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The inhibitory effect of opioids on HepG2 cells is mediated via interaction with somatostatin receptors

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Abstract

Opioids, acting via G-protein coupled membrane receptors, induce analgesia. However their role is not limited to their anti-nociceptive action. They are found in several peripheral tissues acting as negative regulators of cellular processes. Even though that is not fully elucidated, it becomes obvious that opioids exert their effects in close relation to other neuropeptides such as somatostatin. Hepatocellular carcinoma is one tumor, among others, which secrete bioactive peptides while somatostatin analogs exert an inhibitory effect. We have used the human hepatocyte-derived cancer cell line HepG2, in order to examine the effect of opioids on cell growth and their possible mode of action. Our results show that the opioid ethylketocyclazocine (EKC) inhibits cell proliferation and induces apoptosis. This inhibitory effect is not exerted via opioids receptors since it was not reversed by the opioid antagonist diprenorphine and functional opioid receptors were not found on HepG2 cells. On the contrary, we show that EKC binds to somatostatin receptors, and activates a PTP signalling cascade. In this respect, the interaction of opioids with somatostatin receptors on hepatocellular carcinoma cells, and the fact that they are widely used for pain control, may provide some additional clues for the discrepancies during treatment with somatostatin analogues.

Index terms: Opioid (ethylketocyclazocine); Opioid receptors (delta, mu, kappa); Somatostatin receptor; HepG2 cell; Tyrosine phosphatase

1. Introduction

Endogenous opioid peptides arise from three main precursor molecules, namely pro-opiomelanocortin, and pro-enkephalins A and B (pro-dynorphin). They were initially described in nervous tissue, mediating anti-nociception. In subsequent studies, however, opioids have been also found in a number of peripheral normal and neoplastic tissues. It is not clear whether neural tissue-elements or peripheral tissues synthesize and secrete opioids. Opioids are acting through specific receptors, being categorized to three main types, mu (μ), kappa (κ) and delta (δ), and belonging to the superfamily of G-protein coupled membrane receptors (GPCR). A number of opioid receptor subtypes have also been described (see www.AfCS.org). The G-protein coupled membrane receptors (GPCR) superfamily, in addition to opioids includes the somatostatin and other neuropeptide receptors, while functional interactions (see Hatzoglou et al., 2005, for a recent review; Chiodera et al., 1989; Ghosh et al., 1997; Hatzoglou et al., 1996a,b, 1995; Kampa et al., 1997, 2001; Konagaya et al., 1998; Shook et al., 1987), and receptor-hetero-dimerization (Pfeiffer et al., 2002) have been proposed.

Hepatocellular carcinoma represents about 5% of all cancers. In addition, minor survival improvement has been observed in the last fifty years (Bruix et al., 2001). Recently, octreotide (a somatostatin analog) has been proposed for the treatment of advanced hepatocellular carcinoma (Dimitroulopoulos et al., 2002; Kouroumalis et al., 1998; Raderer et al., 2000; Yuen et al., 2002). However, existing data remain controversial. Octeotride

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inhibits the proliferation of HepG2 cells *in vitro*, through binding to specific somatostatin receptors (sst2, 3 and 5) and activation of a phosphotyrosine phosphatases (PTP)-dependent pathway (Notas et al., 2004). Recent reports further indicate that nociceptin/orphanin FQ, an agonist of opioid kappa3 receptor (Horvath et al., 2004; Szalay et al., 2004) or cortistatin (Notas et al., 2004) may be secreted by hepatocellular carcinoma *in vivo* or *in vitro*. However, the effect and interaction of opioids on the development and control of hepatocellular carcinoma has not been investigated so far. In the present work, we show that opioids modulate the growth of the human hepatocellular cancer-derived cell line HepG2, through induction of apoptosis. However, the observed effect was not mediated through opioid, but rather via somatostatin receptors, sharing the same PTP signaling cascade, as previously described in the same cell line (Notas et al., 2004).

2. Materials and methods

2.1. Radiochemicals and chemicals

Somatostatin-14 (SRIF-14) and [^{125}I]Tyr11-SRIF-14 were purchased from BACHEM (Heidelberg, Germany). [^{3}H][D-Pen^{2}, D-Pen^{5}]enkephalin (DPDPE) (S.A. 37 Ci/mmol) and [^{3}H] U69,593 [(+)-(5α,7α,8â)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide] (46 Ci mmol) were from New England Nuclear Co (Perkin Elmer, Boston MA). [^{3}H]diprenorphine (S.A. 29 Ci/mmol) was from Amersham (Buckinghamshire, UK). Octreotide was from Novartis (Basel, Switzerland). Ethylketocyclazocine (EKC) was a gift from Sterling-Winthrop, while diprenorphine was from Reckit and Coleman Co. Alpha$_{S1}$-casomorphin was synthetized by Dr S. Loukas (Demokritos NCBR, Athens, GR)., Unlabeled [D-Ala2, *N*-Me-Phe4,Gly5ol]enkephalin (DAGO) and [D-Ala2,D-Leu5] enkephalin (DADLE) and all other biochemicals were obtained from Sigma-Hellas (Athens, GR). All culture media and serum were from Gibco BRL (Life Technologies, Paisley, UK).

2.2. Cell cultures

The human hepatocyte-derived cancer cell line HepG2 was obtained from DSMZ (Braunschweig, Germany), and cultured in RPMI 1640, supplemented with 10% foetal bovine serum, at 37 °C, 5% CO_2. HepG2 cells were plated in 24-well plates, at an initial density of 2×10^4 cells, with 1.0 ml medium per well. All substances were added to cultures one day after seeding (designated as day 0), in order to ensure uniform attachment of cells at the onset of the experiments. Cells were grown for a total of 6 days, with a change of the medium and the different substances tested on day 3. Growth and viability of cells were measured by the tetrazolium salt assay (MTT) (Mosmann, 1983) and verified by direct measurement of cells by flow cytometry.

2.3. Apoptosis and cell cycle

Cells treated with 10^{-7} M opioids or octreotide for the indicated time-periods were detached with trypsin-EDTA, washed twice with PBS and subjected to a gradient of cold ethanol fixative dilutions, for 10 min each. Fixed cells were resuspended in PBS, at a concentration of 10^6 cells/ml and stained with propidium iodide (0.5 μg/ml), in the presence of DNase free RNaseA (0.2 mg/ml) for 15 min. A four-fold volume of PBS-0.05% BSA was added and samples were analyzed by flow cytometry, using a Beckton-Dickinson FACSArray apparatus (Beckton-Dickinson, Franklin Lakes, NJ, USA) and the CELLQuest (Beckton-Dickinson) and ModFit LT (Verify Software, Topsham, MN, USA) software.

For apoptosis detection, cells treated with opioids or octreotide were trypsinized, washed twice with HBSS and resuspended in staining buffer (10 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.4). Annexin V-FITC (Beckton-Dickinson, Franklin Lakes, NJ, USA) was added, according to the manufacturer's protocol, cells were incubated for 15 min in the dark and apoptotic cells were assayed by flow cytometry (Vermes et al., 1995). For specific experiments, apoptotic bodies measurement was also performed, using the cell death detection ELISA kit (Roche Diagnostics, Mannheim, Germany), based on a quantitative measurement of DNA and histones.

2.4. RT-PCR

Reverse transcription-Polymerase chain reaction (RT-PCR) was performed on total RNA from untreated HepG2 cells as previously described (Notas et al., 2004). The oligonucleotide sequence, annealing temperatures, cycles and product size for each gene-specific primer pair used are shown in Table 1. KOR

Table 1
Primes used in RT-PCR assays of opioid receptors

Primer	UniSTS code		Primer	Tm	Product size
β-actin	109142	F	GGTGGCTTTTAGGATGGCAAG	62,9	161 bp
		R	ACTGGAACGGTGAAGGTGACAG	63,6	
KOR	87561	F	CGTCTCAAGAGCGTCCG	57,6	123 bp
		R	TATGTGAATGGGAGTCCAGC	57,3	
DOR		F	ACCAAGATCTGCGTGTTCCT	57,3	210 bp
		R	CGATGACGAAGATGTGGATG	57,3	
MOR	28223	F	CATGCCATTCCGACCTTC	56,0	100 bp
		R	AGGCACTTTCCTAGAGAATTAGAGC	61,3	

KOR: kappa opioid receptor; DOR: delta opioid receptor; MOR: mu opioid receptor.

and MOR primers were selected from the NCBI UNISTS databank. The primer for DOR was designed with primer3 software, available at http://wwwgenome.wi.mit.edu/genome_software/ other/ primer3.html) and tested by our group. All primers were synthesized by MWG (Ebersberg, Germany). The conditions for amplification were: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C to 60 °C, 30 s at 72 °C, followed by an extension for 7 min at 72 °C. Human hippocampal total RNA-derived cDNA, before and after RNase treatment was used as positive and negative PCR control.

2.5. Ligand binding assays

2.5.1. Displacement binding of somatostatin on HepG2 membranes

HepG2 cells membranes were prepared, as described previously (Liapakis and Thermos, 1992) using 50 mM Tris–HCl, pH 7.4 at 4 °C, containing bacitracin (200 mg/ml), EGTA (1 mM), $MgCl_2$ (5 mM), leupeptin (10 mg/ml), and phenylmethanosulfonyl fluoride (0.1 mM). Membranes (70 μg protein) were incubated with radioactive [^{125}I]Tyr11-somatostatin-14 (1.5 nM; 2000 Ci/mmol), in the presence and absence of non-radioactive ligands. The mixture was equilibrated for 90 min at 25 °C and the binding reaction was terminated by vacuum filtration over GF/C filters that were previously soaked for 60 min in polyethyleneimine (0.1%) and 30 min in bovine serum albumin (1%). The filters were washed 3 times with 5 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4 and counted in a gamma counter (LKB-Wallac, Turku, Finland, 75% efficiency). The iodinated ligand was diluted with Tyr^{11}-somatostatin-14 in order to obtain the desired concentration. Specific binding was defined in the presence of 10^{-6} M somatostatin-14 and represented 39.32±0.03% of the total binding.

2.5.2. Displacement binding assay of opioids on HepG2 cells

Binding assays in whole HepG2 cells (about 10^6 cells/well) were performed as described by Hatzoglou et al. (1996a). Before binding, cells were washed twice with 2 ml of phosphate buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4). Binding was performed in the same buffer, in a total volume of 0.5 ml, containing radioactive opioids, without or with the addition of potential displacers, at a range from 10^{-12} to 10^{-6} M. 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 2N NaOH, and mixed with 4 ml scintillation cocktail (SigmaFluor, Sigma, St Louis, MI). The bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000, Packard), with a 60% efficiency for Tritium.

2.6. Statistics

Statistical comparisons were performed using the paired or unpaired *t*-test and two way analysis of variance) where applicable. Results are expressed as mean±standard error. $P<0.05$ was considered significant.

3. Results

3.1. Opioids inhibit the proliferation of HepG2 cells and induce apoptosis

When HepG2 cells were incubated with the general opioid agonist ethylketocyclazocine (EKC, 10^{-12} to 10^{-6} M), which presents an enhanced selectivity for kappa1 and kappa2 receptors (Castanas et al., 1985a,b), a dose dependent inhibition of cell proliferation was observed (Fig. 1A) with an IC_{50} of 2.7± 1.4 nM. In contrast, other opioid agonists (DAGO and DADLE, acting through mu and delta opioid receptors respectively) exhibit minor inhibitory effects at higher concentrations (Fig. 1A), with IC_{50}s 19.3±1.7 nM and 7.8±0.4 nM respectively. These results point out that EKC is the most potent opioid agonist and its effect might be attributed mainly to its

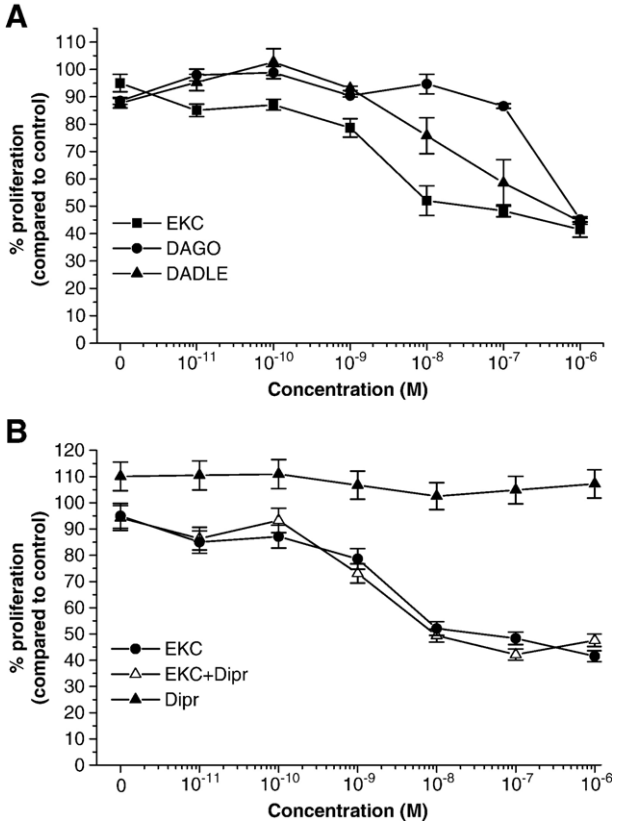

Fig. 1. Opioids decrease cell growth of HepG2 human hepatocellular carcinoma cells. A. HepG2 cells were incubated for six days with the indicated concentrations of different opioids agonists with medium and opioids change at day 3. Cell number was assayed by the MTT assay, as described in Materials and methods. Figure presents the percentage of viable cells under each condition, normalized by the cell number of control (untreated) cells. Mean± SEM of three independent experiments, performed in triplicate. B. The effect of EKC in the absence or in the presence of the opioid antagonist diprenorphine. HepG2 cells were incubated in the absence (control) or the presence of different concentrations of ethylketocyclazocine (EKC, circles), diprenorphine (Dipr, up triangles) or their association (increasing concentrations of EKC in the presence of 10^{-6} M diprenorphine, squares), for 6 days, with medium and agents change at day three. Figure presents the percentage of viable cells under each condition, normalized by the cell number of control (untreated) cells. Mean±SEM of three independent experiments, performed in triplicate.

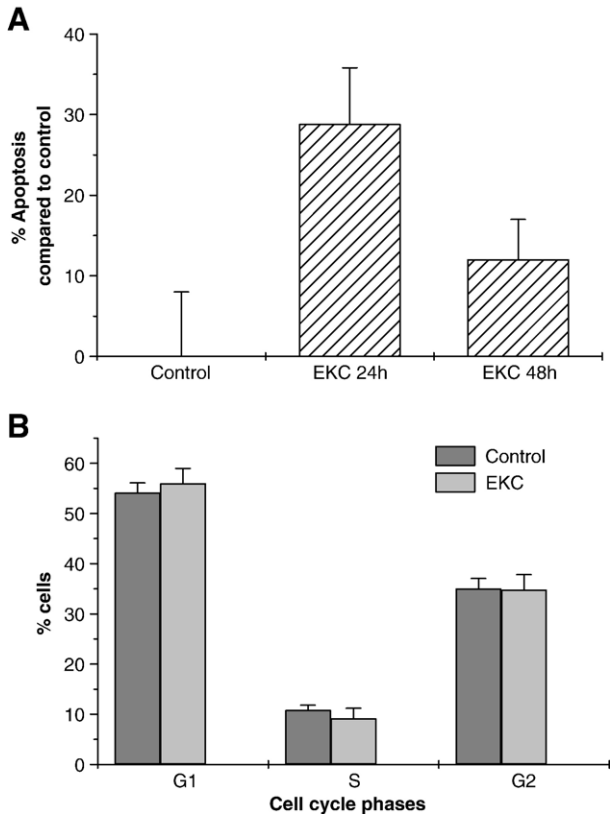

Fig. 2. The effect of EKC on apoptosis (A) and cell cycle (B) of HepG2 cells. HepG2 cells were incubated in the absence or in the presence of 10^{-7} M of ethylketocyclazocine, for 24 or 48 h. Then, cells were removed, stained with propidium iodide (for the determination of cell cycle) or Anexin V-FITC/PI for the detection of apoptotic cells, as described under Materials and methods and analyzed by flow cytometry. Figure presents the Mean±SEM of three independent experiments.

interaction with kappa opioid receptors. However, the addition of the general opioid antagonist diprenorphine (10^{-5} M) did not significantly affect the inhibitory effect of EKC on HepG2 proliferation (IC_{50}s 2.7±1.4 and 1.38±0.5 nM in the absence or in the presence of diprenorphine respectively Fig. 1B). The absence of diprenorphine antagonism suggests that the opioid action might be mediated through a mechanism different from opioid receptors.

In order to further elucidate the effect of EKC on HepG2 cells, we assayed its effect on cell cycle and apoptosis. As shown in Fig. 2A, EKC induced a 28% increase of Annexin V-FITC stained apoptotic cells after two days of incubation. However, after another cell cycle, this apoptotic effect was much attenuated (12% at day 4) (similar results on apoptosis were obtained by the cell death detection ELISA kit). In contrast, no significant alteration of cell cycle was found (Fig. 2B). These results could be explained by a transient action of EKC on apoptosis, or by a specific action of the agent on cells at a specific point of their growth.

3.2. Detection of opioid receptors in HepG2 cells

As indicated above, EKC, compared to DADLE or DAGO, is the most potent opioid agonist, decreasing cell proliferation of HepG2 cells and inducing apoptosis. In order to determine whether opioid receptors are expressed in HepG2 cells, we have performed an RT-PCR assay, in order to reveal opioid receptor transcripts. As shown in Fig. 3A, only kappa opioid receptor mRNA was detected. However, ligand binding experiments (Fig. 3B), with the use of tritiated U69,593, a pure agonist of kappa opioid receptors, or [^{3}H]EKC (not shown), did not show any specific displacement of the radioactive ligand, indicating the absence of a functional kappa opioid receptor site on these cells. In contrast the general antagonistic ligand diprenorphine and the selective delta receptor ligand DPDPE revealed a dose dependent displacement of the tritiated by the non-tritiated homologous agent. The above results could indicate the presence of opioids sites with mu or delta specificity. However, the absence of delta or mu transcripts, the observed effect of delta or mu-selective agonists on cell growth, and the absence of an opiod-receptor related effect (absence of action of the antagonist diprenorphine) prones about the absence of functional receptor site. These data suggest that the antiproliferative and proapoptotic action of opioids might be attributed to its interaction with other membrane receptor systems.

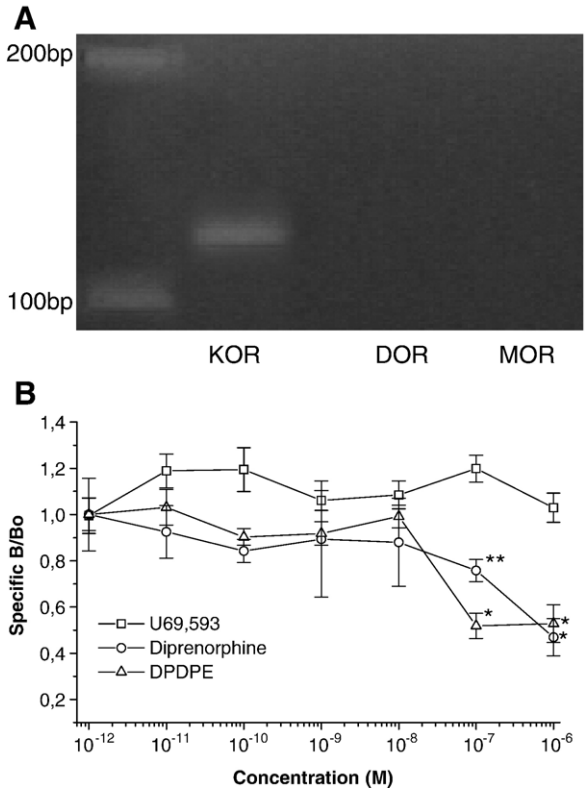

Fig. 3. Detection of opioid receptors on HepG2 cells. Á. Total RNA was extracted from HepG2 cells and RT-PCR for opioid receptors was performed as described under Materials and methods. Figure presents a typical RT-PCR profile for delta (DOR), mu (MOR) and kappa (KOR) opioid receptors, repeated two more times with similar results. B. Displacement binding assays: Assays were performed in whole HepG2 cells as described in Materials and methods section. Cells were incubated with radioactive opioids, in the presence or in the absence of the non radioactive homologous displacers, at a range from 10^{-12} to 10^{-6} M. The results are presented as the ratio of the specific binding in the presence of the displacer to the specific binding in the absence of any displacer (B/B_o). Figure presents the Mean±SEM of three independent experiments.

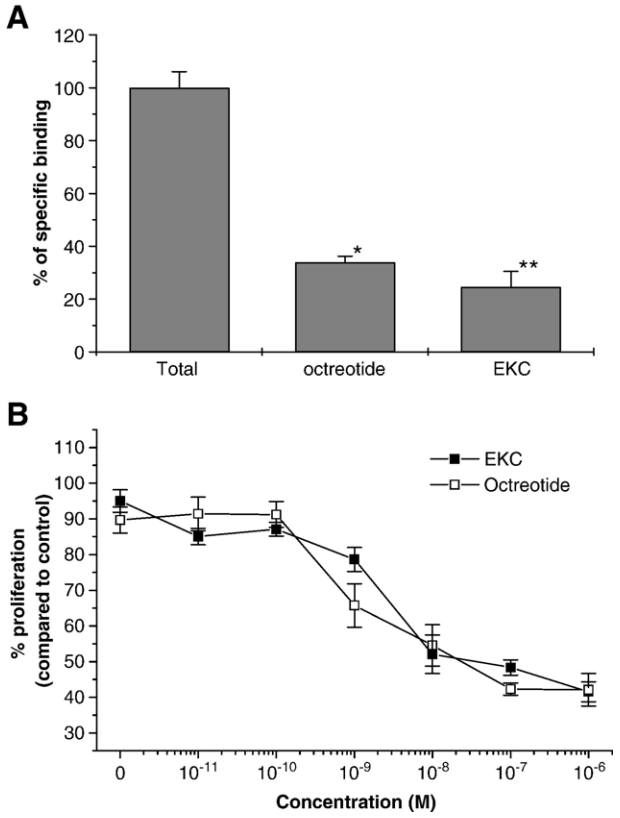

Fig. 4. A. Specifying binding of EKC to somatostatin receptors: Displacement binding experiments were performed in HepG2 cells membranes incubated with radioactive [^{125}I]Tyr^{11}-somatostatin-14 in the presence and absence of octreotide or EKC (10^{-7} M). The results are presented as the percentage of the specific radiolabelled somatostatin binding (100%). Figure presents the Mean±SEM of three independent experiments. B. Antiproliferative effect of EKC and octreotide on HepG2. Cells were incubated in the absence (control) or in the presence of different concentrations of ethylketocyclazocine (EKC, squares), or octreotide (open squares) for 6 days, with medium and agents change at day three. Figure presents the percentage of viable cells under each condition, normalized by the cell number of control (untreated) cells. Mean±SEM of three independent experiments, performed in triplicate.

3.3. EKC binds to somatostatin receptors on HepG2 cells and its effect is mediated by protein tyrosine phosphatases

Several findings indicate that G-protein coupled receptors (GPCRs) do not function as monomeric entities, but form dimmers (both homo- and hetero-dimmers) on the membrane of cells (Bulenger et al., 2005; Terrillon and Bouvier, 2004). Such a hetero-dimerization has been described for adrenergic and opioid receptors (Jordan et al., 2001). Moreover, an interaction between opioid and somatostatin receptors was found on breast cancer cells (Hatzoglou et al., 1995). As functional somatostatin receptor subtypes have been described on HepG2 cells (Notas et al., 2004), we have assayed whether the inhibitory action of EKC could be mediated through such an interaction. As presented in Fig. 4A, EKC displaced, at equimolar concentrations, [^{125}I]Tyr^{11}-SRIF-14 binding on HepG2 membranes to the same extend as octreotide, a non-specific agonist of somatostatin receptors. IC_{50}s were 5.7±1.1 and 7.3±2.3 nM for octreotide and EKC respectively, while no additive effect upon co-incubation with both agents was observed (not shown). In addition, as shown in Fig. 4B, similar IC_{50}s of octreotide and EKC were observed on HepG2 proliferation (1.23±0.6 and 2.8±1.6 nM respectively), suggesting that both opioids and octreotide might act through somatostatin receptors.

To further confirm the above interaction, we have assayed the effect of EKC on the previously reported intracellular pathway triggered by somatostatin analogues, namely the phosphotyrosine phosphatase (PTP)activation (Notas et al., 2004). Indeed, as shown in Fig. 5, a gradual inhibition of PTP by increasing concentrations of sodium orthovanadate, reverted to the same extend the inhibitory effect of octreotide and EKC, indicating a similar intracellular mediation of action. In view of these results, we concluded that the inhibitory action of EKC, the only opioid acting on the proliferation of HepG2 cells is mediated through somatostatin receptors.

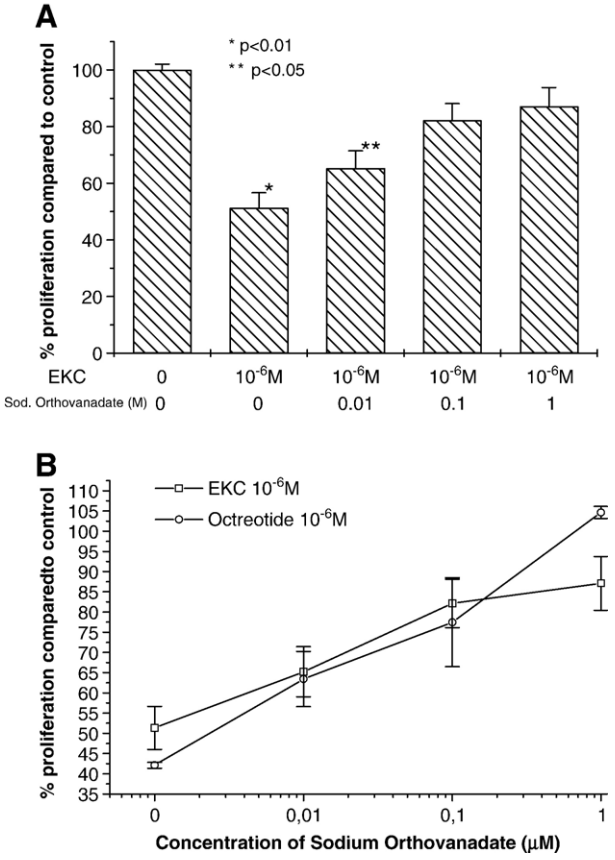

Fig. 5. A. The antiproliferative effect of EKC in the presence a PTPs' inhibitor: cells were incubated with different concentrations of ethylketocyclazocine, in the presence or in the absence of different concentrations of sodium orthovanadate (a PTP inhibitor) for 6 days, with medium and agents change at day three. Figure presents the percentage of viable cells under each condition, normalized by the cell number of control (untreated) cells. Mean±SEM of three independent experiments, performed in triplicate. B. The antiproliferative effect of EKC and octreotide in the presence a PTPs' inhibitor: cells were incubated with 10^{-6} M EKC or octreotide in the absence or presence of different concentrations (10^{-8}–10^{-6} M) of sodium orthovanadate for 6 days, with medium and agents change at day three. Figure presents the percentage of viable cells under each condition, normalized by the cell number of control (untreated) cells. Mean±SEM of three independent experiments, performed in triplicate.

4. Discussion

Endogenous opioid peptides and exogenously administered alkaloids result in the inhibition of the nociceptive stimuli transmission. This effect conducted researchers to investigate whether opioid agonists could mediate similar inhibitory actions on different cell functions, such as cell proliferation and survival. If such an inhibition occurred, opioids could be used for the control of cancer cell proliferation. Indeed, research during the last 20 years showed that opioid agonists inhibit growth and proliferation of a number of neoplastic cell lines, through an activation of opioid receptors (Hatzoglou et al., 2005).

Opioid receptors belong to the superfamily of G-protein coupled membrane receptors. This family includes, in addition to opioids, somatostatin and adrenergic sites, together with a number of orphan receptors, for which no endogenous ligand has been identified. An interesting finding, in addition to a certain homology of receptor structure, is that members of this family can heterodimerize (Bulenger et al., 2005; Terrillon and Bouvier, 2004), to elicit their action, while a cross-reaction of agonists on heterologous members of this family may occur. Indeed, opioids were found to bind to somatostatin receptors and inhibit cell growth in different cancer cell systems. In renal OK cells the inhibitory effect of morphine was mediated via somatostatin receptors (Hatzoglou et al., 1996b). Stirweiss et al. reported that the cyclic pentapeptide, cCD-2 (Tyr-cyclo[d-Orn-Tyr(Bzl)-Pro-Gly]), derived from beta-casomorphin-5, inhibited the growth of SH-SY5Y cells independently of opioid receptors. cCD-2 possessed only low affinity for mu-receptors and its action was exerted by specific binding to somatostatin receptors (sst1) and stimulating the activity of protein tyrosine phosphatases (Stirnweiss et al., 2003). In addition it has been reported that opioids can interact with both receptor systems on the same cell (Hatzoglou et al., 1996a; Kampa et al., 1997, 2001). Finally, it was shown that the SSTR2 and the mu opioid receptor heterodimerize in human embryonic kidney 293 cells, without altering the binding or coupling properties of each receptor (Pfeiffer et al., 2002) but affecting their phosphorylation and desensitization. Our results indicate that such a heterologous interaction occurs on HepG2 hepatocellular carcinoma cells. Indeed, in spite of the absence of functional opioid receptors, the opioid agonist EKC elicits an inhibitory and a proapoptotic action. Further analysis of EKC activity revealed that it is mediated through somatostatin receptors. Indeed, EKC competes for octreotide binding, exhibits a similar antiproliferative effect as octreotide, and shares the same intracellular signalling mechanism, namely phosphotyrosine phosphatase activation.

The effect of opioids on the growth of hepatocyte cancer cells has not been investigated so far. There are only two studies dealing either with their toxicity (Falk et al., 1995; Jairaj et al., 2003) or bioavailability (Kotb et al., 2005) in the liver. In contrast, we have reported recently that HepG2 cells express functional somatostatin receptor subtypes (Notas et al., 2004): sst2 are mainly located intracellularly while sst3 and sst5 located at the membrane level. Somatostatin analogs and the secreted cortistatin inhibit cell growth through interaction with the membrane-located somatostatin receptor subtypes. In addition, a number of studies report that somatostatin analogues inhibit *in vitro* hepatocyte cell growth (Liu et al., 2004; Notas et al., 2004; Wang et al., 2001) and reduce hepatocellular carcinoma xenografts in nude mice (Wang et al., 2001). Moreover, there are clinical trials showing some degree of improvement on the survival rate for patients with hepatocellular carcinoma when treated with octreotide. In this respect, the interaction of opioids with somatostatin receptors on hepatocellular carcinoma cells that we report here, may provide some additional clues during treatment with somatostatin analogues, in view of the use of opioids for pain control. In addition, as we have reported previously, hepatocellular carcinoma cells produce and secrete appreciable amounts of cortistatin (Notas et al., 2004), responsible for somatostatin receptor internalisation and modification of the somatostatin receptor profile expressed on the membranes of these cells. This should be taken into account and it might explain some discrepancies in the treatment of hepatocellular carcinoma with somatostatin analogues.

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References

Bruix, J., Sherman, M., Llovet, J.M., Beaugrand, M., Lencioni, R., Burroughs, A.K., Christensen, E., Pagliaro, L., Colombo, M., Rodes, J., 2001. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J. Hepatol. 35, 421–430.

Bulenger, S., Marullo, S., Bouvier, M., 2005. Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. Trends Pharmacol. Sci. 26, 131–137.

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 delta and mu 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 kappa sites. J. Neurochem. 45, 688–699.

Chiodera, P., Gnudi, A., Bianconi, L., Capretti, L., Fagnoni, F., Volpi, R., Coiro, V., 1989. Naloxone abolishes the inhibiting effect of somatostatin on the release of oxytocin evoked by insulin-induced hypoglycemia in humans. Metabolism 38, 709–711.

Dimitroulopoulos, D., Xinopoulos, D., Tsamakidis, K., Zisimopoulos, A., Andriotis, E., Markidou, S., Panagiotakos, D., Chrysohoou, C., Bazinis, A., Paraskevas, E., 2002. The role of sandostatin LAR in treating patients with advanced hepatocellular cancer. Hepatogastroenterology 49, 1245–1250.

Falk, P.M., Sabater, R.T., Carballo Jr., D.D., 1995. Response of the human hepatic tissue cultures Hep-G2 and WRL-68 to cocaine. J. Pharmacol. Toxicol. Methods 33, 113–120.

Ghosh, S., Geller, E.B., Adler, M.W., 1997. Interaction of cholecystokinin and somatostatin with a selective mu-opioid agonist and mu- and kappa-antagonists in thermoregulation. Brain Res. 745, 152–157.

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

Hatzoglou, A., Bakogeorgou, E., Hatzoglou, C., Martin, P.M., Castanas, E., 1996a. Antiproliferative and receptor binding properties of alpha- and beta-casomorphins in the T47D human breast cancer cell line. Eur. J. Pharmacol. 310, 217–223.

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

Hatzoglou, A., Kampa, M., Castanas, E., 2005. Opioid-somatostatin interactions in regulating cancer cell growth. Front. Biosci. 10, 244–256.

Horvath, A., Folhoffer, A., Lakatos, P.L., Halosz, J., Illyes, G., Schaff, Z., Hantos, M.B., Tekes, K., Szalay, F., 2004. Rising plasma nociceptin level during development of HCC: a case report. World J. Gastroenterol. 10, 152–154.

Jairaj, M., Watson, D.G., Grant, M.H., Skellern, G.G., 2003. The toxicity of opiates and their metabolites in HepG2 cells. Chem. Biol. Interact. 146, 121–129.

Jordan, B.A., Trapaidze, N., Gomes, I., Nivarthi, R., Devi, L.A., 2001. Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proc. Natl. Acad. Sci. U. S. A. 98, 343–348.

Kampa, M., Bakogeorgou, E., Hatzoglou, A., Damianaki, A., Martin, P.M., Castanas, E., 1997. Opioid alkaloids and casomorphin peptides decrease the proliferation of prostatic cancer cell lines (LNCaP, PC3 and DU145) through a partial interaction with opioid receptors. Eur. J. Pharmacol. 335, 255–265.

Kampa, M., Loukas, S., Tsapis, A., Castanas, E., 2001. Receptorphin: a conserved peptide derived from the sequence of the opioid receptor, with opioid displacement activity and potent antiproliferative actions in tumor cells. BMC Pharmacol. 1, 9.

Konagaya, T., Kusugami, K., Yamamoto, H., Nishio, Y., Kaneko, H., Nagai, H., Mitsuma, T., 1998. Effect of intragastric administration of beta-endorphin on thyrotropin-releasing hormone and somatostatin release into gastric lumen of rats. J. Gastroenterol. 33, 27–31.

Kotb, H.I., El-Kady, S.A., Emara, S.E., Fouad, E.A., El-Kabsh, M.Y., 2005. Pharmacokinetics of controlled release morphine (MST) in patients with liver carcinoma. Br. J. Anaesth. 94, 95–99.

Kouroumalis, E., Skordilis, P., Thermos, K., Vasilaki, A., Moschandrea, J., Manousos, O.N., 1998. Treatment of hepatocellular carcinoma with octreotide: a randomised controlled study. Gut 42, 442–447.

Liapakis, G., Thermos, K., 1992. Characterization of [125I]Tyr11-somatostatin binding sites in the rabbit retina. Neuropeptides 21, 13–19.

Liu, H.L., Huo, L., Wang, L., 2004. Octreotide inhibits proliferation and induces apoptosis of hepatocellular carcinoma cells. Acta Pharmacol. Sin. 25, 1380–1386.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Notas, G., Kolios, G., Mastrodimou, N., Kampa, M., Vasilaki, A., Xidakis, C., Castanas, E., Thermos, K., Kouroumalis, E., 2004. Cortistatin production by HepG2 human hepatocellular carcinoma cell line and distribution of somatostatin receptors. J. Hepatol. 40, 792–798.

Pfeiffer, M., Koch, T., Schroder, H., Laugsch, M., Hollt, V., Schulz, S., 2002. Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J. Biol. Chem. 277, 19762–19772.

Raderer, M., Hejna, M.H., Muller, C., Kornek, G.V., Kurtaran, A., Virgolini, I., Fiebieger, W., Hamilton, G., Scheithauer, W., 2000. Treatment of hepatocellular cancer with the long acting somatostatin analog lanreotide in vitro and in vivo. Int. J. Oncol. 16, 1197–1201.

Shook, J.E., Pelton, J.T., Lemcke, P.K., Porreca, F., Hruby, V.J., Burks, T.F., 1987. Mu opioid antagonist properties of a cyclic somatostatin octapeptide in vivo: identification of mu receptor-related functions. J. Pharmacol. Exp. Ther. 242, 1–7.

Stirnweiss, J., Hartrodt, B., Greksch, G., Sturzebecher, U., Bohmer, F.D., Neubert, K., Liebmann, C., 2003. Tyr-c[D-Orn-Tyr(Bzl)-Pro-Gly]: a novel antiproliferative acting somatostatin receptor agonist with mu-opioid receptor-sensitizing properties. Br. J. Pharmacol. 140, 13–22.

Szalay, F., Hantos, M.B., Horvath, A., Lakatos, P.L., Folhoffer, A., Dunkel, K., Hegedus, D., Tekes, K., 2004. Increased nociceptin/orphanin FQ plasma levels in hepatocellular carcinoma. World J. Gastroenterol. 10, 42–45.

Terrillon, S., Bouvier, M., 2004. Roles of G-protein-coupled receptor dimerization. EMBO Rep. 5, 30–34.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods 184, 39–51.

Wang, C., Tang, C., Tang, L., 2001. Inhibition effects of octreotide on the growth of hepatocellular carcinoma in vitro and in vivo. Zhonghua Yixue Zazhi 81, 1194–1197.

Yuen, M.F., Poon, R.T., Lai, C.L., Fan, S.T., Lo, C.M., Wong, K.W., Wong, W.M., Wong, B.C., 2002. A randomized placebo-controlled study of long-acting octreotide for the treatment of advanced hepatocellular carcinoma. Hepatology 36, 687–691.