

# Opioids As Modulators of Cell Death and Survival— Unraveling Mechanisms and Revealing New Indications

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**Abstract**—Opioids are powerful analgesics but also drugs of abuse. Because opioid addicts are susceptible to certain infections, opioids have been suspected to suppress the immune response. This was supported by the finding that various immune-competent cells express opioid receptors and undergo apoptosis when treated with opioid alkaloids. Recent evidence suggests that opioids may also effect neuronal survival and proliferation or migrating properties of tumor cells. A multitude of signaling pathways has been suggested to be involved in these extra-analgesic effects of opioids. Growth-promoting effects were found to be mediated

through Akt and Erk signaling cascades. Death-promoting effects have been ascribed to inhibition of nuclear factor- $\kappa$ B, increase of Fas expression, p53 stabilization, cytokine and chemokine release, and activation of nitric oxide synthase, p38, and c-Jun-N-terminal kinase. Some of the observed effects were inhibited with opioid receptor antagonists or pertussis toxin; others were unaffected. It is still unclear whether these properties are mediated through typical opioid receptor activation and inhibitory G-protein-signaling. The present review tries to unravel controversial findings and provides a hypothesis that may help to integrate diverse results.

## I. Introduction

Stimulation of opioid receptor signaling in neurons produces strong analgesic effects. In addition to these

well recognized effects, various studies suggest that opioids elicit a variety of biological effects that appear to be independent of their analgesic properties and may effect cell survival or proliferation. The complexity of the molecular mechanisms whereby opioids modulate cell survival and cell death has just begun to be fully appreciated. For a long time the study of opioid receptor signaling has been focused on the classical adenylyl

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cyclase/cyclic AMP/protein kinase A (PKA<sup>1</sup>) pathway. However, it has now been realized that this pathway does not sufficiently explain the wide array of biological responses to opioids. These include growth-promoting as well as death-promoting effects that are in part shared with other G<sub>i</sub>-protein-coupled receptors. This review unravels the complexity of potential mechanisms and controversial results and reveals some hypothetical new clinical indications for opioids.

## II. Growth-Promoting and Protective Effects

Nearly 20 years ago, Meriney et al. (1985) reported that morphine administered during embryogenesis prevents apoptosis of ciliary ganglion neurons that normally die during the period of synapse formation. They suggested that this effect was mediated through a neurotrophic mechanism or inhibition of neurotransmission (Meriney et al., 1985). Morphine also prevented peroxynitrite-induced apoptosis in primary astrocytes (Kim et al., 2001) and enhanced the proliferation of endothelial cells (Gupta et al., 2002), kidney fibroblasts (Singhal et al., 1998), and adult hippocampal progenitor neurons (Persson et al., 2003). In addition, dynorphin (KOR agonist) and DOR agonists were found to increase proliferation of prostate cancer cells (Moon, 1988), rat splenocytes (Ni et al., 1999), and neuroblastoma cells (Law and Bergsbaken, 1995; Law et al., 1997), respectively. Opioid receptor antagonists had converse effects in neuroblastoma cells (Zagon and McLaughlin, 1983) and hippocampal progenitors (Persson et al., 2003) but failed to inhibit the growth-stimulating effects of morphine in endothelial cells (Gupta et al., 2002). Table 1 summarizes results of various studies.

Since the first description, it has been repeatedly demonstrated that morphine treatment is associated with

<sup>1</sup>Abbreviations: PKA, protein kinase A; CaM, calmodulin; CaMK, calmodulin kinase; DAMGO, [D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin; DOR,  $\delta$ -opioid receptor; EGF, epidermal growth factor; EGFR, EGF receptor; Erk, extracellular-regulated kinase; G $\alpha$ , GTP-binding protein,  $\alpha$  subunit; G $\beta\gamma$ , GTP-binding protein,  $\beta$  and  $\gamma$  subunit; GDP, G-protein dissociation protein; G<sub>i</sub>, inhibitory pertussis toxin-sensitive G-protein; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; G<sub>z</sub>, inhibitory pertussis toxin-insensitive G-protein; HIV, human immunodeficiency virus; ICAM, intercellular cell adhesion molecule; IL, interleukin; JNK, c-Jun-N-terminal kinase; KOR,  $\kappa$ -opioid receptor; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MKK, mitogen-activated protein kinase kinase; MMP, metalloproteinase; MOR,  $\mu$ -opioid receptor; NF- $\kappa$ B, nuclear factor kappa B; NK cell, natural killer cell; NMDA-R, N-methyl-D-aspartate receptor; NO, nitric oxide; NOS, nitric-oxide synthase; PAR, proteinase-activated receptor; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A (cAMP-dependent protein kinase); PKB/Akt, protein kinase B; PKC, protein kinase C; PTX, pertussis toxin; RGS, regulator of G-protein signaling; Src, non-receptor tyrosine kinase; TGF, tissue growth factor; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; U50,488H, *trans*-( $\pm$ )-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzene acetamide methane-sulfonate hydrate; VEGF, vascular endothelial growth factor.

increased Erk expression and/or phosphorylation in neurons (Ortiz et al., 1995; Berhow et al., 1996; Ma et al., 2001), immune cells (Chuang et al., 1997), and opioid receptor transfected cells (Belcheva et al., 1998; Polakiewicz et al., 1998b; Kramer and Simon, 2000; Schmidt et al., 2000). Erk activation also occurs following treatment with selective DOR and KOR agonists (Hedin et al., 1999; Kam et al., 2004; Narita et al., 2002; Persson et al., 2003). As the Erk phosphorylation cascade ranks among the main signaling pathways involved in mitogenic responses to external stimuli, it may be suggested that Erk is one messenger by which opioids transmit their neuroprotective or survival-promoting effects. The mechanisms by which opioids may cause Erk activation have been extensively studied. These effects are shared by other G-protein-coupled receptors (for review, see Schwindinger and Robishaw, 2001) and are most likely dependent on G-protein-signaling (please see below).

### A. G $\beta\gamma$ -Phosphatidylinositol 3-Kinase Pathway

Opioid receptors of the  $\mu$ ,  $\delta$ , and  $\kappa$  subtypes (MOR, DOR, KOR) are G-protein-coupled receptors (GPCR). Upon agonist binding, the opioid receptor couples to the heterotrimeric pertussis toxin-sensitive inhibitory G-protein (G<sub>i/o</sub>) (Fig. 1). The  $\alpha$  subunit binds GTP and dissociates from G $\beta\gamma$ . This event allows both G $\alpha$ -GTP and free G $\beta\gamma$  to regulate the activities of downstream molecules. G $\alpha$ -GTP is primarily responsible for the well studied classical pathway consisting of an inhibition of adenylyl cyclase, lowering intracellular cAMP levels, and thereby decreasing PKA activity. The action of the inhibitory G $\alpha$ -protein is modulated by RGS (regulator of G-protein signaling) proteins that act as GTPase activating proteins. RGS proteins thereby reduce the lifetime of G $\alpha$ -GTP and accelerate the termination of its effects. Apart from the G $\alpha$ -mediated effects, several reports have underscored the relevance of opioid receptor signaling through G $\beta\gamma$  (Narita et al., 2004). Liberated G $\beta\gamma$  activates specific isoforms of phosphatidylinositol 3-kinase, i.e., PI3K $\gamma$  (Stoyanov et al., 1995; Lopez-Illasaca et al., 1997; Brock et al., 2003) and PI3K $\beta$  (Maier et al., 1999; Czupalla et al., 2003). This event leads to activation of Ras and the Raf/MEK/Erk kinase cascade (Schwindinger and Robishaw, 2001; Kramer et al., 2002; Persson et al., 2003). PI3K is also linked to protein kinase B (PKB/Akt), which exerts anti-apoptotic effects through inhibition of Bad (pro-apoptotic mitochondrial protein) (Dudek et al., 1997; Franke et al., 1997; Polakiewicz et al., 1998b) and activation of NF- $\kappa$ B (Madrid et al., 2000). Hence, G $\beta\gamma$ -mediated PI3K-dependent signaling pathways may contribute to the anti-apoptotic effects of morphine and selective agonists. These effects depend on opioid receptor stimulation and coupling to G<sub>i</sub> because the anti-apoptotic or protective effects of morphine are abolished with the opioid receptor antagonist naloxone (Meriney et al., 1985, 1991) with pertussis toxin (PTX), which inhibits G<sub>i</sub>, and with inhibitors of

TABLE 1  
Summary of growth-promoting and growth-inhibiting effects of opioids

Cell Type	Opioid	In Vitro Concentration and Time	In Vivo Effects	In Vivo Dose	In Vivo or Ex Vivo Effects	Suggested Mechanism	OR Antagonist Inhibition with:	Other Inhibitors	Reference
<b>Immune cells</b>									
Human blood T-lymphocytes stimulated with concanavalin A	$\beta$ -endorphin	$10^{-9}$ – $10^{-10}$ M for 1 h	Stimulation of Con A-induced proliferation			Increase of IL-2	No inhibition with Nx $10^{-6}$ M		van den Bergh et al., 1991; Navolotskaya et al., 2002a
Jurkat T-lymphocytes	Morphine DAMGO	0.01–100 $\mu$ M for 24–48 h	Apoptosis			Increase of ROS, Bcl2 down- and Bax up-regulation	Nix (1 $\mu$ M)	Catalase	Singhal et al., 1999
CEMx174 lymphocytic cells	Morphine	10 $\mu$ M for 12–42 h				Increase of Erk expression and phosphorylation	Nx (10 $\mu$ M)		Chuang et al., 1997
Lymphocytes of splenocytes of morphine-treated BALB/C mice	Morphine	3 $\mu$ M for 24 h	Apoptosis	50 mg/kg single dose	Reduced number of splenocytes at 24 h	Increase of Fas expression	Nx (100 mg/kg single dose)		Yin et al., 1999
Spleen, lung and liver tissue of heroin-treated rats	Heroin			1 mg/kg single dose	Inhibition of LPS-induced iNOS expression		Nix (0.1 mg/kg)		Fecho and Lysle, 2000; Lysle and How, 2000
T- and B-lymphocytes, macrophages in chronically treated mice	Morphine			10–50 mg/kg morphine for 4 days	Atrophy of thymus and spleen decreased Con A/ILPS-induced T- and B-cell proliferation decrease of NK cell cytotoxicity	Decreased cytokine production in macrophages			Lu et al., 1996; Wu et al., 1999
Splenocytes from morphine-treated Lewis rats	Morphine			15 mg/kg single dose	Splenocyte apoptosis decreased Con A-induced splenocyte proliferation	Increase of Con A-stimulated NO release from macrophages		NMMA (NOS antagonist)	Fecho et al., 1994
J774 mouse macrophages	Morphine	0.001–1 $\mu$ M for 24 h	Apoptosis	40 mg/kg every 12 h for 24 h	Increased apoptosis of peritoneal macrophages	Increase of Bax and TGF $\beta$ release		Anti-TGF $\beta$ antibody	Singhal et al., 2000
J774 mouse macrophages	Morphine	1–100 $\mu$ M	Apoptosis inhibition of P-p38 increase of Fas/FasL expression			Inhibition of TGF $\beta$ release, iNOS up-regulation and p53 stabilization	Nix 1 $\mu$ M vs. 0.01 $\mu$ M morphine (only P-p38)	Anti-TGF $\beta$ antibody inhibited with anti-FasL and Casp3, 8, 9 inhibitors	Singhal et al., 2002
J774 mouse peritoneal macrophages from morphine-treated FVB/N mice	Morphine	1 pM–1 nM for 26 h	Apoptosis	20 mg/kg/d for 10 days	Inhibition of macrophage migration into peritoneal cavity and apoptosis	Increase of HO-1 activity and expression		Zinc-protoporphyrin	Patel et al., 2003
Splenocytes from morphine- or heroin-treated C57Bl/6 mice	Morphine heroin			20 mg/kg single dose	Early phase: increase of PHA-stimulated cytokine release (IL-2, TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$ ); late phase: inhibition of cytokine release	Early phase: central-mediated immune stimulation; late phase: peripheral immunosuppression mechanism			Pacifici et al., 2000b
Splenocytes from $\beta$ -endorphin-treated rats	$\beta$ -endorphin			1–10 ng/rat i.t. single dose	Reduction of PHA-stimulated proliferation	Endocrine-mediated mechanism	No inhibition with Nx 5 mg/kg i.p.		Panerai et al., 1995
Thymocytes from morphine-treated B6C3F1 mice	Morphine	Up to 100 $\mu$ M for 18 h	No effect	20 mg/kg for 4 days	Atrophy of thymus and spleen apoptosis of thymocytes particularly CD4 <sup>+</sup> /CD8 <sup>+</sup> cells	Increased glucocorticoid plasma concentrations	Nx 1/7th of morphine dose	Glucocorticoid receptor antagonist	Freier and Fuchs, 1996; Fuchs and Pruett, 1993; Kim et al., 1999
Macrophages	Morphine	0.05 and 50 $\mu$ M for 2 h	0.05 $\mu$ M decrease of LPS-induced IL-6 and TNF $\alpha$ release			0.05 $\mu$ M NF- $\kappa$ B activation, 50 $\mu$ M NF- $\kappa$ B inhibition	Nx 0.1 and 100 $\mu$ M		Roy et al., 1998
Hematopoietic stem cells	Morphine enkephalin	1–10 $\mu$ M for 3–60 min	No effect on proliferation			Increase of MAPK activity (Erk, Elk phosphorylation)	Nx (100 $\mu$ M)		Rozenfeld-Granot et al., 2002
Human neutrophils and monocytes	Morphine Met-enkephalin DAMGO	0.01–100 nM for 1–1.5 h	Inhibition of chemokine-induced (MIP-1 $\alpha$ , RANTES, IL-8) chemotaxis			Heterologous desensitization of CXCR1 and CXCR2 through release G $\beta$ y	Nx (equimolar to $\mu$ -agonist)	PTX	Grimm et al., 1998a; 1998b

TABLE 1 Continued  
Summary of growth-promoting and growth-inhibiting effects of opioids

Cell Type	Opioid	In Vitro Concentration and Time	In Vivo Effects	In Vivo Dose	In Vivo or Ex Vivo Effects	Suggested Mechanism	OR Antagonist Inhibition with:	Other Inhibitors	Reference
Human peripheral blood monocytes	DAMGO morphine	100 nM to 1–100 nM for up to 96 h	Increase of chemokine expression MCP-1, RANTES, and IP-10 and LPS- and PHA-induced TGFβ release	90–120 mg/kg/day in cancer patients	Reduction of NK cytotoxicity, increase of LAK cell development	Increase of prolactin release	μ-selective antagonist		Chao et al., 1992; Wetzel et al., 2000a
Human NK and LAK cells	Morphine			15 mg/kg/day for 7 days	Reduced ConA and PHA-stimulated NK cell activity and splenocyte proliferation	Central mediated effect		Bromocriptine	Provinciali et al., 1996
NK cells and splenocytes of morphine-treated rats with CCI	Morphine		CCI per se had no significant effect						Tsai et al., 2000
Splenocytes	β-endorphin deficiency				Increased splenocyte proliferative response and cytokine production in β-endorphin-deficient mice				Refajo et al., 2002
<b>Neurons and glia</b>									
Primary rat neonatal astrocytes	Morphine	200 μM for 24 h	Reduction of peroxynitrite-induced cell death						Kim et al., 2001
Isolated rat adult hippocampal progenitors	β-endorphin-selective DOR agonists	48 h	Increase of mitosis and DNA content						Persson et al., 2003
Dopaminergic neurons of rat brain neuron-glia cultures	Dynorphin	10 <sup>-15</sup> M	Reduction of LPS-induced apoptosis						Liu et al., 2001
Primary astrocytes from newborn mouse striatum, hippocampus, and cortex	Morphine	1 μM for 6 days	Inhibition of proliferation (H <sup>3</sup> -thymidine incorporation)						Stiene-Martin et al., 1991; Stiene-Martin and Hauser, 1993
Neurons (mouse striatum)	Morphine	0.01–1 μM for 24 h	Increases HIV Tat protein-induced cell death; morphine alone has no effect at 10 μM for 7 days						Gurwell et al., 2001
U87 astrocytoma cell line and human primary astrocytes	Morphine	0.01–0.1 μM for 24 h	Down-regulation of IL-8 and MIP-1β; increase of chemokine receptor expression (CCR3, CCR5, and CXCR2)						Mahajan et al., 2002
Microglia neurons	Morphine	1 μM for 2–7 days	Apoptosis, astrocytes are resistant						Hu et al., 2002
NG108-15 nerve cells	Buprenorphine	100 μM for 4–12 h	Apoptosis						Kugawa et al., 1998; Mao et al., 2002
Spinal cord neurons in rats made tolerant to MOR (GABA-ergic)	Morphine		Effects of morphine associated with high degree of tolerance and dependence	10–20 μg i.t. twice daily for 7 days	Apoptosis, reduced antinociception in tolerant rats	Stimulation of NMDA-R-mediated caspase activation, Bcl2 down-regulation and Bax up-regulation		Caspase-3 inhibitor	
Brain tissue	Morphine		No effect	Increasing doses up to 100 mg/kg every 12 h	Fas up- and Bcl-2 down-regulation decrease of neurofilament				Boronat et al., 2001
Ciliary ganglion neurons of the chick embryo	Morphine	100 μM repeatedly		70–700 nM/day embryonic day 7–14	Inhibition of apoptosis, no general effect on growth	Inhibition of synaptic transmission, inhibition of ACh release			Meriney et al., 1985; 1991
<b>Endothelial cells and fibroblasts</b>									
Kidney fibroblasts	Morphine	1–100 pM 0.01–100 μM for 48 h	Low dose: increase of proliferation (H <sup>3</sup> -thymidine incorporation); high dose: apoptosis						Singhal et al., 1998

TABLE 1 Continued  
Summary of growth-promoting and growth-inhibiting effects of opioids

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Endothelial cells	Morphine	200 nM – 1 $\mu$ M within minutes	Yasodilation (no effect with fentanyl which is no $\mu$ 3-agonist)			$\mu$ 3-receptor-mediated NO release	Nx		Stefano et al., 1995; Bilfinger et al., 1998; Fimiani et al., 1999b; Gupta et al., 2002
HDMEC in vivo; MCF-7 breast cancer cells in nude mice	Morphine	0.01–100 $\mu$ M for 48 h (Akt, Erk phosphorylation within minutes)	Increase of proliferation, tube formation and inhibition of apoptosis (cytotoxic effects at $\geq 1$ mM)	0.714–1.43 mg/kg/day for 38 days	Increase of tumor growth (MCF-7 breast cancer) and angiogenesis	G $\beta$ /PI3K-mediated activation of Akt and eNOS subsequently NO-induced Erk activation	No inhibition with Nx (equimolar to morphine)	PTX L-NAME (NOS-inhibitor) PD98059 (MEK inhibitor)	
Glomerular epithelial cell	Morphine	0.01 $\mu$ M for 48 h	Increase of proliferation			Increase of home oxygenase activity		Hemin, antioxidants	Patel et al., 2003
Glomerular epithelial cell	Morphine	1–100 $\mu$ M for 48 h	Increase of apoptosis			Decrease of home oxygenase activity		Zinc-protoporphyrin, antioxidants	Patel et al., 2003
<b>Tumor cells</b>									
K562 tumor and human peripheral blood mononuclear cells	Morphine	0.001–1 $\mu$ M for 24 h	Increase of proliferation of tumor cells, decrease with peripheral blood mononuclear cells (H $\beta$ <sup>+</sup> , thymidine incorporation)						Sergeeva et al., 1983
U1690 small cell lung cancer	$\beta$ -endorphin	0.001–1 $\mu$ M for 4 days	Increase of proliferation (cell number)				Not inhibited with Nx		Melzig et al., 1995
PC12 lung cancer, HL-60 leukemia, neuroblastoma, KATO III gastric cancer, SEKI melanoma, SKNO1 leukemia	Morphine	1500–5000 $\mu$ M for 1–4 days	Inhibition of proliferation (MTT assay, counting viable cells)						Suoka et al., 1996
Neuroblastoma, PC-9 lung cancer, U251 glioblastoma, HL-60 colon carcinoma	KT-90 morphine	50–200 $\mu$ M 1–10 mM for 48 h	Apoptosis, inhibition of TPA or okadaic acid-induced TNF $\alpha$ release						Suoka et al., 1996; 1998
neuroblastoma in A/Jax mice	Naltrexone				0.1 mg/kg/day, 10 mg/kg/day	Inhibition of NF- $\kappa$ B			
SK-N-SH neuroblastoma cells	Etorphine	10–100 $\mu$ M for 24 h	Inhibition of proliferation (H $\beta$ <sup>+</sup> -thymidine incorporation) apoptosis no effect with morphine		Low dose: decrease of tumor growth and prolonged survival; high dose: increase of tumor growth and reduced survival	Involvement of ORL receptors		PTX ICE-inhibitor	Yin et al., 1997
SK-N-SH neuroblastoma cells	DAMGO morphine	0.1–2 (10) $\mu$ M for 24 h (5–15 min for P-Akt)	Prevention of cell death induced by serum deprivation			G $\beta$ -mediated PI3K/Akt activation	Inhibition with Nx 50 $\mu$ M vs. 1 $\mu$ M DAMGO	Inhibited with PI3K inhibitor LY294002	Iglesias et al., 2003
LCNaP, DU145, PC3 prostate cancer	Morphine, etorphine, various peptides	0.1–1 nM for 4–5 days	Reduction of metabolically active cells (tetrazolium salt assay)			Binding to atypical binding sites	Diprenorphine 1 $\mu$ M: potentiation or no effect		Kampa et al., 1997
DU145 prostate cancer	$\kappa$ -agonist dynorphin A	0.1 $\mu$ M for 72 h	Increase of proliferation (counting viable cells), morphine: no effect			Subcellular compartment shifts of CaM	No inhibition with Nx		Moon, 1988
SCLC cell lines in vivo: SCLC N417 non-SCLC N466	D- and S-methadone	0.1 $\mu$ M for 5 days	Inhibition of proliferation (MTT assay, counting of viable cells)		10 mg/kg/day for 20 days	Atypical binding sites, mRNA, and protein de novo synthesis involved	Nx (0.1 $\mu$ M) only for short term exposure (6 h)	Cycloheximide actinomycin D PTX no effects	Maneckjee and Minna, 1992
H69, N417 SCLC, H157 non-SCLC Lung cancer tissue specimens	Morphine	0.01–1 $\mu$ M for 24 h	Inhibition of proliferation (MTT assay)			Inhibition of protein kinase C	Nx (100 nM vs. 10 nM morphine)	Nicotine	Maneckjee and Minna, 1990, 1994
	Morphine	1 $\mu$ M within minutes				$\mu$ 3-receptor-mediated NOS activation, NO release	Nx		Fimiani et al., 1999a
T47D breast cancer	$\kappa$ -agonists	0.01–1 $\mu$ M for 1 h	Inhibition of NOS activity and NO release (no effect with specific $\mu$ and $\delta$ agonists)			Direct interaction of internalized opioid with NOS	Diprenorphine no effect (1 $\mu$ M)		Kampa et al., 2001

TABLE 1 Continued  
Summary of growth-promoting and growth-inhibiting effects of opioids

Cell Type	Opioid	In Vitro Concentration and Time	In Vivo Effects	In Vivo Dose	In Vivo or Ex Vivo Effects	Suggested Mechanism	OR Antagonist Inhibition with:	Other Inhibitors	Reference
MCF-7 breast cancer	Morphine DADLE ( $\delta$ -agonist) cyclazocine ( $\kappa$ -agonist)	0.01–0.1 $\mu$ M for up to 6 days	Inhibition of proliferation (tetrazolium salt assay, counting viable cells)				Nx (100 nM)	Lack of estrogen in culture	Manekjee et al., 1990
MCF-7, MDA-MB231 breast cancer HT-29 colon cancer in vivo: cells injected s.c. in nude mice	Morphine DAMGO	10–2000 $\mu$ M for 24 h	G <sub>1</sub> cell cycle arrest, inhibition of proliferation (counting of viable cells) and apoptosis	10–30 mg/kg/day for 3 weeks	Inhibition of tumor growth (MCF-7 and MDA-MB231) no effect on HT-29 tumors	p53 phosphorylation and stabilization, up-regulation of p53-dependent p21, Bax, Fas	Nx no effect (equimolar concentrations in vitro and 1/10th of morphine dose in vivo)	No effect with PTX, forskolin, 8-Br-cAMP; inhibition of apoptosis with inhibitors of caspase 3, 8, 9, and Fas/Fusion protein	Tegeder et al., 2003
T47D breast carcinoma	Morphine, etorphine, DADLE	0.001–0.1 $\mu$ M for 5 days	Inhibition of proliferation (H <sup>3</sup> -incorporation)				Diprenorphine 1 $\mu$ M (except for effects of morphine)		Hatzoglou et al., 1996
EL-4 leukemia in C57B6 mice, sarcoma 180 in ddY mice	Morphine	500–1000 $\mu$ M (EL-4, P388, Meth-A, MM-46 cells) for 48–72 h	Decrease of viability/proliferation (MTT assay) of tumor cells attenuated ConA-induced lymphocyte proliferation	10 mg/kg/day for 10 days	Increase of tumor growth	General immunosuppression with morphine or alterations of hormone secretion	Nx (1 mg/kg/day for 10 days)		Ishikawa et al., 1983
Walker 256 carcinosarcoma cells injected i.v. in SD rats	Morphine			5 mg/kg single dose	Increase of metastatic tumor growth	Short lasting suppression of NK cell cytotoxicity	Nx (5 mg/kg single dose)	Pentacozine	Simon and Arbo, 1986
Colon 26-L5 carcinosarcoma cells in vivo: BALB/c mice injected i.v.	Morphine	0.035–0.35 $\mu$ M for 24 h	No effect on cell proliferation, inhibition of adhesion and invasion	10 mg/kg/day for 6 days	Reduced lung metastases	Inhibition of MMP-2 and 9 production (not inhibited with naloxone)	Nx (equimolar concentrations to MOR)		Haramaya et al., 2002
MADBI00 mammary adenocarcinoma cells injected i.v. in Fisher 344 rats	Morphine			5 mg/kg 3 doses (pre, post and 5 h post surgery)	Attenuated surgery-induced enhancement of metastatic growth	Inhibition of post-surgical pain, thereby reduction of stress and stress induced immunosuppression			Page et al., 1993
Colon cancer cells 5 h after surgery i.p. in Fisher 344 rats	Morphine			20 mg/kg/day for 3 days	Reduced liver metastasis	Enhanced NK cell activity at the time of tumor cell injection			Yeager and Colacchio, 1991
B16-16 melanoma cells injected into hindpaw in C57Bl mice	Morphine	0.35–3500 $\mu$ M for 48 h	No effect up to 350 $\mu$ M, inhibition of proliferation at 3500 $\mu$ M	10 mg/kg/day for 6 days	Inhibition of local tumor growth and lung metastasis, similar effect achieved with nerve ligation	Pain reduction prevents stress-induced alterations of anti-tumor immune response			Sasamura et al., 2002
HSC-2 and HSG human oral cancer cell lines	Codenone, morphine	10–500 $\mu$ M for 24 h	Inhibition of proliferation (MTT assay), apoptosis					Acetyl-cysteine	Kawase et al., 2002
<b>Transfected cells</b> CHO stably transfected with MOR	DAMGO	0.1–10 $\mu$ M within minutes					Nx (10 $\mu$ M)	PTX	Polakiewicz et al., 1998b
CHO stably transfected with MOR	Morphine	0.1–10 $\mu$ M within 1 h (mRNA)	Increase of c-fos and junB expression and AP-1 DNA binding activity					PTX PD98059 (MEK-inhibitor)	Shoda et al., 2001
HEK293 cells stably transfected with MOR or mutant MOR	DAMGO	1 $\mu$ M 5–60 min	Erk phosphorylation					Inhibition with inhibitors of PKC and CaMK	Belcheva et al., 2001

ConA, concanavalin A; LPS, lipopolysaccharide; Nx, naloxone; Ntx, naltrexone; CCl<sub>1</sub>, chronic constriction injury; ICE, interleukin-converting enzyme; CHO, chinese hamster ovary; iNOS, inducible NOS; eNOS, endothelial NOS; DADLE, [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin.

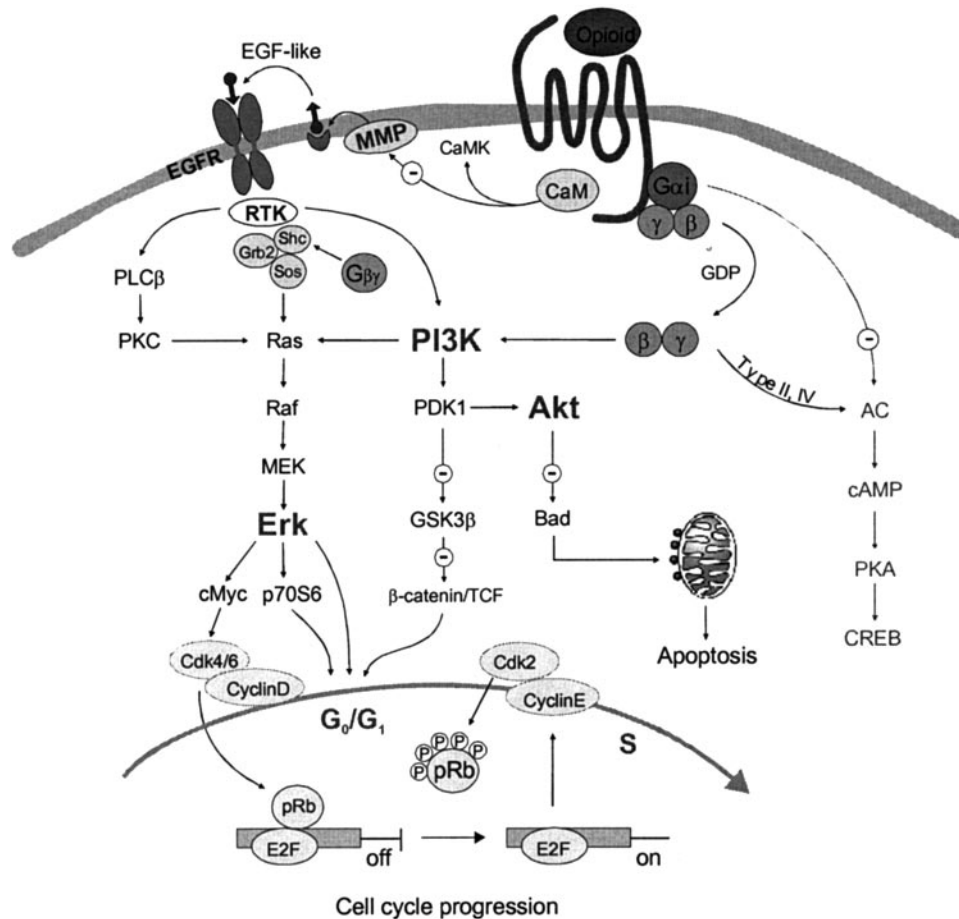


FIG. 1. Possible mechanisms to explain growth promoting and protective effects. Upon agonist binding,  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors couple to the heterotrimeric pertussis toxin-sensitive inhibitory G-protein ( $G_{i0}$ ). The  $\alpha$  subunit binds GTP and dissociates from  $G\beta\gamma$ . This event allows  $G\alpha_i$ -GTP to inhibit adenylyl cyclase (AC) resulting in a decrease of cAMP and inhibition of PKA. Free  $G\beta\gamma$  relocates to the membrane and then anchors and activates PI3K  $\gamma$  or  $\beta$ . PI3K can activate protein kinase B (PKB/Akt) and the Ras/Raf/MEK/Erk pathway thereby mediating anti-apoptotic and mitogenic signals, respectively. Alternatively,  $\mu$  opioid receptor activation initiates mitogenic signals through release of endogenous membrane bound growth factor-like molecules. This effect probably depends on a CaM-mediated disinhibition of membrane bound MMP. Such activated MMPs allow shedding of endogenously expressed EGF-like ligands, which then interact with EGFR and initiate the Erk cascade. Erk acts directly as a transcription factor and as an upstream kinase for p70S6 kinase and p90RSK (ribosomal S6 kinase) and regulates cell cycle transition from G $_1$  to S phase.

PI3K such as wortmannin and LY294002 (Polakiewicz et al., 1998b; Kim et al., 2001).

Activation of the PI3K/Erk or PI3K/Akt pathways through  $G\beta\gamma$  subunits is not specific for opioid receptors but probably a common feature of G-protein-coupled receptors (for review, see Schwindinger and Robishaw, 2001). However, whether activation of this pathway ultimately results in growth or proliferation probably depends on several other factors, such as  $G\beta$ - and  $G\gamma$ -subtype composition, action of the respective  $G\alpha$ , RGS proteins, concomitant signals, and cell type. Stimulation of various receptors that are coupled to an inhibitory G-protein ( $G_{i0}$ ) such as receptors for chemokines (CXCR4, CCR2) (Barbero et al., 2002, 2003), dopamine (D2) (Ghahremani et al., 2000; Narkar et al., 2001), serotonin (5HT1) (Adayev et al., 2003), cannabinoids (CB1, CB2) (Esposito et al., 2002; Gomez Del Pulgar et al., 2002; Molina-Holgado et al., 2002), lysophosphatidic acid (Edg1–3) (Deng et al., 2002; Li et al., 2003; Takahashi et al., 2003), and sphingosine-1-phosphate (S1P1,

S1P3) (Grey et al., 2002; Takuwa et al., 2002) have been found to promote cell growth or counteract apoptotic signals. These effects were partly attributed to  $G\beta\gamma$ -mediated activation of mitogen-activated protein kinases (MAPKs). On the other hand, particularly cannabinoids also induce apoptosis and inhibit tumor growth (De Petrocellis et al., 1998; Sanchez et al., 2001; Casanova et al., 2003; Massi et al., 2004). Hence, cannabinoids share the ability to evoke dual effects with opioids. This suggests that although  $G\beta\gamma$  activation is theoretically part of G-protein activation with all GPCRs, cell survival may be effected differently, and the outcome depends on the simultaneous effects of  $G\alpha$ . This idea is supported by several reports that demonstrate that  $G\alpha$  but no  $G\beta\gamma$  mutants have the potential for tumor cell transformation (Vara Prasad et al., 1994; Xu et al., 1994; Voyno-Yasenetskaya et al., 1994; Wong et al., 1995; Edamatsu et al., 1998; Dermott et al., 1999; Adarichev et al., 2003). Also emphasizing the role of  $\alpha$  subunits,  $\delta$ -opioid receptor stimulation was reported to

cause a  $G\beta\gamma$ -independent Erk1/2 stimulation through  $G\alpha_0$  in neuronal cells (Zhang et al., 2003b).

The MAPK activation mediated through  $G\beta\gamma$  is likely to be terminated after receptor desensitization. Such desensitization occurs rapidly for opioid and cannabinoid receptors in the presence of respective agonists and will probably also rapidly terminate the  $G\beta\gamma$ -mediated mitogenic signal. Since the efficiency of receptor desensitization differs among agonists (Alvarez et al., 2002) a weak "desensitizer" such as morphine (Alvarez et al., 2002) is probably more likely to stimulate cell growth than a strong desensitizer such as methadone. In support, several studies show growth-stimulating effects of morphine, but there are no such reports with methadone so far. In addition, inhibition of ciliary neuron apoptosis with morphine did not occur if equally high doses of morphine were administered each day but only if the daily dose was stepwise increased to overcome receptor desensitization (Meriney et al., 1985). Furthermore, growth-promoting effects of opioids in vitro were only observed at very low concentrations of the respective opioid [in the range of  $10^{-15}$  (Moon, 1988; Liu et al., 2001) to  $10^{-12}$  M (Singhal et al., 1998)] except for endothelial cells where higher concentrations up to  $10^{-4}$  M were used (Gupta et al., 2002) (Table 1). It is unclear how picomolar concentrations of morphine or other opioids may promote cell growth although the binding affinity to opioid receptors is in the nanomolar range with  $K_d$  values about 0.1–10 nM (Wolozin and Pasternak, 1981; Johnson and Pasternak, 1983; Meunier et al., 1983). It seems unlikely that as yet unidentified high-affinity binding sites for opioids exist on non-neuronal cells that specifically mediate mitogenic signals. In tumor cells, morphine causes activation of the inhibitory G-protein at concentrations  $\geq 10$  nM with a maximum at 1  $\mu$ M (Tegether et al., 2003) suggesting that the binding affinity to these opioid receptors is rather low compared with opioid receptors from brain tissue, which is more suggestive of low-affinity binding sites.

#### B. Calmodulin—Epidermal Growth Factor Receptor Pathway

Apart from the  $G\beta\gamma$ /PI3K pathway,  $\mu$ -opioid receptor-induced GPCR signaling can cause Erk activation through cross-activation of the epidermal growth factor receptor (Belcheva et al., 2001). Epidermal growth factor receptor (EGFR) activation apparently occurs via a plasma membrane bound metalloproteinase (MMP), which is involved in the processing of EGF-like precursor molecules that are anchored to the outer surface of the plasma membrane (Fig. 1). The activity of this MMP is inhibited by membrane bound calmodulin (CaM); however, upon opioid receptor activation, CaM is released from the opioid receptor and relocated to intracellular compartments (Wang et al., 2000). The metalloproteinase thereby becomes active and causes shedding of endogenously expressed EGF-like ligands, which ac-

tivate the EGF receptor and subsequently the Erk cascade. Notably, calmodulin kinase II (CaMK-II), which is activated by CaM upon agonist binding to the opioid receptor (Lou et al., 1999), can desensitize opioid receptors (Mestek et al., 1995; Koch et al., 1997) and inhibition of CaMK-II in rat hippocampus attenuates morphine tolerance (Fan et al., 1999) suggesting that the CaM/EGFR-mediated growth stimulatory effect of morphine diminishes once receptor desensitization occurs. The observed cross-activation of EGFR is not restricted to EGFR, but also occurs with other receptor tyrosine kinases such as the fibroblast growth factor receptor (Belcheva et al., 2002). EGFR cross-activation however, does not contribute to  $\delta$ - or  $\kappa$ -opioid receptor-mediated activation of the Erk cascade (Belcheva et al., 2002; Kramer et al., 2002). On the other hand, EGFR cross-activation is not specific for  $\mu$ -opioid receptors but was also observed following stimulation of other  $G_i$ -coupled receptors (Buchanan et al., 2003; Pai et al., 2003; Hart et al., 2004; Sales et al., 2004; Schafer et al., 2004; Tanimoto et al., 2004) as well as receptors coupled to  $G_s$  (Bertelsen et al., 2004) or  $G_q$  (McCole et al., 2002; Cheng et al., 2003) again suggesting that this growth stimulatory signaling pathway is a common option of G-protein-coupled receptors.

#### C. $\mu_3$ -Opioid Receptor—Nitric Oxide Pathway

In endothelial cells, growth-promoting effects of morphine and Erk phosphorylation were inhibited with PTX and the nitric-oxide synthase (NOS) inhibitor L-NAME (*N<sup>o</sup>*-nitro-L-arginine methyl ester). That means that both the inhibitory G-protein and nitric oxide (NO) are involved (Gupta et al., 2002). Among the several known endogenous pro-angiogenic factors, only vascular endothelial growth factor (VEGF) has been shown to depend on nitric oxide for Erk phosphorylation (Ziche et al., 1997; Murohara et al., 1998) suggesting that morphine either acts in a fashion similar to that of VEGF or alternatively that morphine causes cross-activation of the VEGF receptor as has been demonstrated for EGFR (Belcheva et al., 2001). The opioid receptor might be linked to nitric oxide release through the  $G\beta\gamma$ /PI3K/Akt pathway since Akt stimulates endothelial NOS (Dimmeler et al., 1999; Fulton et al., 1999). On the other hand, nitric oxide release from immune cells is specifically evoked by activation of the morphine-selective and opioid peptide-insensitive  $\mu_3$  receptor (Magazine et al., 1996). This receptor is also expressed on endothelial cells (Stefano et al., 1995; Bilfinger et al., 1998) and was recently suggested to be more close to chemokine than opioid receptors. Hence, morphine-induced NO release from endothelial cells may also result from  $\mu_3$  receptor activation (Fimiani et al., 1999b), which might contribute to morphine-evoked vasodilation (Stefano et al., 1995; Bilfinger et al., 1998) and angiogenesis (Gupta et al., 2002). This hypothesis might explain the failure of naloxone to inhibit the pro-angiogenic effects of mor-



phine (Gupta et al., 2002) because naloxone has lower affinity to  $\mu_3$  than morphine (Stefano et al., 1995; Fimiani et al., 1999a). Recently,  $\mu_3$  receptor expression has also been demonstrated in normal lung tissue and lung cancer, and its activation resulted in nitric oxide release (Fimiani et al., 1999a). Since nitric oxide may cause tumor growth inhibition or prevention as well as tumor progression (Wink et al., 1998) the role of  $\mu_3$  receptor-mediated NO release for tumor growth is still unresolved. Effects that are caused by nitric oxide may contribute to the inconclusive results obtained with morphine in studies investigating tumor growth. Because morphine-evoked apoptosis in splenocytes (Fecho et al., 1994) was inhibited by cotreatment with a NOS antagonist it may be suggested that a  $\mu_3$  receptor-mediated increase of NO release contributes to morphine-evoked immunosuppression.

### III. Death-Promoting and Antiproliferative Effects

#### A. Hormone-Mediated Effects

It has been suggested that the immunosuppression that occurs during chronic opioid use might be caused by central hormone-mediated mechanisms. Plasma levels of cortisol (Kim et al., 1999) and prolactin (Provinciali et al., 1996) are actually elevated during chronic treatment with morphine. Because glucocorticoid receptor antagonists inhibit the depletion of splenocytes or thymocytes in morphine-treated mice, this idea of a hormone-mediated effect is further supported (Fuchs and Pruett, 1993). In addition, bromocriptine, which inhibits prolactin release, restores natural killer (NK) cell cytotoxicity in morphine-treated cancer patients (Provinciali et al., 1996).

However, in contrast to the view that opioids impair immune functions, morphine prevents post-surgical immunosuppression by alleviating the stress caused by the painful procedure (Page et al., 1993). This mechanism was suggested to be responsible for a reduction of post-surgical metastatic colonization of adenocarcinoma cells in morphine-treated animals (Page et al., 1993). Importantly, morphine had no effect on metastasis without prior surgery in this study, indicating that the postoperative pain stress was a crucial factor in promoting the metastatic spread. The idea that inhibition of pain prevents tumor growth is supported by a study in mice where melanoma cells were injected into a hind paw. This caused hyperalgesia at the injected site. Treatment with morphine as well as neurectomy of the sciatic nerve innervating the inoculated region reduced local tumor growth and lung metastasis (Sasamura et al., 2002).

However, in contrast to these reports, a single dose of morphine administered after intravenous injection of sarcoma cells was found not to inhibit but enhance metastasis (Simon and Arbo, 1986). A single dose of morphine obviously is not sufficient to provide the favorable

pain and stress reduction required to suppress metastasis. A single dose however, may well attenuate NK cell-mediated tumor cell killing when it is injected during the NK-sensitive period. This may then facilitate metastatic colonization. Hence, the resulting outcome is determined by neuroimmunological interactions and by direct effects on tumor and immune cells. In a leukemia study in mice, for example, morphine increased tumor cell proliferation *in vivo* although it inhibited proliferation of the same cells *in vitro* (Ishikawa et al., 1993).

#### B. Chemokine- and Cytokine-Mediated Effects

In addition to the hormone-mediated "indirect" modulation of cell proliferation, opioids modify T- and B-cell responses (Guan et al., 1997; Shahabi et al., 2000; Beagles et al., 2004; Roy et al., 2004), macrophage and microglial activity (Belkowski et al., 1995; Hu et al., 2000; Hu et al., 2002), chemotaxis (Szabo et al., 2002), cell migration (Patel et al., 2003), and natural killer cell cytotoxicity (Hsueh et al., 1996; Boyadjieva et al., 2001; Yeager et al., 2002) by modifying cytokine and chemokine release (Belkowski et al., 1995; Alicea et al., 1996; Kong et al., 1997; Wetzel et al., 2000b; Sacerdote, 2003), respective receptor expression (Zhang and Rogers, 2000), and chemokine receptor responsiveness (Grimm et al., 1998a; Rogers et al., 2000; Szabo et al., 2002, 2003; Chen et al., 2004) (for review, see McCarthy et al., 2001 and Rogers and Peterson, 2003). These effects do not necessarily affect survival of the opioid-stimulated cell but may modify the course of inflammatory and infectious diseases, such as HIV infection, and thereby survival of the organism. Interestingly, MOR stimulation may cause opposite effects on chemokine receptor expression and/or HIV susceptibility (Peterson et al., 1999; Guo et al., 2002; Li et al., 2002; Suzuki et al., 2002a) than KOR and DOR agonists, which were both found to suppress HIV infection (Chao et al., 1996, 2000; Sharp et al., 1998a, 2001). Morphine for example increases the expression of CCR5 (Miyagi et al., 2000; Guo et al., 2002; Mahajan et al., 2002), which is a co-receptor required for HIV to enter target cells (Liu et al., 1996) and thereby facilitates HIV cell penetration (Mahajan et al., 2002; Suzuki et al., 2002a). In contrast, the KOR agonist U50,488H inhibits the expression of CXCR4, another HIV co-receptor, in CD4+ T-cells (Lokensgard et al., 2002). This effect is associated with a decrease of HIV susceptibility (Chao et al., 1996; Peterson et al., 2001; Lokensgard et al., 2002; Gekker et al., 2004). The release of pro-inflammatory chemokines such as RANTES (regulated on activation normal T-cell expressed and secreted, CCL5), monocyte chemoattractant protein-1, (MCP-1, CCL2), and interferon inducible protein-10 (IP-10, CXCL10) is increased with the MOR agonist DAMGO in blood mononuclear cells (Wetzel et al., 2000b). In contrast, U50,488H inhibits monocyte chemoattractant protein-1 release from astrocytes (Sheng et al., 2003). U50,488H also reduces the lipopolysaccharide-stimu-

lated release of pro-inflammatory cytokines including IL-1, IL-6, and TNF $\alpha$  from macrophages in vitro (Belkowski et al., 1995; Alicea et al., 1996). However, there are also functional overlaps between MOR and KOR. For example, IL-12 release from mouse peritoneal macrophages was reduced with both the MOR agonist DAMGO and the KOR agonist U50,488H (Sacerdote, 2003). Morphine had biphasic (Pacifi et al., 2000a) or conflicting effects on cytokine release (Raghavendra et al., 2002; Sacerdote, 2003; Roy et al., 2004). In most studies, immune-modulating effects were inhibited with opioid receptor antagonists and decreased in part with prolonged treatment (Sacerdote, 2003), suggesting that the effects are mediated through "typical" opioid receptors and are subject to opioid tolerance.

Opioids may further modulate responses to chemokines by blocking G-protein-coupling to chemokine receptors. Chemokines, on the other hand, may likewise desensitize opioid receptors. This phenomenon, called cross-desensitization (Rogers et al., 2000; Chen et al., 2004), occurs in immune cells (Zhang et al., 2003a) and in the central nervous system (Szabo et al., 2002) and is likely due to enhanced phosphorylation of the respective other receptor (Rogers et al., 2000) and/or receptor heterodimerization (Suzuki et al., 2002b; Chen et al., 2004). Cross-desensitization of CCR5 by opioids is associated with a decrease in susceptibility to HIV-1 infection (Sharp et al., 2001; Szabo et al., 2003). As chemokine receptors are also expressed by tumor cells (Burger et al., 2003; Jankowski et al., 2003; Manes et al., 2003; Robinson et al., 2003; Fernandis et al., 2004) the opioid-chemokine interaction may not only affect immune cell-mediated tumor defense mechanisms but may also directly modulate the growth or metastatic potential of tumors that express both kinds of receptors.

A cross-desensitization does not specifically occur between opioid and chemokine receptors. It has also been observed between opioid and cannabinoid receptors (Shapira et al., 2003) and is common to many members of the GPCR superfamily (for review, see Chuang et al., 1996). However, specific kinases that preferentially phosphorylate certain G-protein-coupled receptors ensure a certain hierarchy in the cross-desensitization process.

### C. Apoptosis Associated with Tolerance

The growth-inhibitory or apoptosis-inducing effects of morphine in neurons and immunocytes are directly associated with morphine tolerance (Wu et al., 1999; Mao et al., 2002) or receptor desensitization as assessed by a lack of morphine-stimulated GTPase activity at concentrations that inhibit tumor growth (Teveder et al., 2003). Drugs that prevented the development of morphine tolerance in rats also prevented cell death (Fecho et al., 1994; Mao et al., 2002) and vice versa (Mao et al., 2002). This close association between apoptosis and receptor desensitization suggests that uncoupling of the receptor

from G<sub>i</sub> or receptor internalization rather than G<sub>i</sub> activation may be the key event in initiating opioid-evoked cell death or cell cycle arrest. Although this close association has not been directly shown in terms of tumor cell growth, a comparison of dosing schedules supports this hypothesis (Table 1). Tumor suppression basically occurs after chronic high dose opioid administration in many instances (Maneckjee and Minna, 1992; Harimaya et al., 2002; Sasamura et al., 2002; Teveder et al., 2003). By contrast, tumor-enhancing effects with morphine occur after a single dose (Simon and Arbo, 1986) or at relatively low daily doses (Gupta et al., 2002) that are much lower than the ED<sub>50</sub> for antinociceptive effects, which range between 0.5 and 20 mg/kg depending on the model and strain or species used (Grognet et al., 1983; Elmer et al., 1998; Zong and Pollack, 2000). Hence, such low concentrations of morphine are unlikely to result in substantial receptor desensitization (Alvarez et al., 2002). Comparison of high and low doses of morphine in vitro also reveals dual concentration-dependent effects, i.e., mitogenesis at low and growth inhibition or apoptosis at higher concentrations (Singhal et al., 1998; Gupta et al., 2002).

### D. Is Cell Death Initiated by Receptor Desensitization

The responsiveness of opioid receptors is reduced upon ongoing or repeated exposure to opioid agonists. This agonist-dependent desensitization is referred to as homologous desensitization. It involves a phosphorylation of the receptor through various GPCR kinases (GRK) (Zhang et al., 1998; Schulz et al., 2002), subsequent uncoupling from the G-protein (Whistler and von Zastrow, 1998; Liu et al., 1999), binding to  $\beta$ -arrestin 2 (Zhang et al., 1998; Law et al., 2000; McDonald et al., 2000; Pierce and Lefkowitz, 2001; Xiang et al., 2001) and sequestration into clathrin-coated vesicles with help of the GTPase dynamin (Chu et al., 1997; Li et al., 1999). In addition to agonist-specific desensitization, functions of GPCRs are regulated by agonist-independent mechanisms, namely heterologous desensitization. In this case, the initial phosphorylation is mediated through various second messenger-activated protein kinases such as protein kinase C (PKC) (Shahabi and Sharp, 1993; Ueda et al., 2001; Xiang et al., 2001), CaMK-II (Mestek et al., 1995), MAPK (Polakiewicz et al., 1998a; Schmidt et al., 2000) and tyrosine kinases (Pak et al., 1999). Hence, inhibition of kinases involved in receptor phosphorylation and desensitization should abolish the antiproliferative effects of morphine if the hypothesis of "desensitization-dependent growth inhibition" is true. Indeed, inhibition of PKC activity was shown to attenuate the development of opioid tolerance (Narita et al., 1996; Aley et al., 2000; Narita et al., 2001) and to restrict NMDA-R signaling (Mao et al., 1994, 1995), which has been proposed to account for morphine-induced cell death in spinal cord neurons (Mao et al., 2002). PKC inhibition also prevents a morphine-induced cell cycle

arrest in breast cancer cells (I. Tegeder, unpublished observations).

Receptor internalization initiated by phosphorylation of the receptor through GRKs is  $G_i$ -protein-dependent and therefore subject to inhibition with PTX (Pak et al., 1999). On the other hand, heterologous desensitization involves  $G_i$ -protein-independent kinases and is therefore not reversed with PTX (Pak et al., 1999) but by, for example, the tyrosine kinase inhibitor genistein (Pak et al., 1999) or the PKC inhibitor calphostin (Ueda et al., 2001). Hence, depending on the kinase primarily involved in receptor desensitization and internalization, PTX may antagonize the pro-apoptotic effects of morphine in some cells (Yin et al., 1997) but not in others (Maneckjee and Minna, 1992; Tegeder et al., 2003). These controversial findings therefore do not contradict the hypothesis of an "internalization/desensitization-dependent growth inhibition". The failure of PTX to antagonize the effects of morphine in some studies might also indicate that  $G_z$  rather than  $G_i$  is involved, because in contrast to all other members of the  $G_i$  subfamily,  $G_z$  is no substrate for the pertussis toxin-sensitive ADP-ribosyl transferase (Ho and Wong, 2001). Although  $G_z$  resembles  $G_i$  both in terms of receptors and effectors it exhibits some unique biochemical and regulatory properties. For example,  $G_z$  interacts with p21-activated kinase, G-protein-regulated inducers of neurite outgrowth, and the Eya2 transcription cofactor. These targets may constitute the link between  $G_z$  and downstream regulators of cellular development, survival, proliferation, differentiation, and even apoptosis (Ho and Wong, 2001). Irrespective of  $G_i$  or  $G_z$  signaling however, one would expect that desensitization-dependent effects are antagonized with naloxone because it is generally accepted that opioid receptor antagonists reduce opioid receptor desensitization and internalization (Crain and Shen, 1995; Shen and Crain, 1997); however, results with naloxone were also conflicting. The effects of morphine were abolished in some studies (Maneckjee et al., 1990; Roy et al., 1998; Yin et al., 1999; Rozenfeld-Granot et al., 2002) but not effected in others (Maneckjee and Minna, 1992; Hatzoglou et al., 1996; Kugawa et al., 1998; Gupta et al., 2002; Tegeder et al., 2003). Particularly the inhibition of tumor cell growth by morphine was mostly not antagonized with naloxone (Maneckjee and Minna, 1992; Hatzoglou et al., 1996; Kugawa et al., 1998; Tegeder et al., 2003). Atypical binding sites were therefore suggested to be involved (Maneckjee and Minna, 1992; Hatzoglou et al., 1996), which might have a low affinity to naloxone. In support, inhibitory effects of naloxone were found in several studies where concentrations were 2 to 10 times higher than those of morphine (Stiene-Martin and Hauser, 1993; Maneckjee and Minna, 1994; Roy et al., 1998; Hu et al., 2002; Rozenfeld-Granot et al., 2002) whereas failure often occurred at equimolar or lower concentrations (Maneckjee and

Minna, 1992; Hatzoglou et al., 1996; Kugawa et al., 1998; Tegeder et al., 2003).

### E. Direct Effects of Internalized Agonist

It has been suggested that opioid agonists are internalized together with their receptors and might directly modulate the function of intracellular signaling molecules. In particular,  $\kappa$  agonists were reported to bind directly to the reductase part of active dimeric NO synthases, thereby acting as noncompetitive inhibitors of the enzymes (Kampa et al., 2001). This may represent a potential explanation for internalization-dependent effects; however, it is unclear whether opioids may act as direct enzyme modifiers in vivo or whether high intracellular concentrations of an internalized agonist may have direct toxic effects.

### F. $\beta$ -Arrestin As Signal Transducer

It has been recognized that  $\beta$ -arrestins not only act as adapter molecules that target GPCRs to clathrin-coated pits for endocytosis but might have novel functions as GPCR signal transducers (McDonald and Lefkowitz, 2001). Recent reports suggest that they bind directly to Src family kinases and components of the Erk and c-Jun-N-terminal kinase 3 (JNK3) MAPK cascades. By linking these proteins to the GPCR,  $\beta$ -arrestins can confer distinct enzymatic activities upon the receptor, which may lead to signals that are important for the regulation of cellular growth and differentiation. This may provide an explanation for the observed morphine-evoked activation or up-regulation of various members of the MAPK family including Erk1/2, p38, and c-Jun-N-terminal kinase (Ma et al., 2001; Singhal et al., 2001). It has been reported that mice lacking  $\beta$ -arrestin 2 experience enhanced morphine-induced analgesia and do not become tolerant to morphine (Bohn et al., 1999, 2002). In addition,  $\beta$ -arrestin 2 was found to be up-regulated in the brain of rats made tolerant to opioids (Hurle, 2001) emphasizing the importance of this scaffolding protein for  $\mu$ -opioid receptor desensitization (Whistler and von Zastrow, 1998). Although the binding partners recruited by  $\beta$ -arrestin 2 upon opioid receptor phosphorylation have not yet been identified, studies with other GPCRs may provide an idea of potential interactions.

Stimulation of proteinase-activated receptor-2 (PAR2) induces the assembly of receptor/ $\beta$ -arrestin/Raf/Erk complexes that lead to a cytosolic retention of activated kinases and promote growth inhibitory effects associated with PAR2 activation (DeFea et al., 2000). In yeast two hybrid screens and brain lysates,  $\beta$ -arrestin was found to bind the tissue restricted isoform of JNK3. The complexes of  $\beta$ -arrestin and JNK3 also contain upstream JNK activators (McDonald et al., 2000; McDonald and Lefkowitz, 2001). The formation of this complex therefore retains JNK3 in the cytosol and increases its overall activity by facilitating the interaction with its

upstream partners. JNK3 is up-regulated in rat brain during chronic morphine treatment and withdrawal (Fan et al., 2003), and a targeted deletion of JNK3 protected mice from neuronal apoptosis (Kuan et al., 2003) suggesting that this JNK isoform may contribute to the cell death-promoting effects of morphine. JNKs are among several kinases that phosphorylate p53 at N-terminal serine residues leading to a stabilization of the protein. Therefore, JNK activation might constitute a signaling pathway by which morphine causes the observed p53 phosphorylation and stabilization (Singhal et al., 2002; Tegeder et al., 2003). The effects of morphine on p53 may also be mediated through the ubiquitin ligase Mdm2 which ensures rapid p53 degradation under homeostatic conditions. If p53 phosphorylated in response to various kinds of cell stress, Mdm2 can no longer bind to p53 and hence, p53 is stabilized and acts as key regulator of cell proliferation and cell death. As reported recently, Mdm2 expression is modified in certain brain regions in morphine-treated rats (Jiang et al.,

2003). In addition,  $\beta$ -arrestin 2 directly interacts with Mdm2 (Shenoy et al., 2001) and regulates its ubiquitin ligase activity. Hence, there are several potential mechanisms that may link opioid receptors to p53 and other proteins involved in cell cycle control and apoptosis (Fig. 2).

#### IV. Summary of Mechanisms of Action

Opioid receptor signaling has been implicated in the regulation of cell proliferation and cell death in various cells expressing opioid receptors. It emerged that growth-promoting effects occur at low concentrations or single doses of opioids and are probably mediated in part through  $G\beta\gamma$ -mediated activation of PI3K/Akt and Ras/Erk cascades. Alternatively,  $\mu$ -opioid-induced mitogenesis may be mediated through cross-activation of growth factor receptors. On the other hand, growth inhibitory effects occurred at chronic opioid treatment or relatively high in vitro concentrations and are closely associated

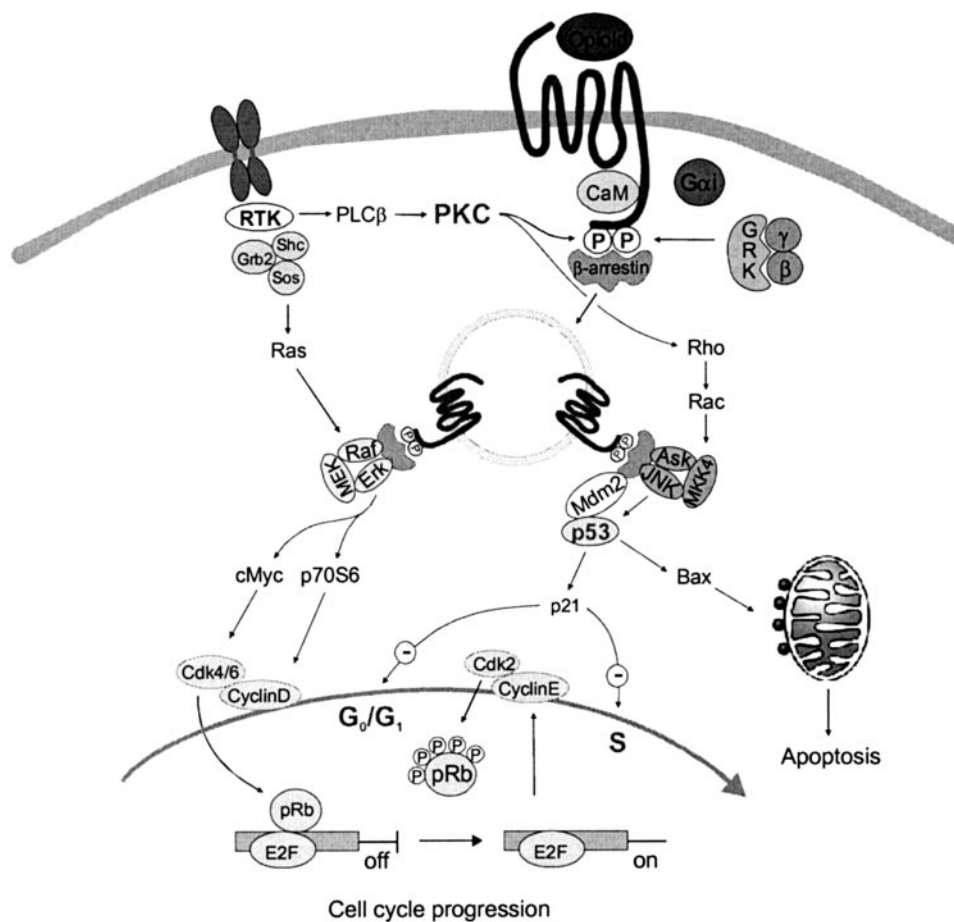


FIG. 2. Possible mechanisms for death-promoting and antiproliferative effects of morphine. Repeated or ongoing activation of opioid receptors leads to opioid tolerance. This desensitization involves a phosphorylation of the receptor through GRKs or other kinases such as PKC and tyrosine kinases, subsequent uncoupling from the G-protein, binding to  $\beta$ -arrestin 1 or 2 and sequestration of the receptor into clathrin-coated vesicles with help of the GTPase dynamin. Apart from targeting the opioid receptor to clathrin-coated pits to allow for endocytosis,  $\beta$ -arrestins bind directly to Src family kinases and components of the Erk and JNK3 MAPK cascades. By recruiting these proteins to the GPCR  $\beta$ -arrestins can confer distinct enzymatic activities upon the receptor. The assembly of receptor/ $\beta$ -arrestin/Raf/Erk complexes can lead to a cytosolic retention of Erk and inhibition of its growth-promoting effects. Scaffolding of JNK with its upstream kinases Ask (apoptosis signal-regulating kinase) and MKK4/7 may increase its overall activity. Additionally,  $\beta$ -arrestin interacts with Mdm2, which regulates p53 ubiquitination.

with opioid receptor desensitization and internalization. Desensitization-dependent effects might be mediated through direct effects of internalized agonist, through “withdrawal” of a growth-promoting signal or through  $\beta$ -arrestin that acts as a scaffolding protein for Src family kinases and MAPKs. By recruiting these proteins to the receptor,  $\beta$ -arrestin can confer distinct enzymatic activities upon the opioid receptor and thereby mediate growth inhibitory or apoptotic signals.

## V. Clinical Implications

### A. Inhibition of Inflammation with Endogenous Opioids

The diverse immune modulating effects observed with selective agonists at  $\mu$ ,  $\delta$ , and  $\kappa$  receptors suggest that endogenous opioids with different receptor specificities also accomplish different tasks. The question arises whether it might be possible to exploit or enhance certain features of endogenous opioids to achieve desired effects such as tumor suppression, inhibition of chronic inflammation, and increased resistance toward viral infection such as HIV.

There are several studies that suggest that endogenous opioids have similar effects on immune cell functions as opioid alkaloids or synthetic agonists. The MOR-selective endomorphin-1 for example potentiates HIV expression in brain cell cultures (Peterson et al., 1999). A similar effect was observed with  $\beta$ -endorphin, which is an agonist at  $\mu$  and  $\delta$  receptors (Sundar et al., 1995), whereas the KOR agonist dynorphin inhibited cytokine induced up-regulation of HIV expression (Chao et al., 1995). Endomorphin-1 was further reported to alter IL-10 and IL-12 release from T-cells (Azuma and Ohura, 2002) and IL-8 production in colon cancer cells (Neudeck and Loeb, 2002). Most studies addressing the effects of  $\beta$ -endorphin on immunocytes found immunosuppressive effects in the form of inhibition of proliferation or reduction of pro-inflammatory cytokine release (Garcia et al., 1992; Panerai et al., 1995; Bhardwaj et al., 1996; Ientile et al., 1997; Hosoi et al., 1999; Takeba et al., 2001). For example,  $\beta$ -endorphin inhibited TNF $\alpha$ , IL-1 $\beta$ , and matrix metalloproteinase-9 expression in synovial epithelial cells from patients with rheumatoid arthritis (Takeba et al., 2001). Leu- and Met-enkephalin (DOR agonists) had similar effects (Takeba et al., 2001). Absence of  $\beta$ -endorphin in  $\beta$ -endorphin-deficient mice was associated with increased splenocyte proliferation and lipopolysaccharide-stimulated IL-6 and TNF $\alpha$  production in spleen macrophages (Refojo et al., 2002). Cell proliferation in spleen and bone marrow was also increased in  $\mu$ -opioid receptor knockout mice (Tian et al., 1997). However, in contrast to these immunosuppressive effects,  $\beta$ -endorphin increased the number of natural killer cells in the spleen (Kowalski, 1997), enhanced conditioned NK cell activity (Mandler et al., 1986; Hsueh et al., 1995) and concanavalin A-induced proliferation

(van den Bergh et al., 1991; Navolotskaya et al., 2002b), IL-2 production (van den Bergh et al., 1991), and Ca<sup>2+</sup> mobilization in T-cells (Shahabi et al., 1996). The latter effect was shared by Met-enkephalin and inhibited with the DOR-specific antagonist naltrindole (Shahabi et al., 1996), indicating that this effect was probably mediated through the  $\delta$  receptor. Met-enkephalin also stimulated T-cell proliferation (Singh et al., 1999) probably by activating Erk-signaling pathways (Sharp et al., 1998b; Shahabi et al., 1999; Kramer et al., 2002); however, it induced apoptosis in leukemia cells (Mernenko et al., 1996). Dynorphin A, a specific KOR agonist (Chavkin et al., 1982), enhanced the mitogen-induced proliferative response and interleukin-2 production of rat splenocytes. In addition, dynorphin protected cardiomyocytes from ischemia or hypoxia-induced injury (Cao et al., 2003).

It is difficult to define a general rule of how endogenous opioids will modify the immune response in a given situation because of these partly conflicting results. However, based on the literature it appears reasonable to suggest that the release of endogenous opioids is aimed at inhibiting exaggerated inflammatory reactions (Gironi et al., 2000; Takeba et al., 2001; Philippe et al., 2003) and inflammatory pain (Cabot et al., 1997, 2001; Machelska et al., 2002) whereas NK cell-mediated defense mechanisms against tumor cells or invading microorganisms are supported (Hsueh et al., 1995, 1996; Kowalski, 1997; Boyadjieva et al., 2001; Yeager et al., 2002).

Immune cells express various endogenous opioids (Przewlocki et al., 1992; Mousa et al., 2001; Rittner et al., 2001; Jessop et al., 2002). In case of inflammation or tissue injury, they migrate from the circulation to the inflamed tissue (Mousa et al., 2001; Brack et al., 2004). This process is controlled by adhesion molecules such as ICAM (Machelska et al., 2002) and selectin (Machelska et al., 1998) on leukocytes and vascular endothelium. In this way, recruited immunocytes release endogenous opioid peptides at the site of inflammation to reduce the pain (Stein et al., 1990; Schafer et al., 1994; Machelska et al., 1998; Cabot et al., 2001) and to stop the proliferation and cytokine release from immunocytes thereby acting as “anti-inflammatory” molecules. Anti-selectin and anti-ICAM antibodies inhibit the migration and thereby cut the supply of endogenous opioid peptides at the site of inflammation (Machelska et al., 1998, 2002). As a result, the pain intensifies. However, whether opioid peptide-releasing immunocytes can be specifically directed into desired tissues or whether the release of different opioid peptides can be orchestrated in a desired fashion has not yet been evaluated.

### B. Morphine Plus Naloxone As Add-On for Cancer Treatment

The observed tumor-suppressive effects of morphine, which were mostly not antagonized with naloxone, sug-

gest the intriguing possibility that morphine might be useful as an adjunct in cancer therapy not only to reduce cancer pain but also tumor growth. Since pain treatment in cancer patients mostly requires chronic high dose opioid therapy, tolerance will surely occur. Therefore, opioid tolerance-dependent growth inhibition is much more likely in this setting than single or low dose-associated mito- and angiogenesis (Gupta et al., 2002). However, it has to be considered that a general suppression of the immune system might jeopardize the favorable outcome. Interestingly, there is only one study demonstrating accelerated tumor growth upon chronic morphine treatment at reasonable doses (Ishikawa et al., 1993). This was associated with general immunosuppression (Ishikawa et al., 1993). The unfavorable effects of morphine in this case might be due to the type of cells that were leukemia and sarcoma cells (Ishikawa et al., 1993) whereas most of the “successful” in vivo studies employed adenocarcinoma cells (Maneckjee et al., 1990; Maneckjee and Minna, 1990, 1994; Hatzoglou et al., 1996; Kampa et al., 1997; Sasamura et al., 2002; Tegeder et al., 2003). Apart from a possible unfavorable immunosuppression, morphine treatment leading to tolerance might be associated with apoptosis of GABAergic inhibitory spinal cord neurons (Mao et al., 2002) compromising endogenous mechanisms to restrict pain signaling. This is particularly disadvantageous in cancer patients suffering from serious pain. Since naloxone was able to prevent neuronal apoptosis (Mao et al., 2002) whereas it mostly failed to antagonize morphine-induced apoptosis of tumor cells (Maneckjee and Minna, 1992; Hatzoglou et al., 1996; Kampa et al., 1997; Tegeder et al., 2003) chronic high dose morphine plus low dose naloxone treatment might be a therapeutic option that possibly combines favorable tumor suppression with reduced neuronal toxicity. The results obtained in previous in vivo experiments suggest that this issue is worth being addressed in clinical studies.

### C. Subanalgesic Morphine to Accelerate Healing Processes

The observed mito- and angiogenic properties of low subanalgesic doses of morphine (or other opioids) might possibly be exploited for treatment of some types of tissue injury to support or accelerate healing processes. A study that investigated the effects of morphine on stress ulcers in the stomach of rats supports this hypothesis because it showed that morphine not only prevented stress ulceration but also promoted the regeneration of gastric mucosa and accelerated ulcer healing (Cho et al., 2003). There was no change of the number of microvessels in this study. However, the dose of 2 to 8 mg/kg is in the range of effective antinociceptive doses for rats and therefore probably was too high to facilitate angiogenesis. The endogenous MOR and DOR agonist  $\beta$ -endorphin was reported to stimulate the expression of cytokeratin 16 (Bigliardi-Qi et al., 2000) and transforming growth

factor  $\beta$  type II (Bigliardi et al., 2003) in human skin organ cultures. Both, CK16 and TGF $\beta$  type II receptor are not expressed in normal skin but appear in regenerating epithelial cells of the epidermis during wound healing. TGF $\beta$  is one of the most important factors for the maturation of granulation tissue and the epithelization of the defect. Hence, the effects of  $\beta$ -endorphin on CK16 and TGF $\beta$  further indicate a role of opioids for wound healing. The hypothetical potential of subanalgesic doses of morphine or other opioid alkaloids to speed up healing processes, however, has not yet been addressed with in vivo studies.

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