absence of any correlation between the type of opioid binding sites and catecholamine production suggests that the two phenomena might have no direct relationship.

κ opioid agonists are the most potent opioid effectors in normal adrenal chromaffin cells and in pheochromocytomas. Indeed, the κ opioid agonists U-50488H and dynorphin (1–13) are strong inhibitors of acetylcholine-evoked catecholamine release from isolated adrenal chromaffin cells. The inhibitory effect of U-50488H is antagonized by diprenorphine and MR-2266, two opioid antagonists with high affinity for κ opioid receptors (Dumont and Lemaire, 1985). Furthermore, the endogenous κ opioid receptor agonist dynorphin (1–13) is 1000-fold more potent than Leu^5-enkephalin in inhibiting nicotine-induced endogenous catecholamine secretion from bovine chromaffin cells (Marley et al., 1986). Interestingly, dynorphin (1–13) is more potent in inhibiting noradrenaline release than adrenaline, whereas [Leu^5]-enkephalin possesses the opposite selectivity (Marley et al., 1986). In the PC12 rat pheochromocytoma cell line, κ opioids are definitely the most potent inhibitors of dopamine release and suppressors of their proliferation (Venihaki et al., 1996a,b). Thus, it is possible that κ opioid agonists may represent the most potent endogenous opioid regulators of adrenal chromaffin cells while the δ opioid agonists, although the most abundant, may have exert a limited biological role. Conversely, pentapeptide enkephalins may also act via κ opioid binding sites because their local concentration, especially after

stimulation of chromaffin cells, can be high in the intracellular space (it has been calculated to be at the micromolar range) (Castanas et al., 1985a,b). At such high concentrations, pentapeptide enkephalins have been shown to interact with κ opioid sites (Castanas et al., 1985a,b). Finally, it should be noted that, as has been previously reported (Castanas et al., 1985b), the opioid peptides [Met^5]-enkephalin-Arg^6-Gly^7-Leu^8, and [Met^5]-enkephalin-Arg^6-Phe^7 which are produced in parallel to the pentapeptide enkephalins as a result of the splicing of proenkephalin A in the adrenal medulla, are preferential ligands of κ opioid binding sites (Castanas et al., 1985b).

As in the central nervous system, opioids inhibit catecholamine release from adrenal chromaffin cells. It is postulated that this effect of opioids is not only pharmacological but also physiological, taking place locally with chromaffin cell-derived opioids. Indeed, it is now classical knowledge that opioids are produced by adrenal chromaffin cells and that they are co-stored and co-secreted with catecholamines (Wilson et al., 1980; Klein et al., 1982; Douglas et al., 1986). Adrenal chromaffin cells produce the two major opioid precursor proteins proenkephalin A and prodynorphin (Margioris, 1993). Post-translational processing of these proteins gives rise to a multitude of opioid peptide agonists which exert their biological effects via different types of opioid binding sites (Castanas et al., 1985a,b). κ sites are the most biologically important since they are implicated in the inhibition of the proliferation of several types of malignant cells including those from breast (Hatzoglou et al., 1996a,b), prostate (Kampa et al., 1997), and uterus (Hatzoglou et al., 1995a,b). Our data suggest that in pheochromocytomas, the κ_1 type of opioid binding site is the most numerous. The predominance of κ receptors in human pheochromocytomas may be related to the paracrine regulation of neoplastic cell proliferation. Since the KAT45 human pheochromocytoma cell line expresses mainly κ opioid binding sites, an apparent characteristic of human pheochromocytomas, it may prove to be valid in vitro model for the evaluation of the role of opioids in these tumors.

Acknowledgements

The present work was supported by grants from the University of Crete Research Committee, The Hellenic Anticancer Society and the Greek Ministry of Health (KESY), the General Secretariat of Research and Technology (GGET), Medicon Hellas, Varelas, and DELTA Dairy products grants.

References

Attali, B., Guarderes, C., Mazarguil, H., Audigier, Y., Cros, J., 1982. Evidence for multiple κ binding sites by use of opioid peptides in the guinea pig lumbo-sacral spinal cord. Neuropeptides 3, 53–64.

Bourhim, N., Cantau, P., Giraud, P., Castanas, E., 1993. Comparative thermodynamics of opioid receptor ligand interaction in the bovine adrenal medulla membranes-evidence of opioid site heterogeneity. Comp. Biochem. Physiol. C 105, 435–442.

Bunn, S.J., 1991. Localisation and pharmacological characterisation of [^{3}H]bremazocine binding in the bovine adrenal medulla. Neurosci. Lett. 125, 139–142.

Bunn, S.J., Dunkley, P.R., 1991. Opioid inhibition of nicotine-induced $45Ca^2(+)$-uptake into cultured bovine adrenal medullary cells. Biochem. Pharmacol. 41, 715.

Bunn, S.J., Marley, P.D., Livett, B.G., 1988. The distribution of opioid binding subtypes in the bovine adrenal medulla. Neuroscience 27, 1081–1094.

Castanas, E., Giraud, P., Audigier, Y., Drissi, R., Boudouresque, F., Conte-Devolx, B., Oliver, C., 1983. Opiate binding sites on bovine adrenal medulla and six human pheochromocytomas. Life Sci. 33, 295–298.

Castanas, E., Giraud, P., Audigier, Y., Drissi, R., Boudouresque, F., Conte-Devolx, B., Oliver, C., 1984. Adrenal medullary opiate receptors. Pharmacological characterization in bovine adrenal medulla and a human pheochromocytoma. Mol. Pharmacol. 25, 38–45.

Castanas, E., Bourhim, N., Giraud, P., Boudouresque, F., Cantau, P., Oliver, C., 1985a. Interaction of opiates with opioid binding sites in the bovine adrenal medulla: I. Interaction with δ and μ sites. J. Neurochem. 45, 677–687.

Castanas, E., Bourhim, N., Giraud, P., Boudouresque, F., Cantau, P., Oliver, C., 1985b. Interaction of opiates with opioid binding sites in the bovine adrenal medulla: II. Interaction with κ sites. J. Neurochem. 45, 688–699.

Douglas, B.H.D., Duff, R.B., Thureson-Klein, A.K., Klein, R.L., 1986. Enkephalin contents reflect noradrenergic large dense cored vesicle populations in vasa deferentia. Regul. Pept. 14, 193–210.

Dumont, M., Lemaire, S., 1985. Interaction of dynorphin with κ opioid receptors in bovine adrenal medulla. Neuropeptides 6, 321–329.

Fallo, F., Boscaro, M., Sonino, N., Mantero, F., 1988. Effect of naloxone on the adrenal cortex in primary aldosteronism. Am. J. Hypertens. 1, 280–282.

Hatzoglou, A., Romain, S., Dalapascha, H., Castanas, E., 1994. Acidification reveals a greater number of epidermal growth factor receptors in human membrane preparations. Clin. Chim. Acta 227, 97–109.

Hatzoglou, A., Ouafik, L.H., Bakogeorgou, E., Thermos, K., Castanas, E., 1995a. Morphine cross-reacts with somatostatin receptor SSTR2 in the T47D human breast cancer cell line, and decreases cell proliferation. Cancer Res. 55, 5632–5636.

Hatzoglou, A., Gravanis, A., Zoumakis, E., Margioris, A., Castanas, E., 1995b. Identification and characterization of opioid binding sites present in the Ishikawa human endometrial adenocarcinoma cell line. J. Clin. Endocrinol. Metab. 80, 418–423.

Hatzoglou, A., Bakogeorgou, E., Stournaras, C., Emmanuel, D., Castanas, E., 1996a. Identification and characterization of opioid and somatostatin binding sites in the kidney oposum (OK) cell line, and their effect on growth. J. Cell. Biochem. 63, 410–421.

Hatzoglou, A., Bakogeorgou, E., Castanas, E., 1996b. The antiproliferative effect of opioid agonists on the T47D human breast cancer cell line, is partially mediated through opioid receptors. Eur. J. Pharmacol. 296, 199–207.

Hirokami, M., Togashi, H., Matsumoto, M., Yoshioka, M., Saito, H., 1994. The functional role of opioid receptors in acetylcholine release in the rat adrenal medulla. Eur. J. Pharmacol. 253, 9–15.

Kampa, M., Bakogeorgou, E., Hatzoglou, A., Damianaki, A., Martin, P.-M., Castanas, E., 1997. Opioid alkaloids and casomorphin peptides decrease prostatic cancer cell line (LNCap, PC3 and DU145) proliferation through a partial interaction with opioid receptors. Eur. J. Pharmacol. 335, 255–266.

Karl, M., Saviolakis, G.A., Gravanis, A., Chrousos, G.P., Margioris, A.N., 1996. The PC12 rat pheochromocytoma cell line expresses the prodynorphin gene and secretes the 8 kilodalton dynorphin product. Regul. Pept. 61, 99–104.

Klein, R.L., Wilson, S.P., Dzielak, D.J., Yang, W.H., Viveros, O.H., 1982. Opioid peptides and noradrenaline co-exist in large dense-cored vesicles from sympathetic nerve. Neuroscience 7, 2255–2261.

Mansour, A., Lewis, M.E., Khachaturian, H., Akil, H., Watson, S.J., 1986. Multiple opioid receptor subtypes in the pituitary–adrenal axis: a cross-species study. NIDA Res. Monogr. 75, 311–314.

Margioris, A.N., 1993. Opioids in neural and non-neural tissues. Trends Endocrinol. Metab. 4, 163–168.

Marley, P.D., Mitchelhill, K.I., Livett, B.G., 1986. Effects of opioid peptides containing the sequence of Met^5-enkephalin or Leu^5-enkephalin on nicotine-induced secretion from bovine adrenal chromaffin cells. J. Neurochem. 46, 1–11.

Munson, P.J., Rodbard, D., 1980. LIGAND: a versatile computerized approach to characterization of ligand-binding systems. Anal. Biochem. 107, 220–239.

Pesce, G.O., Cruciani, R.A., Munson, P.J., Rodbard, D., 1990. Computer modeling of subtypes of κ opioid receptors in adrenal medulla. Eur. J. Pharmacol. 182, 429–439.

Venihaki, M., Gravanis, A., Margioris, A.N., 1996a. The proliferation of PC12 rat pheochromocytoma cells is affected by opioids in a type-specific and naloxone-reversible manner. Peptides 17, 413–419.

Venihaki, M., Gravanis, A., Margioris, A.N., 1996b. Opioids inhibit dopamine secretion from PC12 rat pheochromocytoma cells in a naloxone-reversible manner. Life Sci. 58, 215–223.

Venihaki, M., Ain, K.B., Gravanis, A., Margioris, A.N., 1998. KAT45, a noradrenergic human pheochromocytoma cell line producing corticotrophin-releasing hormone. Endocrinology 139, 713–722.

Wilson, S.P., Klein, R.L., Chang, K.J., Gasparis, M.S., Viveros, O.H., Yang, W.H., 1980. Are opioid peptides co-transmitters in noradrenergic vesicles of sympathetic nerves?. Nature 288, 707–709.