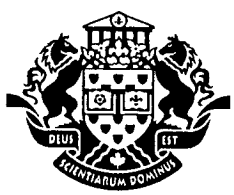

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FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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Characterization, identification and purification of a high affinity binding site for H4-(86-100) peptide in membrane preparations of rat alveolar macrophages

By

Andréanne Bonhomme

Dissertation submitted to Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology

Under the supervision of
Dr. Simon Lemaire and
Dr. Irma Lemaire

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ABSTRACT

Rat alveolar macrophages express specific binding sites for C-terminal fragments of histone H4. The present study was aimed at identifying and characterizing the receptor for the C-terminal fragment 86 to 100 of histone H4, an antinociceptive peptide structurally similar to histogranin. A high affinity low capacity binding site for [I^{125}]H4-(86-100) was found in the macrophage membrane preparation with a K_d of 12.3 ± 0.8 nM and a B_{max} of 9.6 ± 0.3 pmol/mg protein. The binding was time- and protein concentration-dependent and sensitive to trypsin treatment. H4-(86-100), histogranin and related C-terminal histone H4 fragments were tested for their ability to compete with the binding of [I^{125}] H4-(86-100). HN-(8-10) and HN-(6-9) did not show any significant inhibition at 10^{-5}M, suggesting that the minimal active core is HN-(7-10) and the most potent peptide being HN-(7-15). [^{125}I]H4-(86-100) binding was insensitive to μ, δ and κ opioid ligands. Dynorphin A-(1-13) and nociceptin significantly inhibited [^{125}I]H4-(86-100) binding, but H4-(86-100) did not affect the binding of [^{3}H]nociceptin, suggesting that the effects of these peptides on the binding may be allosteric. A close correlation (0.85) was observed between the abilities of C-terminal histone H4 peptides to compete with the binding of [^{125}I]H4-(86-100) to alveolar macrophage membranes and block pain in the mouse writhing test, suggesting that such receptor may be involved in the antinociceptive effects of the peptides. The inhibitory effect of GTP analogs (GTP-γ-S and Gpp(NH)p) and pertussis toxin on membrane preparations and the finding that H4-(86-100) potently (10^{-8} M) inhibited forskolin-stimulated cAMP levels in cultured alveolar macrophages suggest the involvement of a G_iPCR. Gel electrophoresis of cross-linked bound

radiolabelled ligand revealed two binding proteins, 30 kDa and 54 kDa, whose detection was increased by stimulation of macrophages with interferon gamma (IFN_{γ}). Affinity chromatography and SDS-PAGE gel electrophoresis revealed a major protein band identified as β-actin trypsin digests. The 54 and 30 kDa protein band requires further investigation.

LIST OF ABBREVIATIONS

AC	= adenylyl cyclase
ATP	= adenosine triphosphate
B_{max}	= maximal number of binding sites
cAMP	= cyclic adenosine monophosphate
CGRP	= calcitonin gene-related peptide
CNS	= central nervous system
COX	= cyclooxygenase
δ	= delta
DSLET	= [D-Ser^2, Leu^5]enkephalinyl-Thr
GPCR	= G-protein coupled receptor
κ	= kappa
HN	= histogranin
HPLC	= high performance liquid chromatography
i.c.v.	= intracerebroventricular injection
IC_{50}	= 50% inhibitory concentration
IFNγ	= interferon gamma
i.p.	= intraperitonial
i.t.	= intrathecal injection
K_d	= equilibrium dissociation constant
K_i	= inhibitory constant
LPS	= lipopolysaccharide
MA	= macrophage

μ	= mu
OGP	= osteogenic growth peptide
ORL_1	= opioid-like receptor 1
PBL	= peripheral blood lymphocytes
PGs	= prostaglandins
PGE_2	= prostaglandin E_2
SP	= substance P
VIP	= Vasoactive intestinal peptide

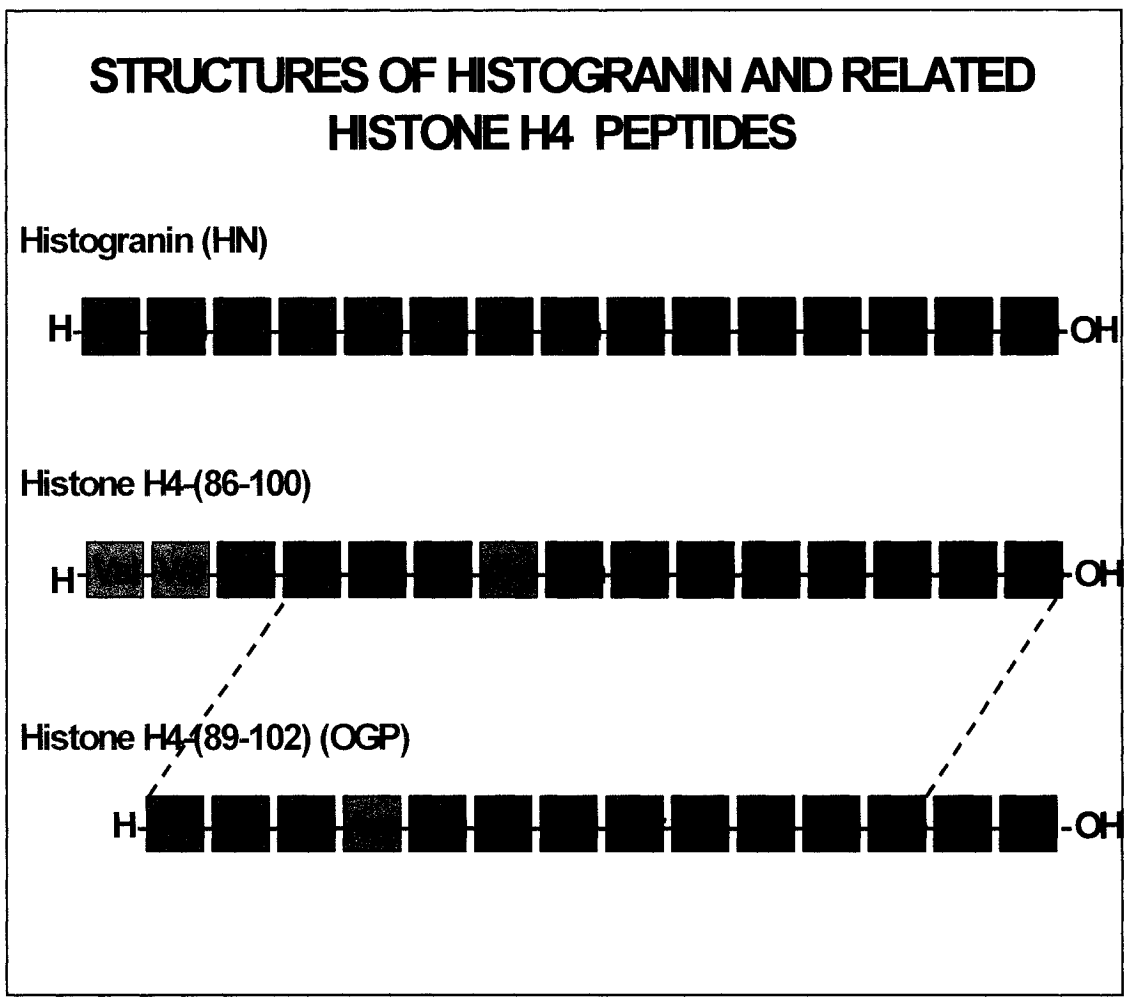
1. INTRODUCTION

1.1 Preamble

The research interest of our laboratory has recently been focused on the isolation, structural identification, synthesis and determination of biological activity of HN. HN was first isolated from the bovine adrenal medulla. Its structure corresponds to the C-terminal fragment 86 to100 of histone H4 (H4-(86-100)) with modifications in position 86, 87 and 92 (Figure 1). The peptide was first coined for its *in vivo* modulation of N-methyl-D-aspartate (NMDA)-induced convulsions in mice (Shukla et al., 1995). Recently, HN and related histone H4-(86-100) were also found to display non-opioid analgesic activities (Lemaire et al., 1997; Ruan et al., 2000). The peptides were then shown to be potent in blocking pain in mouse writhing and tail-flick assays suggesting that they may be considered as endogenous non-opioid antinociceptive peptides. The search of the gene coding for HN in the bovine adrenal medulla was unsuccessful up to now, but it led to the discovery of a histone H4 gene variant (H4-v.1) (Gendron et al., 1998). The discovery of H4-v.1 provided the first evidence for the presence of a polyadenylated histone H4 mRNA in a vertebrate species whose expression may give rise by translation to the production of peptides with potential extranuclear functions, since H4-(86-100) and OGP do not contain the hydrophobic N-terminal signal sequence of histone H4 that allows the protein to enter into the nucleus after its synthesis. Tissues that play a primordial role in immune defence such as the lung, spleen and lymph nodes express high levels of H4-v.1 mRNA and show

high contents of the C-terminal histone H4 peptides H4-(86-100) and OGP. Furthermore, the expression of H4-v.1 mRNA is lipopolysaccharide (LPS)-inducible in rat alveolar MA and its induction correlates with an increase in the synthesis of H4-(86-100) and OGP, but not whole histone H4 nor core H4 mRNA, (Poirier et al., unpublished data). Isolated rat alveolar MA were also found to be a good target for the action of C-terminal histone H4 related peptides, since nanomolar concentrations of H4-(86-100), HN and OGP were shown to inhibit LPS-induced prostaglandin E_2 (PGE_2) release from primary cultures of rat alveolar MA (Lemaire et al., in preparation). In agreement with this, specific binding of H4-(86-100) was observed *in situ* in cultured MA (Lemaire et al., 2000; 2001). In parallel studies, a high affinity binding site for HN and related peptides was found on human peripheral blood mononuclear lymphocytes (PBML) whose binding characteristics corresponded to the pain relieving effects of the peptides (Lemaire et al., 1993a). Since PBML is a mixed cell population containing a significant proportion of monocytes as potential precursors of MA, we herein formulated the concept that the C-terminal histone H4 peptides and HN may exert their pain relieving activity by acting on a specific receptor on brain and/or peripheral MA. The elucidation of the nature of this interaction constitutes the matter of the present study.

FIGURE 1. Comparison of the structure of HN with those of the C-terminal histone H4 peptides H4-(86-100) and OGP. The area within the dashed lines indicates the common sequence between OGP and H4-(86-100). The amino acids highlighted in red denote the differences between HN and H4-(86-100).


STRUCTURES OF HISTOGRANIN AND RELATED HISTONE H4 PEPTIDES
Histogranin (HN)
H-
-OH
Histone H4-(86-100)
H-
-OH
Histone H4-(89-102) (OGP)
H-
-OH

1.2 Extranuclear and/or extracellular roles of histone derived peptides

Histones are nuclear proteins known to play a role in the folding of DNA during DNA replication and cell division (Tsevtkov et al., 1989). The level of histone mRNA/protein within the cell varies depending on the status of the cell cycle: higher in the S phase but lower in non-dividing differentiated cells (Wu et al., 1981). The increased expression of the different groups of histones needed during chromosome replication involves a complex regulating system (Wu et al., 1986). The mRNAs coding for nuclear histones differentiate from other species of mRNA as they do not contain a poly-A tail at the 3' end but instead, have a 3' end palindrome sequence. This unique design assures a rapid passage of the histone mRNA through the nuclear membrane by a mechanism that is assisted by a membrane transporter dependent upon the nuclear demand for histone production (Birchmeier et al., 1984).

However, variant genes exist for all types of histones located at sites distant from core histone genes and expressed in tissue specific, cell cycle-independent and inducible manners. Two types of polyadenylated mRNA histones have been isolated: those which do not contain the palindromic sequence but instead are polyadenylated at their 3' end include human H3.3 (Wells and Kedes, 1985), $H1^{o}$ (Alonso et al., 1988) and avian H5 (Krieg et al., 1982a; Krieg et al., 1982b); and those which contain both the histone 3' consensus palindrome and the poly(A) tail include mouse H1-var.1 (Cheng et al., 1989), H2a (Moss et al., 1994), human H2A.X (Mannironi et al., 1989; Ivanova et al., 1994) and H2B.1 (Collart et al., 1992). Recently, the first example of mammalian histone H4 cDNA variant, H4-

v.1, was cloned and sequenced in our laboratory from a bovine adrenal medullary cDNA phage library (Gendron et al., 1998). H4-v.1 is a histone cDNA variant with a consensus palindromic sequence and a polyadenylated 1.1 kb downstream extension. The relative high expression of H4-v.1 in tissues (lungs, spleen, lymph nodes) that contain high levels of the C-terminal histone H4 peptides H4-(86-100) and OGP (Poirier et al., unpublished data) suggests a role in the synthesis of histone H4 derived peptides with extranuclear and/or extracellular functions.

1.2.1 Osteogenic Growth Peptide

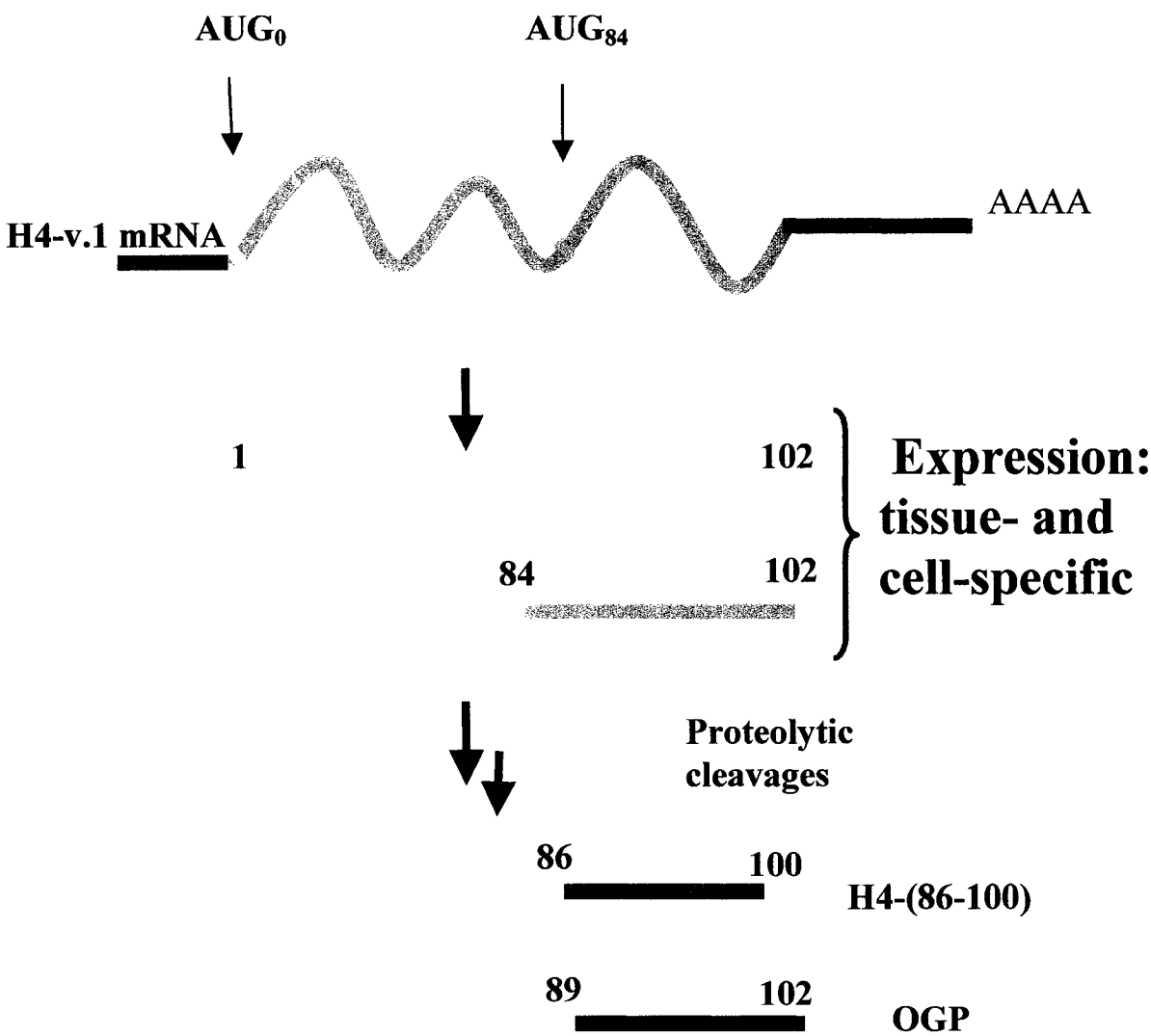
Various studies suggest that C-terminal histone H4 peptides exist and possess extranuclear and/or extracellular functions. For instance, osteogenic growth peptide (OGP), a 14 amino acid peptide corresponding to the C-terminal fragment 89 to102 of histone H4 (Bab et al., 1992), is believed to be involved in the regeneration of bone marrow (Bab et al., 1992). *In vitro*, OGP regulates stromal-cell proliferation and differentiation and *in vivo* the peptide is formed after the osteogenic phase of post ablation bone marrow regeneration (Bab et al., 1992), being mainly found in human and mammalian blood plasma as a molecule non-covalently bound to α_2-macroglobulin (Gavish et al., 1997). OGP appears to represent a new class of compound involved in the systemic control of osteoblast proliferation and differentiation, but it can also regulate the stromal cell expression of hemopoietic factors such as macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 6 and the stem cell factor (Bab and Einhorn, 1993).

Recently, Bab et al., (1999) have indicated that two methionine starting codons comprised within the coding region of histone H4 mRNA can be used to initiate translation. The translation initiated at the first starting codon generates the synthesis of whole histone H4 (102 amino acid protein), whereas that initiated at the second starting codon forms a short C-terminal peptide that does not contain the signal sequence in histone H4 for its entry into the nucleus and thus may constitute a precursor for C-terminal histone H4 peptides with extranuclear function. These conclusions were suggested by Bab et al., 1999 who observed that a mutation on the first initiation codon in the coding sequence of a histone H4 cDNA CAT-reporter gene construct is still able to generate the synthesis of a short histone H4 sequence attached to the CAT-reporter protein. This protein was shown on western blot to have a molecular weight corresponding to that of H4-(84-102)-CAT-reporter protein as expected from the position of the alternate AUG codon in histone H4 mRNA (Figure 2). Therefore, these authors proposed that the peptide H4-(84-102) generated from translation initiation at the second starting codon could be a direct precursor for OGP (histone H4-(89-102)) and other C-terminal histone H4 peptides (Figure 2). Bab et al., (1999) indicated that the abundance of the translated small H4 peptide depended upon the cell type in which the poly A-containing H4 gene construct was introduced. Interestingly, cell type dependence is an expression characteristic of histone gene variants (Harris et al., 1991), suggesting that the formation of H4-(84-102) as a precursor of antinociceptive C-terminal histone H4 peptides may depend upon the expression of the variant H4-v.1 mRNA, which itself is polyadenylated and is largely

expressed in rat alveolar MA along with the synthesis of H4-(86-100) and OGP upon LPS stimulation (Poirier et al., 2000 and unpublished data). Poirier et al., (unpublished data) demonstrated that the incubation of the synthetic peptide H4-(84-102) with proteolytic enzymes extracted from alveolar MA (aminopeptidase, carboxypeptidase) generated H4-(86-100) and OGP (Figure 2).

Although these evidences suggest a possible mechanism of synthesis for the C-terminal histone H4 peptides, no evidence was obtained to indicate how these peptides were secreted. A possible mechanism of secretion for C-terminal histone H4 peptides could be the lysosomal pathway already used for the secretion of cytokines. For instance, IL-1α and IL-1β are synthesised as precursor proteins and stored into lysosomal-like vesicles and proteolytically cleaved at the membrane level before being released in the extracellular milieu as biologically active cytokines (Koyabashi et al., 1988; Brody and Durum, 1989; Singer et al., 1995). Procytokines and pro C-terminal histone H4 peptides lack the hydrophobic signal sequence of conventional secreted proteins and they are being released from macrophages, a cell type that contains well-developed lysosomal vesicles but no secretory granule. Therefore, depending on the cell type from which they are being released, at least in macrophages, C-terminal histone H4 peptides and cytokines may use the same non-granular constitutive route of secretion for their actions at both peripheral and central sites.

FIGURE 2. Synthesis of C-terminal histone H4 peptides via translation starting at the alternative AUG codon (Bab et al., 1999; Poirier et al., unpublished data). According to Bab et al., 1999, the precursor of C-terminal histone H4 peptides may be generated from the translation of histone H4 mRNA at the alternative AUG codon (second codon) since the H4-(84-102) does not contain the N-terminal sequence for its entry into the nucleus and the mutation of the first initiation codon results in the formation of H4-(84-102). Poirier et al. (unpublished data) observed that the stimulation by LPS of the production of H4-v.1 mRNA variant in macrophages correlates with an increase in immunoreactive H4-(86-100) but not whole histone H4 nor core H4 mRNA. Moreover, in vitro treatment of synthetic H4-(84-102) with macrophage proteolytic enzymes converts the compound into H4-(86-100) and OGP as identified by mass spectrometry.


AUG0
AUG84
H4-v.1 mRNA
AAAA
1
102
84
102
Expression:
tissue- and
cell-specific
Proteolytic
cleavages
86
100
H4-(86-100)
89
102
OGP

1.2.2 Other histone-derived peptide fragments

Other C-terminal fragments of histone H4 have been reported to have extranuclear and/or extracellular functions. The autoimmune disease, lupus erythematosus, is characterized by the presence of nuclear materials in blood plasma (Robert, 2001). These materials comprise nucleosomes, histones, nucleohistones and histone-derived peptides (Salaman et al., 2001). Systemic Lupus Erythematosus (SLE) is a chronic inflammatory disease often fatal, which can affect many organs/tissues such as the kidney, brain, lung and heart (Bonney, 2001). Histone H4 derived peptides have been shown to serve as epitopes to initiate autoimmune reactions leading to the formation of autoantibodies and the development of glomerulonephritis in lupus prone mice (Decker et al., 2000; Lu et al., 1999). According to Decker et al. (2000), the C-terminal H4 peptide H4-(88-99) is the best epitope in histone H4 to initiate T cell responses. In this case, the T cell sensitization to histones was an important factor in the development of lupus and the disease state in lupus prone mice was retarded or even prevented by inducing tolerance to the histone H4 fragment H4-(88-99).

Besides being involved in lupus related diseases, particular attention regarding an antimicrobial role for histone-derived peptides or fragments was devoted. Antimicrobial peptides are effectors of immediate defence in innate immunity where their expression can be induced during infection and inflammation (Frohm et al., 1997; Stolzenberg et al., 1997). For instance, salmon histone derived peptides such as H1 histones, were shown to inhibit the growth of

antibiotic-supersusceptible *Salmonellla enterica* serovar Typhimurium as well as *Aeromonas salmonicida* and *Vibrio anguillarum* (Patrzykat et al., 2001). On the other hand, Buforin I and Buforin II present on the luminar surface of the intestine, are histone H2A-derived antimicrobial peptides, responsible for penetration of the outer and inner membranes of bacteria, ultimately resulting in either a disruption of the cell membrane or its permeabilization (Park et al., 2000, Kim et al., 2000). Therefore, structure analysis of Buforin antimicrobial peptides permits the design of potent and efficient molecules to combat bacterial infection. Recently, Agerberth et al., (2000) have isolated an active antimicrobial fragment of histone H2B, while identifying antibacterial components in human T and NK cells under stimulation with interleukin-2. Their findings suggested that antimicrobial peptides derived from histone H2B are effector molecules generated from T and NK cells in host defence response and inflammation.

1.2.3 Histogranin and H4-(86-100)

HN, a pentadecapeptide first isolated in our laboratory from bovine adrenal medulla (Lemaire et al., 1993b), was shown to possess 80% homology to portion 86 to 100 of histone H4 (Figure 1). Immunoreactive-HN (ir-HN) was shown to be present in various rat tissues including the pituitary, adrenal gland, lung, spleen, blood plasma and isolated alveolar MA (Lemaire et al., 1993b; Lemaire et al., 1995). The presence of HN in the pituitary and its release from the adrenal medulla first suggested a neuroendocrine function, but the relative high concentration of the peptide in the lungs and isolated alveolar MA, suggested a

role in the modulation of immune functions (Lemaire et al., 1994). On the other hand, H4-(86-100) is also present in various rat tissues and its relative high concentration in the bone marrow, lymph nodes and spinal cord suggests a role in inflammatory processes and pain perception (Poirier et al., 2000). In isolated rat alveolar MA, the levels of H4-(86-100) are increased by LPS stimulation along with H4-v.1 mRNA but not whole histone H4 protein (102 amino acids protein) nor core histone H4 mRNA (non-polyadenylated H4 mRNA), suggesting that the production of H4-(86-100) may depend upon the specific expression of the variant H4-v.1 mRNA (Poirier et al., 2000).

1.2.3.1 Antinociceptive effects of Histogranin and H4-(86-100)

In 1990, Sagen and colleagues showed that the transplantation of adrenal chromaffin cells into the rat spinal subarachnoid space could alleviate chronic and neuropathic pain induced by hindpaw administration of formalin and sciatic nerve injury (Hama and Sagen, 1994). Recently the same research team found that the analgesic effects of the transplanted adrenal chromaffin cells in rat models of chronic pain and inflammation were mimicked by the adrenomedullary peptide HN. Thus, they showed that relatively low doses (nmolar) of the chemically stable analog of HN, [Ser^1]HN, blocked pain induced by formalin (Siegan and Sagen, 1997), peripheral neuropathy (Siegan et al., 1997) and direct intrathecal application of NMDA (Hama et al., 1999; Siegan et al., 1996). Recently, HN and the C-terminal histone H4 peptide H4-(86-100) were found to display non-opioid analgesic activities in two mice models of pain. Thus, intracerebroventricular

(i.c.v.) administration of these peptides in mice blocked writhing induced by intraperitoneal (i.p.) administration of acetic acid and tail-flick induced by radiant heat in dose- and structure-dependent manners (Lemaire et al., 1997; Ruan et al., 2000).

1.2.3.2 Histogranin and H4-(86-100) binding sites

In rat brain membranes, specific binding sites were found for [^{125}I][Ser^{1}]HN with the characteristics of a receptor, e.g. high affinity, trypsin sensitivity, ligand specificity, saturability and binding reversibility (Rogers and Lemaire, 1993). However, the abilities of HN and related peptide analogs and fragments to compete with [^{125}I][Ser^{1}]HN binding did not correspond to their abilities to inhibit pain in the mouse writhing test, but rather to their abilities to block convulsions induced by NMDA in mice (Lemaire et al., 1993b). The activities of the peptides in the binding and convulsion assays depended upon the integrity of the 15 amino acid peptide, whereas analgesia was better produced by the HN fragment HN-(7-15), HN-(7-10) being the minimal active core (Ruan et al., 2000). A role of the dopamine D2 receptor in the supraspinal analgesic effects of HN and related peptides was suggested by their reversal with the D2 receptor antagonist raclopride (Ruan et al., 2000) and the modulation of [^{3}H]raclopride binding to rat brain membranes by HN and related peptides (Ruan and Lemaire, 2001). However, the interaction of HN with the D2 site was suggested to be indirect since sulpiride did not affect the binding of [I^{125}][Ser^{1}]HN to rat brain membranes (Rogers and Lemaire, 1993).

In another study, it was found that human PBML, containing a mixed population of lymphocytes and monocytes, expressed specific high affinity binding sites for [^{125}I][Ser^{1}]HN (Lemaire et al., 1993a). In contrast with the rat brain receptor, the binding of the peptide to PBML membranes was not affected by polyamines (NMDA receptor modulators) and showed structural binding requirements comparable to those found for the analgesic activity of the peptides (Ruan et al., 2000). In parallel studies, we recently demonstrated that rat spleen membranes express a high level of high affinity sites for [^{125}I]H4-(86-100) (Poirier et al., 2000). Again, this suggests that C-terminal histone H4 peptides may play an important role in the regulation of the immune functions of spleen cells which are largely represented by lymphocytes and macrophages.

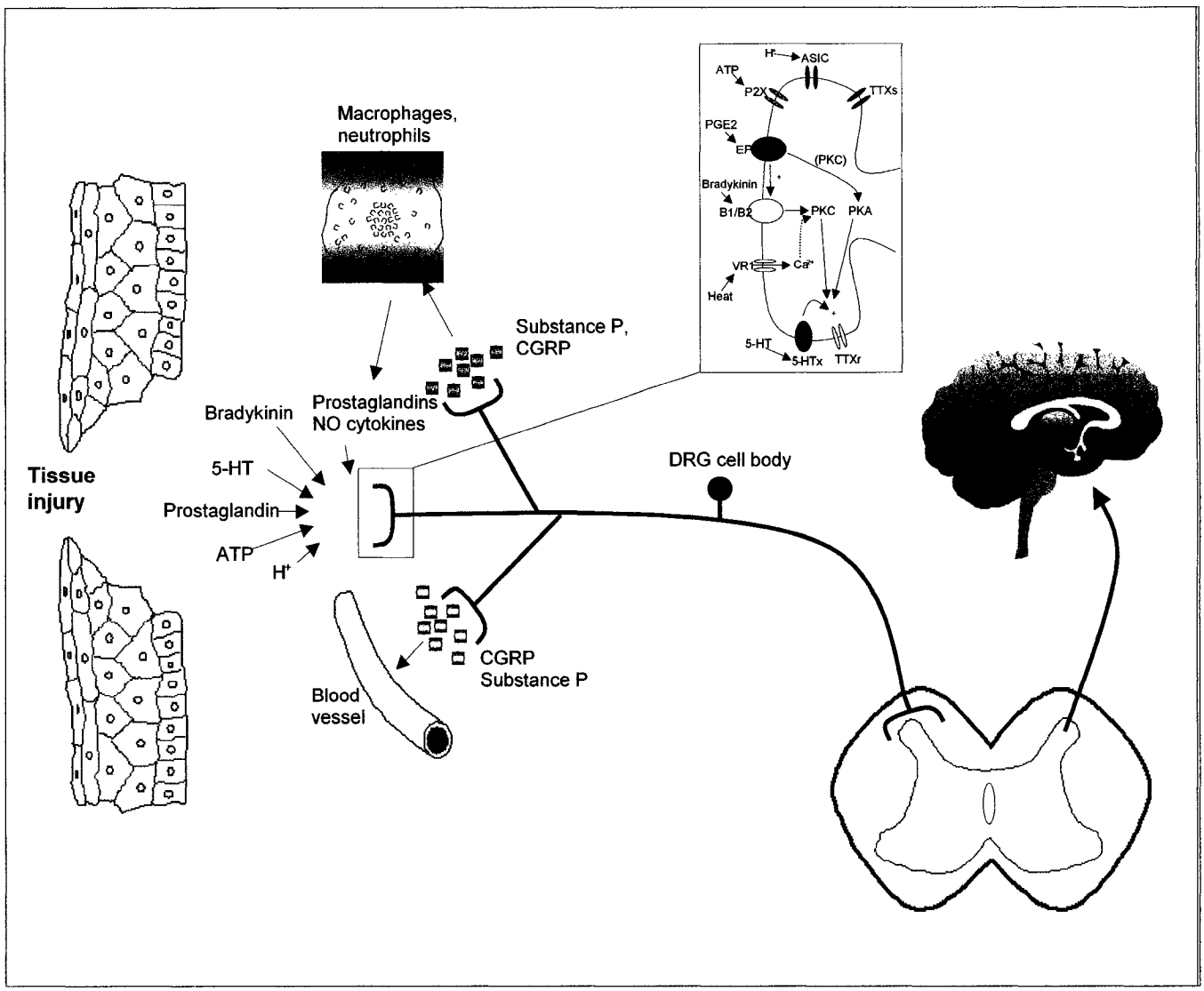
1.3 Possible role of macrophages in the antinociceptive effects of C-terminal histone H4 peptides

1.3.1 Interaction between the immune and central nervous systems

The interaction between components of the nervous system and multiple target cells of the immune system has been receiving increasing attention. For instance, nociceptive neurons involved in pain transmission are depolarized by various chemical mediators of pain including H^+, bradykinin (BK), (serotonin) 5-HT, ATP, PGs, which are released by macrophages in different area of tissue injury or inflammation (Figure 3) (Basbaum and Woolf, 1999). Acute and chronic peripheral inflammation is accompanied by increases in interleukins, PGE_2 and nitric oxide (NO) production, and elevated releases of excitatory amino acids

(glutamate), substance P (SP), BK, and calcitonin gene related peptide (CGRP) (Ahluwalia and Perretti, 1994) (Figure 3). For instance, PG increases the sensitivity of pain receptors to BK, one of the most potent pain-producing agents (Lombardo and Wilson, 1997). BK exerts its biological effects through the activation of two transmembrane GPCR, denoted BK B_1 and B_2. (Couture et al., 2001) (enlargement in Figure 3). Consequently, the activation of sensory nerve terminal by BK also causes the release of neuropeptides such as SP, neurokinin A, CGRP (Geppetti et al., 1990). These peptides can also mediate a number of local pro-inflammatory effects on MA and contribute to nociceptor sensitization and hyperalgesia. Thus, SP and CGRP stimulate vascular endothelial cells to release the smooth muscle relaxant NO and thereby increase tissue blood flow and inflammation (Dray and Perkins, 1993). Furthermore, these neuropeptides released by sensory nerve terminals under pain or inflammation can directly modulate MA immune functions and regulate cytokines and PGs production, antigen presentation and antibody formation in response to inflammation (Ho et al., 1997).

FIGURE 3. The molecular complexity of the nociceptors is illustrated by its response to inflammatory mediators at the site of tissue injury. Injury or tissue damage releases bradykinin and PGs, which activate or sensitize nociceptors. Activation of nociceptors leads to the release of SP and αCGRP. SP can act on immune cells such as MA releasing furthermore PGs, cytokines or NO or act on blood vessel. The enlargement of the peripheral terminal of nociceptors showing the various ligand-gated ion channels and GPCR, and the inflammatory mediators that binds directly to them. BK receptors are driven and up-regulates by PGE_2, which activate protein kinase A (PKA) resulting in sensitization process and pain activation. 5-HT, serotonin; ATP, adenosine triphosphate; CGRP, calcitonin gene-related peptide; TTXs, tetrodotoxin sensitive sodium channel; VR1, vanniloid receptor 1; DRG, dorsal root ganglia. Adapted from figure 1 in Basbaum and Woolf, 1999 and figure 24-7 in Basbaum and Jessel, 2000.


Macrophages,
neutrophils
Substance P,
CGRP
Bradykinin
Prostaglandins
NO cytokines
5-HT
Prostaglandin
ATP
H+
Tissue
injury
DRG cell body
CGRP
Substance P
Blood
vessel
H+
ASIC
ATP
P2X
TTXs
PGE2
EP
(PKC)
Bradykinin
B1/B2
PKC
PKA
VR1
Ca2+
Heat
5-HT
5-HTx
TTXr

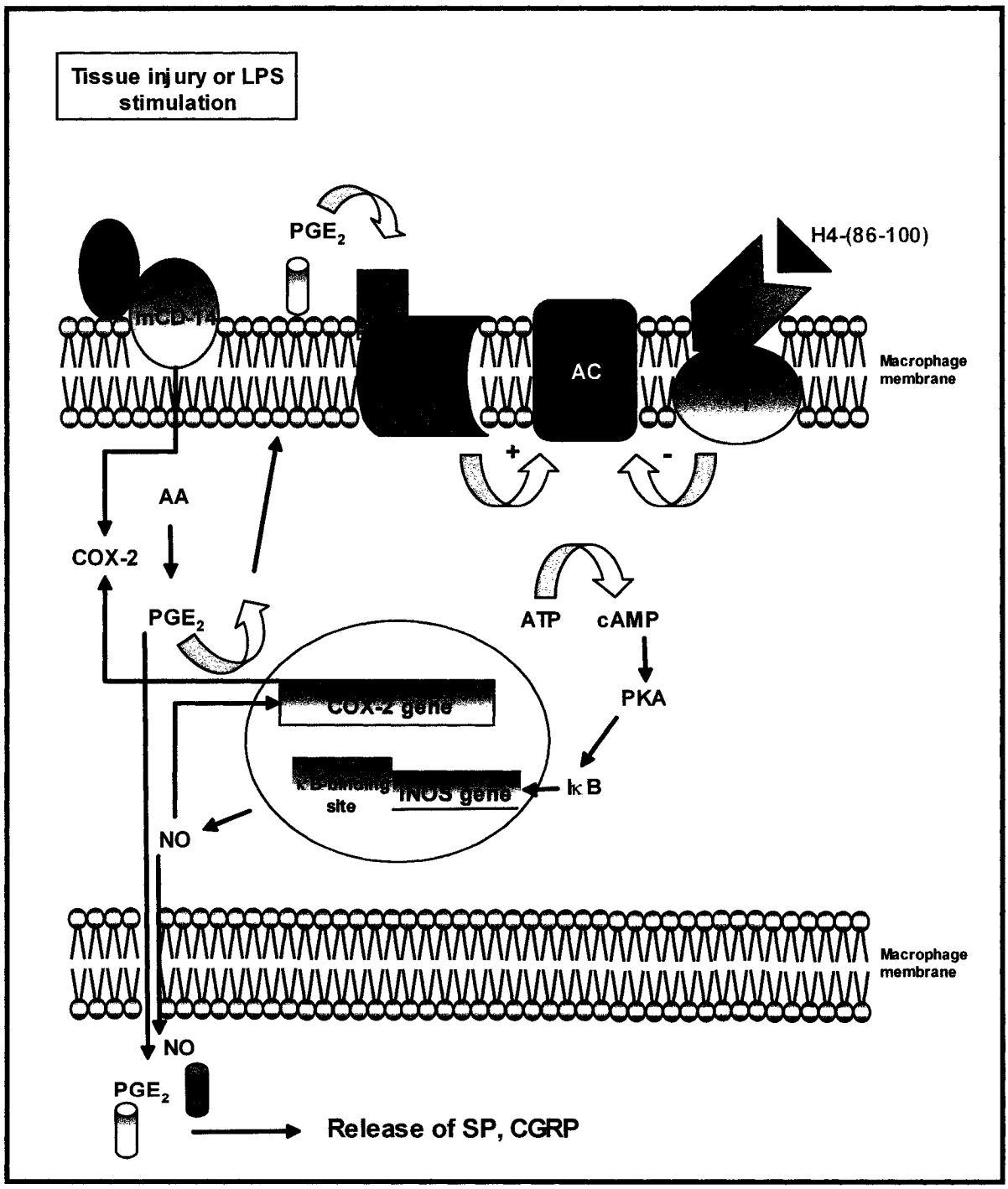
1.3.2 Role of macrophages in immune responses

A successful and rapid immune response to infectious or noxious agents responsible of inflammation is dependent on the activation of an appropriate set of immune functions largely displayed by MA. MA act in cooperation with lymphocytes to protect the host against unwanted invaders such as microorganisms or tumor cells (Tse and Rosenthal, 1988). MA are important secretory cells (Henson et al., 1988), play a pivotal role in antigen presentation and lymphocyte activation (Tse and Rosenthal, 1988) and exert cytotoxicity against tumor cells (Adams and Hamilton, 1988). MA are highly regulated and modulated by circulating peptides and hormones which themselves can evoke the secretion of a large variety of inflammatory mediators termed "cytokines" (Balkwill and Burke, 1989). Cytokines are extremely potent, acting at picomolar concentration and play a prominent role in cell-cell communication during inflammation. The knowledge of the mechanisms that regulate MA functions is pivotal in the understanding of immune response to inflammation. Thus, a modulation of the production of PGE_2 by some modulatory agents such as H4-(86-100) in LPS-stimulated alveolar MA may involve a cascade of events comprising the interaction of the compound with a specific receptor possibly of the category of GPCR followed by changes in the synthesis of specific intracellular signal transducers such as cAMP, modulation of LPS-induced production of cyclooxygenase-2 (COX-2) and PGE_2 (Lemaire and Ouellet, 1996; Hayashi et al., 1991) (Figure 4).

This is supported by the observation that the sustained induction of COX-2 expression during inflammation is due to the persistent presence of elevated intracellular concentrations of cAMP (Hinz et al., 2000). For that reason, Hinz et al., (2000) were able to establish an autoregulation of COX-2 expression in human monocytes via a positive feedback mechanism by PGE_2, which increases the formation of cAMP (Figure 4). Since the inflammatory molecule PGE_2 interacts with a GPCR (Boie et al., 1997), more precisely a G_s protein, it is capable of increasing cAMP formation resulting in an increase in COX-2 expression and an amplification of the production of pro-inflammatory agents (Figure 4). The present study will address the elucidation of the first step of the cascade of events, e.g. the characterization of the interaction of H4-(86-100) with a specific receptor on alveolar MA membrane and the isolation and structure identification of specific binding protein(s).

FIGURE 4. Schematic representation of the signalling pathway for LPS or substances released from tissue injury inducing the production of PGE_2 via a cAMP-dependent activation cascade. The LPS induces COX-2 enzyme expression, PGE_2 formation, cAMP production via a G_s-protein coupled adenylate cyclase mechanism, and PKA activation or release of pain/inflammation agents such as SP, CGRP and glutamate. The activation of PKA can induce furthermore the expression of the COX-2 gene by stimulation of NF-κB-binding site, initiating iNOS expression and NO release, therefore amplifying the PGE_2 formation. The modulation of pain and inflammation by H4-(86-100) and C-terminal histone H4 peptides could result in a mechanism involving a G_iPCR.

EP, prostaglandins E_2 receptor; G_s, G_s-protein coupled receptor; AC, adenylate cyclase; H4-R, Histone H4 receptor; G_i, G_i-protein coupled receptor; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CGRP, calcitonin gene-related peptide; NO, nitric oxide; iNOS gene, nitric oxide synthase gene; COX-2, cyclooxygenase 2; AA, arachidonic acid. Diagram adapted from Vanegas and Schaible, (2001) and Chen et al., (1999).


Tissue injury or LPS stimulation
PGE2
H4-(86-100)
mCD-14
AC
Macrophage membrane
+
-
AA
COX-2
PGE2
ATP
cAMP
PKA
COX-2 gene
site
iNOS gene
IκB
NO
Macrophage membrane
NO
PGE2
Release of SP, CGRP

1.3.3 Effects of C-terminal histone H4 peptides on macrophage functions: possible role of a G_i protein-coupled receptor

GPCR and cAMP have preponderant roles in the modulation of the metabolism of arachidonic acid in MA in response to inflammatory stimuli (Figure 4). El-Shenawy et al., (2002) have recently demonstrated that the anti-inflammatory and antinociceptive properties of melatonin in rat are due to its interaction on a specific receptor on MA. Mouse peritoneal MA revealed to display a PTX-sensitive melatonin signal transduction pathway that involves a G_iPCR-mediated inhibition of AC (Garcia-Perganeda et al., 1999). Their results suggest that the modulatory effects of melatonin on pain and inflammation may depend upon an interaction of the peptide with a G_iPCR on MA, resulting in a decrease in cAMP and subsequent blockade of NF-κB-mediated COX-2 induction.

Our recent findings which indicate that LPS can induce the expression of H4-v.1 mRNA and the synthesis of H4-(86-100) and OGP in isolated rat alveolar MA (Poirier et al., unpublished data) combined with the knowledge that C-terminal histone H4 peptides inhibit LPS-induced secretions of PGE_2 from the alveolar MA (Lemaire et al., in preparation) point to the importance of studying the nature of the receptor(s) involved in the effects of C-terminal histone H4 peptides. As shown in figure 4, the proposed mechanism of action for H4-(86-100) to inhibit LPS-evoked PGE_2 production and secretion is via the stimulation of a specific G_iPCR followed by an inhibition of AC and a decrease in cAMP levels. This hypothetical mechanism of action of H4-(86-100) on MA is proposed on the basis of the recent findings of Gabarin et al., (2001) which indicate that the mitogenic

effects of OGP and related C-terminal histone H4 peptides on osteoblasts involve pertussis toxin-sensitive stimulation of MAP kinase-signaling cascade. Therefore, C-terminal histone H4 peptides are more likely acting on a G_iPCR.

2. HYPOTHESIS AND SPECIFIC AIMS

The experimental evidence mentioned above suggests a role for H4-v.1 and C-terminal histone H4 peptides in the modulation of alveolar MA functions. The research proposal is based on the hypothesis that H4-(86-100) and related peptides may affect the release of PGE_2 from LPS-stimulated MA by a mechanism which involves an interaction of the peptide with a specific G_iPCR, followed by a change in AC activity resulting in a decrease in cAMP levels and possible modulation of the production of enzymes involved in the production of pain factors including PGE_2 and congeners.

Thus, the overall aim of this work was to verify if H4-(86-100) and related peptides interact with a specific receptor on macrophage membranes and if such putative specific interaction possesses the characteristics of G-protein coupled receptors (GPCR), common to various peptide hormones and neuropeptides.

Hence, the specific aims were:

(1) To perform a pharmacological characterization of [^{125}I]H4-(86-100) binding to rat alveolar MA membranes (protein nature, binding affinity, capacity, specificity, and reversibility);

(2)To verify if the binding characteristics of C-terminal histone H4 peptides to rat alveolar MA membranes correlate with the analgesic activity of the peptides;

(3) To determine if the H4-(86-100) binding site(s) can be classified in the receptor category of $GPCR_s$ (effects of salts, guanine nucleotides, sensitivity to pertussis toxin, receptor size, link to adenylate cyclase) and if they are inducible under stimulatory conditions (LPS or IFN_γ);

(4) To isolate, purify and sequence the specific H4-(86-100) binding protein(s).

3. MATERIALS AND METHODS

3.1 Materials

H4-(86-100), OGP, dynorphin-A (1-13), [Ser^1]HN and other HN related peptides were synthesized in our laboratory as previously described (Lemaire et al., 1986a; Prasad et al., 1995) by the solid phase procedure (Merrifield, R.B, 1983). RH-28 antibody was raised against [Ser_1]HN as described by Lemaire et al., 1993b. Carrier-free NaI^{125} and [*Leucyl*-^{3}H]Nococeptin were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Diethylenetriamine, spermidine, neurotensin, nociceptin, and naloxone were purchased from Research Biochemicals Inc. (Natick, MA, USA) and SP, vasoactive intestinal peptide (VIP) and [D-Ser^2, Leu^5]enkephalinyl-Thr (DSLET) were obtained from Peninsula Laboratories (Belmont, CA, USA). Morphine was provided by Hoffman-Laroche Ltd (Vaudreuil, Qc). U-50488H was purchased from the UpJohn Company (Kalamazoo, MI). LPS, trypsin, leupeptin, pepstatin, bacitracin, captopril, bestatin, thiorphan, forskolin, (Gpp(NH)p), (GTP-γ-S), guanine 5'-o-(2-thiodiphosphate) (GDP-β-S), BSA, polyethylenimine (PEI), polyethyleneglycol-8000 (PEG-8000), trifluoroacetic acid (TFA), poly-L-lysine and gamma-globulins were obtained from Sigma Chemicals Co. (St-Louis, MO, USA). Adenosine-5'-triphosphate (ATP) was purchased from Boehringer Mannhein (Germany) and disuccinimidylsuberate (DSS), PTX-B (B Oligomer), PTX-A (A Promoter), thymidine, and β-nicotinamide-adenine dinucleotide (NAD) were obtained from Calbiochem (La Jolla. CA, USA). For cell culture, culture medium RPMI 1640 and FBS were obtained from Wisent (Qc, Canada), and gentamicin sulfate and

IFNγ were purchased from Schering Canada Inc. (Canada) and Gibco BRL, respectively. Biotrak cellular communication assays, cAMP enzymeimmunoassay (EIA) system (RPN 225) and HiTrap Streptavidin Affinity column were purchased from Amersham Pharmacia Biotech. 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) was obtained from Pierce, USA and the Microcon YM-30 columns (Ultracel-YM regenerated cellulose membrane) were obtained from Millipore, Nepean, Ontario, Canada.

3.2 Iodination of H4-(86-100), [Ser^1]HN and OGP

Peptides were iodinated with 1 mCi carrier free NaI^{125} by the chloramine-T procedure (Hunter and Greewood, 1962) for 30 seconds at room temperature and passed trough a Sep-Pak C18 cartridge which was previously conditioned with 20 ml acetonitrile (ACN) 100% and 20 ml of TFA 0.1%. The peptide product was eluted with 3 ml of 60 % ACN in 0.1% TFA and the labelled peptide was separated from the non labelled peptide by HPLC using a reverse phase μ-Bondapak C18 column (Waters). The HPLC column was run at 1 ml per min and the peptide was eluted with gradient of ACN (15 to 60% in 0.1% TFA) between 10 and 60 min. Five μl aliquots of each fraction were used to monitor the presence of the radioactive peptide. The radioactive peptide peak was collected and the concentration of the peptide ([^{125}I]H4-(86-100), [^{125}I][Ser^1]HN and [^{125}I]OGP) was determined by a radioimmunoassay (Lemaire et al., 1986b). In a typical assay for the measurement of the concentration of radiolabelled or non-radiolabelled H4-like peptides, radioimmunoassays with anti-HN (RH-28) were performed by

incubating at 4°C for 20 hours the following components: (1) 100 µl of buffer A (0.15 M phosphate buffer, pH 7.4, containing 0.1% BSA, 0.9% NaCl and 0.01% sodium azide [NaN_3]) in the presence or absence of different concentrations (10^{-11} to 10^{-7} M) of the non-labelled peptide; (2) 100 µl of the radiolabeled peptide (20,000 cpm: standard curve; 300,000 or 600,000 cpm for the determination of the concentration of the radiolabelled peptide) in buffer A containing 0.05% poly-L-lysine and (3) 100 µl of antiserum RH-28 (from rabbit; diluted 1:5000 in buffer A). The antiserum had a good cross-activity for the three iodinated peptides. After incubation, the antigen-antibody complex was precipitated by the addition of 100 µl of 0.5% gamma-globulins in buffer A and 500 µl of 20% polyethyleneglycol (PEG) in buffer A and centrifuged at 2000 rpm (Beckman centrifuge) for 30 minutes. The supernatant was discarded and the pellet monitored for radioactivity. The concentration of the radiolabeled peptide was calculated according to the standard curve established with the displacement of non-labelled peptide (10^{-9} to 10^{-4} M). The concentration of the radiolabelled peptide varied from 50 to 250 nM in various preparations.

3.3 Bronchoalveolar lavages of rats

Male Wistar rats weighing between 250-275 g were purchased from Charles River Canada Inc. (St-Constant, Canada). The animals were obtained from pathogen-free colony, shipped behind filter barriers, and housed in isolated temperature-controlled hoods. Animals were injected intraperitoneally with sodium pentobarbital (1 ml/rat) prior to be submitted to the following procedures:

abdominal aorta incision (bleeding), thorax cage incision (lung exposure) and trachea cannulation with a valve system permitting the entrance and exit of liquid from the lung (Lemaire, 1985). The bronchoalveolar lavage was based on the technique referred in Lemaire (1991). Essentially, the exposed lungs were injected with a total volume of 49 ml of phosphate-buffered saline (PBS) pH 7.4 in aliquots of 7 ml (7 lavages in total). Lung massages were performed for two different interval periods: 4 times 4 minutes followed by 3 times 2 minutes. The liquid (PBS containing the alveolar MA) was recovered after each interval period with a syringe attached to the valve system. After its total recovery (≈ 47 ml, 96%), the fluid was centrifuged (IECCentra-8R Centrifuge) at 1000 RPM (200 X g) for 5 minutes at 4°C. The cell pellet was resuspended at 3.5 x 10^6 cells per ml in PBS and counted in a hemocytometer chamber. The average of alveolar MA obtained from one rat varied between 7 to 8 x 10^6 cells. The viability (average 99.0%) was determined by trypan blue exclusion. The alveolar MA were frozen at –70°C until needed for membrane preparation.

3.4 Membrane preparation of rat alveolar macrophages for binding assay and cross-linking experiments

Alveolar MA membranes were prepared according to the technique described for the preparation of bovine aortic endothelial cells (Bernier et al., 1995) with the following modifications: cells obtained from 3 rats were broken first by thawing followed by 10-repeated passages trough a needle (18G 11/2) and then homogenised by six strokes with a thigh pestle putter in 10 ml of ice-cold 50

mM Tris-HCl buffer, pH 7.4 (buffer B) containing protease inhibitors (leupeptin 10 μg/ml, pepstatin 10 μg/ml). After centrifugation at 27000 X g for 15 minutes at 4°C, the pellet was resuspended in ice cold buffer B containing 0.1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Protein concentration was determined by the method of Lowry (1951) using BSA as standard. Membranes from rat alveolar MA were always freshly prepared for each assay, since freezing and storage of membranes at – 70°C led to appreciable loss of binding activity.

3.5 Membrane preparation of rat spleen for receptor cross-linking and purification assay

Eight male Wistar rats were decapitated and their whole spleen were rapidly removed and homogenized in 40 ml of ice-cold Tris-HCl buffer 50 mM, pH 7.4 containing 5 nM EDTA, 0.2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin (buffer C) using a Beckman polytron (Rogers and Lemaire, 1993). The homogenate was centrifuged at 1000 X g for 30 minutes at 4°C to remove nuclear material. The supernatant was then centrifuged at 27,000 X g for 30 minutes at 4°C. The resulting pellet was resuspended in 40 ml of buffer C, incubated at 37°C for 30 minutes and centrifuged for another 30 minutes at 27,000 X g. The resulting pellet was homogenized and incubated on ice in 40 ml of buffer C supplemented with 0.3 M KCl for 60 minutes at 4°C and centrifuged at 27,000 X g for 30 minutes at 4°C. The pellet was washed with 40 ml of buffer C two times by homogenization and centrifugation, and the final membrane pellet was resuspended in 10 ml of buffer C containing 1 % CHAPS for solubilization of the

membrane proteins as described under the section 3.12. To establish the optimal concentration of CHAPS for solubilization of membrane proteins, spleen membrane preparations were incubated in presence of different concentrations (0.1, 0.2, 0.3, 0.4 and 1.0%) of CHAPS for 1 hour at 4°C and centrifuged at 100,000 X g for 1 hour. The solubilized proteins were then incubated in the presence of the labelled peptide as indicated above followed by the addition of cold buffer C containing the appropriate concentration of CHAPS to stop the reaction. After binding, the excess labelled peptide was eliminated by passing the solution on Microcon YM-30 filters followed by rinsing with buffer C and binding measurement according to section 3.7.

3.6 Alveolar macrophage culture and stimulation with inflammatory agents

Alveolar MA obtained from bronchoalveolar lavages were incubated in RPMI 1640 medium supplemented with 0.5% dialysed FBS, 0.005% gentamycin and 0.8% Hepes (Lemaire, I., et al., 1996) at 1 x 10^6cells/ml in the absence or presence of LPS (1 μg/ml) (Lemaire, I., et al., 1996) or IFNγ (100 U/ml) (Li, C.Y., et al., 1998) as indicated for 24 h at 37°C in humidified atmosphere containing 5% CO_2. After the incubation, cells were harvested in 2 ml of PBS for membrane preparations (see above description of alveolar MA membrane preparations).

3.7 [^{125}I]H4-(86-100) binding

Binding assays, unless indicated, were performed with modifications of previously described methods (Lemaire, 1993b; Makman and Dvorkin,1997). Briefly, the equilibrium binding assays were performed at 4°C for 45 minutes in 0.5 ml of 50 mM Tris-HCl buffer pH 7.4 containing protease inhibitors (leupeptin 10 μg/ml, pepstatin 10 μg/ml) (buffer B) and 0.015 mg of membrane proteins. Binding experiments were performed in duplicates, with [^{125}I]H4-(86-100) (1nM) in the presence or absence of 20 μM H4-(86-100). In saturation binding experiments, rat alveolar MA membrane preparations (0.015 mg proteins) were incubated in duplicate with increasing concentrations of [^{125}I]H4-(86-100) (0.1-32.0 nM) in the absence and presence of the cold ligand (20 μM). Competition experiments were performed with 1 nM of [^{125}I]H4-(86-100) in the presence of various concentrations (10^{-9} -10^{-5}M) of H4-(86-100) or one concentration (10^{-7} or 10^{-6} or 10^{-5}M) of various peptides and non-peptides, as indicated. Binding experiments were terminated by the addition of 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4 supplemented with 6.6% polyethyleneglycol and filtration under reduced pressure through 934-AH Whatman filters (Rogers, C., and Lemaire, S., 1993) pre-treated for one hour with 2.0% PEI in 50 mM Tris-HCl, pH 7.4 at room temperature. The filters were washed four times with 3 ml of 50 mM Tris-HCl, pH 7.4 supplemented with 6.6% polyethyleneglycol and the filters were transferred into polypropylene tubes and counted with a gamma counter at 75% efficiency (Beckman). In all binding experiments, specific [^{125}I]H4-(86-100) binding was obtained by subtraction the non-specific binding measured in the presence of 20

μM unlabelled H4-(86-100) from the total binding. In order to monitor the effects of PTX on the binding of the radiolabelled peptide, membranes (100 μg) were incubated for 30 minutes at 30°C with 0.25 μg PTX-A or 1 μg PTX-B (inactive form) in the presence of 20 μM NAD, 10 mM thymidine, 0.5 mM ATP, 150 mM $NaPO_4$, pH 7.0 (total volume of 200 μl) (Shreeve et al., 2000). Toxins were eliminated by centrifugation prior to the binding assay.

3.8 [*Leucyl*-^{3}H]Nociceptin binding

Binding of [*Leucyl*-^{3}H]Nociceptin, unless indicated, was performed with modifications of previously described methods (Makman and Dvorkin, 1997). Briefly, in equilibrium binding assays, membranes (15 μg) were incubated at 22°C for 30 minutes in 0.5 ml of 50 mM Tris-HCl buffer pH 7.4 containing 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1% BSA, and a cocktail of inhibitors (bacitracin, 25 μM; captopril, 10 μM; bestatin, 30 μM; thiorphan, 0.3 μM). In all binding experiments, specific [*Leucyl*-^{3}H]Nociceptin binding was obtained by subtraction the non-specific binding measured in the presence of 50 μM unlabelled nociceptin from the total binding. Binding experiments were carried out in duplicates with [*Leucyl*-^{3}H]Nociceptin (0.75 nM) (Dumont and Lemaire, 1998). The reaction was stopped by the addition of 2 ml of ice cold buffer and filtration under reduced pressure through 934-AH filters pre-treated for 1 hour with 0.5% PEI. Filters were incubated for 14-16 hours in 10 ml of liquid scintillation solution and counted for 2 minutes in a scintillation counter (1414 Wallac Winspectral, Wallac in Turku,

Finland). [*Leucyl*-^{3}H]Nociceptin binding was also monitored for comparison of binding displacements by nociceptin and H4-(86-100) at 10^{-5} M.

3.9 Alveolar macrophage stimulation and cyclic AMP measurement

Alveolar MA obtained from bronchoalveolar lavages were incubated in a 96 well plate at 1 x 10^{6}cells/ml of RPMI 1640 medium supplemented with 0.5% dialysed FBS, 0.005% gentamycin, and 0.8% Hepes for 20 hours at 37°C in humidified atmosphere containing 5% CO_2. After the incubation, cells were stimulated for 30 minutes with H4-(86-100) (10^{-8}M), nociceptin (10^{-8}M), dynorphin A (1-13) (10^{-8}M), SP (10^{-8}M), VIP (10^{-8}M), forskolin (10^{-8}M) and a mixture of forskolin and each one of the peptides at 10^{-8}M (Delgado et al., 1999). The culture medium was discarded and the cells were resuspended in a lytic buffer (0.25 % solution of dodecyltrimethylammonium bromide) included in the ELISA kit (cAMP EIA system, Amersham Pharmacia Biotech) for 15 minutes at room temperature. The lytic buffer permitted the extraction of intracellular cAMP. The amounts of intracellular cAMP were quantified using the procedures indicated in the ELISA kit method for measurement of cAMP. This system combined the use of a peroxidase-labelled cAMP conjugate, a specific antiserum, which is immobilized on to pre-coated microtitre plates, and a one-pot stabilized substrate solution. The assay is based on competition between unlabelled cAMP and fixed quantity of peroxidase-labelled cAMP, for a limited number of binding sites on a cAMP specific antibody (Albano et al., 1974, Volker et al., 1985). The kit allowed the simple and rapid extraction of cAMP and its measurement in cultured cells.

After the addition of 150 µl of the enzyme substrate containing 3,3',5,5'-tetramethylbenzidine (TMB)/hydrogen peroxide, the plate was incubated at room temperature for 1 hour before the addition of 100 µl of 1.0 M sulphuric acid. The optical density (OD) was measured with a Plate Reader version 2.01 for windows program in a micro-plate reader (Packard SpectraCount) at 450 nM within 30 minutes.

3.10 Covalent cross-linking of iodinated peptides to rat alveolar macrophage or spleen membranes

For covalent cross-linking experiments with [^{125}I]H4-(86-100), [^{125}I][Ser^{1}]HN or [^{125}I]OGP, binding was effected using the same conditions as described above except for the use of eppendorf tubes (1.5 ml). After 45 minutes of incubation with the radiolabelled peptide at 4°C in 0.5 ml of buffer B, the samples were centrifuged at 11000 RPM in a microcentrifuge for 15 minutes at 4°C. Membranes were washed twice with 1 ml of buffer B, resuspended in 300 µl 50 mM Tris-HCl, pH 7.4 containing a final concentration of 1 mM of DSS and incubated for 20 minutes at room temperature (Bernier et al., 1995). One ml of ice-cold buffer B was then added to stop the linkage reaction and the membranes were washed twice with 1 ml of buffer B followed by centrifugation. The pellet was resuspended in 15 µl a protein denaturing buffer containing 50 mM Tris/HCl, pH 6.9, 5% mercaptoethanol, 10% glycerol (v/v), 2% sodium dodecyl sulphate (SDS) (w/v), and 0.1% bromophenol (w/v) and incubated for 15 minutes at 60°C. The denatured protein material was analyzed by SDS-PAGE. Covalent cross-linking

with [^{125}I]H4-(86-100) was also realized on proteins solubilized from spleen membranes. The excess of labelled peptide was eliminated by passage of the solution through Microcon YM-30 filters before subjecting to the cross-linking DSS reaction.

3.11 SDS-PAGE and autoradiography of [^{125}I]H4-(86-100) binding protein

Electrophoresis was carried out in the presence of SDS and mercaptoethanol as described by the method of Laemmli (1970). Denaturated proteins (60-80 μg) were subjected to electrophoresis on 10% polyacrylamide gel at 150 volts for 2 hours. The gel was fixed and stained with 0.25% Coomassie brilliant blue in a mixture of 45% methanol/10% acetic acid for 30 minutes and distained overnight with the same solution without the Coomassie brilliant blue. The gel was dried for two hours at 80°C and exposed for 3 to 7 days to a Kodak X-mat AR-5 films. Binding proteins were revealed by autoradiography (McLean et al., 1989).

3.12 Solubilization and purification of H4-(86-100) binding proteins from rat spleen using a HiTrap Streptavidin affinity columns

In general, the method for purification of the receptor was based on the ability of H4-(86-100)-NH-CO-SS-biotin to bind to the solubilized receptor and to be captured thereafter by a HiTrap Affinity resin attached to Streptavidin (see Figure 5) (Marie et al., 1990). The H4-(86-100) binding proteins were eluted from the affinity column with mercaptoethanol by breakage of the disulphide (SS) bond

between biotin and H4-(86-100), therefore releasing the binding proteins under an attached form to H4-(86-100).

The various steps for the purification of the membrane H4-(86-100) binding sites were as follows:

A) H4-(86-100) was conjugated to a spacer arm-NH-CO-SS-biotin prior to its binding to the solubilized membrane proteins (Hochhaus et al., 1988). The conjugation was performed according to the instructions provided in the EZ-Link NHS-SS-Biotin kit (Pierce, USA). Biotinylation of the peptide was made on the side-chain of Lys in position 91 of H4-(86-100) using sulfosuccinimidyl 2-(biotiamido) ethyl-1-3'dithioproprionate (10^{-3} M) in water (Diamandis and Christopoulos, 1991).

B) Membrane preparations obtained from 8 rat spleens were incubated in the presence of buffer C containing CHAPS 1% for 1 hour at 4°C (Zhang et al., 1998). The membranes were then centrifuged at 100,000 X g for 1 hour at 4°C and the supernatant containing the solubilized proteins (12 mg) was subjected to the previously described binding procedure for 45 minutes at 4°C using H4-(86-100)-NH-CO-SS-biotin (10^{-7}M) as the ligand. The binding solution was loaded onto a HiTrap Streptavidin Affinity column (Amersham Pharmacia Biotech; column dimensions i.d. x h, 0.7 x 2.5 cm; column volume, 1 ml; binding capacity: 300 nmol biotin) previously washed with 10 ml of CHAPS 1% in buffer C and incubated overnight at 4°C with rotation (Marie et al., 1990). In a separate set of experiments, the affinity chromatography was performed with HiTrap Streptavidin affinity columns

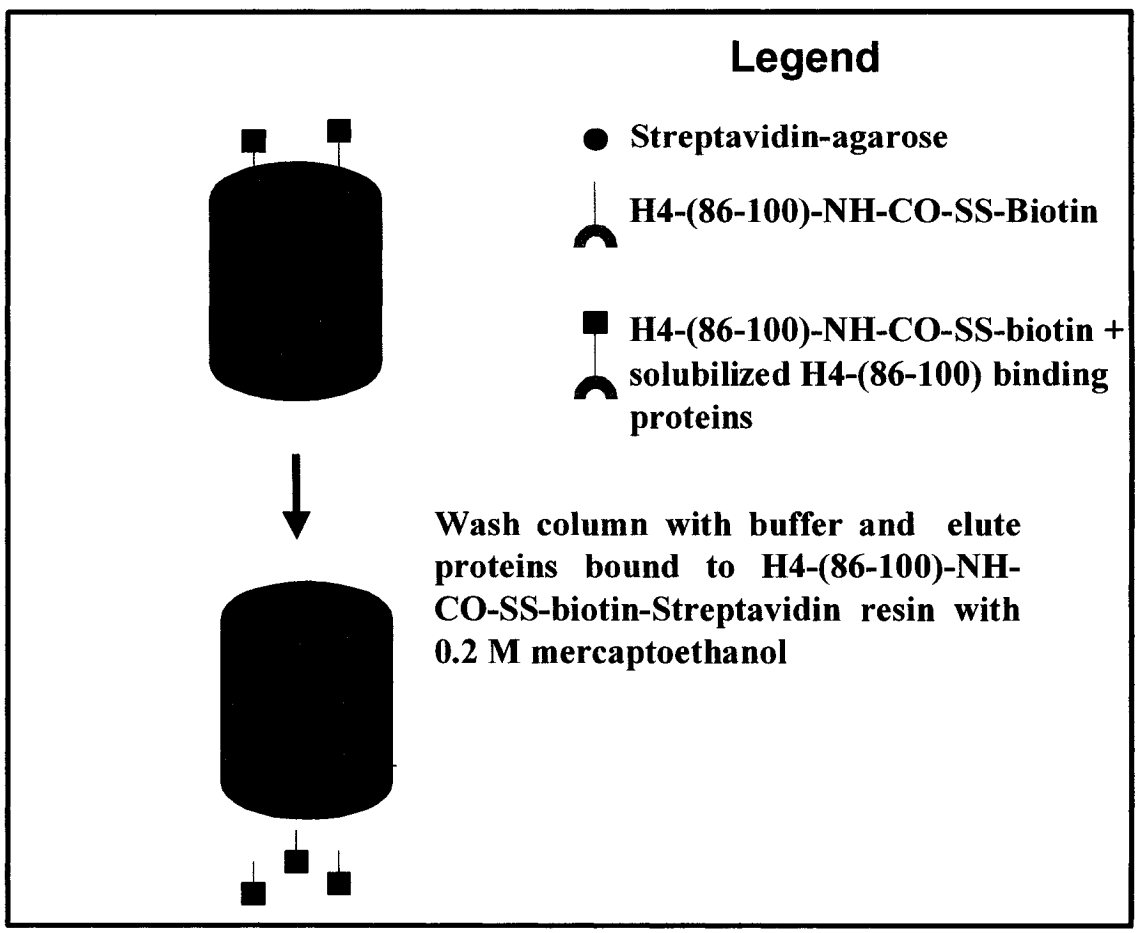
that had been preincubated for one hour with CHAPS 1% in buffer C in the presence (control) or absence (test) of a saturating amount (10 μmoles) of biotin. A second control set of experiments was realised by passing through HiTrap Streptavidin affinity columns membrane proteins that had been subjected to binding to H4-(86-100)-NH-CO-SS-biotin (10^{-7}M) in the absence (test) or presence (control) of a saturating concentration (10^{-4}M) of H4-(86-100).

B) The unbound material was first removed from the column by lavage with 50 ml of ice-cold (4°C) 1% CHAPS in buffer C.

D) The H4-(86-100) binding proteins were eluted with 10 X 0.5 ml fractions of 0.2 M mercaptoethanol and concentrated by passage through Microcon filter YM-30 before being subjected to electrophoresis on SDS gels (10% polyacrylamide) (Hagiwara et al., 1992).

FIGURE 5. Schematic representation of steps used for the purification of H4-(86-100) binding proteins in rat spleen membranes.

Solubilized membrane proteins (CHAPS) were preincubated with NH-SS-Biotin for 45 minutes at 4°C and loaded onto a HiTrap Streptavidin-agarose column for overnight incubation at 4°C. The column was washed with 1% CHAPS in buffer C and the S-S link between H4-(86-100) and biotin was broken with 0.2 M mercaptoethanol for the separation of the binding proteins associated to the ligand from the Streptavidin-biotin resin (Marie et al., 1990 and Hagiwara et al., 1992).


Legend
Streptavidin-agarose
H4-(86-100)-NH-CO-SS-Biotin
H4-(86-100)-NH-CO-SS-biotin + solubilized H4-(86-100) binding proteins
Wash column with buffer and elute proteins bound to H4-(86-100)-NH-CO-SS-biotin-Streptavidin resin with 0.2 M mercaptoethanol

3.13 Purification and detection of binding proteins by analytical gel electrophoresis

Parts (1/40) of the fractions eluted from the affinity column were denaturated by a 15 minutes incubation at 60°C in a buffer containing 50 mM Tris/HCl, pH 6.9, 5% mercaptoethanol, 10% glycerol (v/v), 2% SDS (w/v), and 0.1% bromophenol (w/v). The denatured material was first subjected to analytical electrophoresis on 10% polyacrylamide gel, at 150 volts for 2 hours. Electrophoresis was carried out in the presence of SDS and mercaptoethanol as described in the method of Laemmli (1970). The gel was fixed for 4 hours at room temperature in a mixture of ethanol:acetic acid:H_2O (30:10:60) with gentle shaking and rinsed twice with 20 ml of 30% ethanol for 30 minutes and twice with 20 ml of H_2O for 10 minutes. The gel was then emerged in a solution of silver nitrate 0.1% in H_2O for approximately 30 minutes and rinsed on both sides under a stream of deionised H_2O. The silver stain reaction was quenched by washing the gel in 1% acetic acid for a few minutes and then several times with H_2O. Positively stained protein bands were imaged on a flat-bed scanner (Hewlett Packard)

3.14 Preparative SDS gel isolation and sequencing of the major H4-(86-100) binding protein

The fraction eluted from the affinity column that contained H4-(86-100) binding proteins were concentrated by filtration through Microcon YM-30 (exclusion size $\leq$ 30 kDa) and separated by electrophoresis on a preparative polyacrylamide (10%) SDS gel (dimensions; 16 cm X 16 cm, W X L) according to

the method of Laemmli (1970). The electrophoresis was run for 6 hours at 100 volts. The polyacrylamide gel was fixed and stained with 0.25% Coomassie brilliant blue in a mixture of 45% methanol/10% acid acetic for 30 minutes and distained overnight with the same solution without the Coomassie brilliant blue. The 54 kDa protein band was cut out from the gel and sent to Service Proteomique de l'Est du Quebec for sequence identification. The samples were subjected to tryptic digestion, followed by mass spectrometry on Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) for first glance identification. The first glance identification on mass spectrometry provided molecular weights of tryptic fragments. The tryptic digest was also subjected to HPLC for separation of the peptides prior to their separate identification by mass spectrometry.

3.15 Data analysis

The equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) were generated from Scatchard plot analysis using the curve-fitting computer program GraphPad PRISM (GraphPad Software, Inc, San Diego CA, USA). The inhibition constant (K_i) was calculated according to the equation: $K_i = IC_{50}/(1+[L]/K_d)$, wherein [L] is the concentration of [^{125}I]H4-(86-100) and K_d its equilibrium dissociation constant (Cheung and Prusoff, 1973). All values for statistical significance in inhibiting the binding of [^{125}I]H4-(86-100) (Table 1, Figure 6B, 11 and 14B) or [*Leucyl*-^{3}H]Nociceptin (Figure 9) were determined using one-way ANOVA analysis of variance followed by Dunnett's comparison test. Statistical significance of changes in cAMP levels indicated by peptides or

forskolin (Figure 13) was determined using one-way analysis of variance followed by Bonferonni comparison test. All data represent the mean ± Standard Error (S.E) of three experiments conducted in duplicate.

4. RESULTS

4.1 Protein dependency of [^{125}I]H4-(86-100) binding

Alveolar MA membrane preparations from male Wistar rats were used for the characterization of [^{125}I]H4-(86-100) binding. The binding increased linearly with respect to protein up to a concentration of 0.04 mg/ml (Figure 6A). At higher membrane concentrations, the binding was no longer linear. A concentration of 0.03 mg/ml (e.g. 15 μg/ assay) was used in all subsequent binding tests in order to measure the maximal amount of specific binding in the linear (or valid) portion of the binding curve. The background in this test was measured in the presence of a saturating (10^{-5}M) concentration of H4-(86-100). The background increased linearly without forming a plateau at the higher concentrations of membrane proteins (not shown). It was subtracted from the total binding (binding performed in the absence of the cold ligand) to provide the specific binding as shown in figure 6A. The protein nature of the binding site was confirmed by experiments measuring the binding of [^{125}I]H4-(86-100) to rat alveolar MA membrane preparations pre-treated with trypsin (10 μg/mg of membrane protein; Figure 6B). Trypsin pre-treatment lowered the specific binding activity down to 5% of control. On the other hand, [^{125}I]H4-(86-100) binding to membranes pre-treated with heat-denatured trypsin plus soybean trypsin inhibitor represented 95% of that observed in membranes untreated with the proteolytic enzyme (control).

4.2 Time and temperature dependencies

The binding of [^{125}I]H4-(86-100) was found to be dependent upon the time and temperature of incubation (Figure 7). At 4°C, maximum binding was reached within 40-45 minutes and was slightly reduced after 60 minutes. Increasing the temperature to 22°C induced a reduction in maximal binding activity. Since protease inhibitors were present in the binding experiments, it is difficult to presume that the decrease in the binding activity was due to degradation of the peptide, although this possibility cannot be excluded. It is also possible that higher temperature favours a higher dissociation rate or changes the equilibrium between the percents of associated and dissociated peptide. Subsequent binding studies, except when indicated, were conducted at 4°C for 45 minutes.

4.3 Saturation binding activity

Saturation binding experiments with concentrations ranging from 0.10 to 32.0 nM of [^{125}I]H4-(86-100) revealed the presence of a high affinity binding site (Figure 8A). [^{125}I]H4-(86-100) binding displayed dependency upon the concentration of the radioligand and reached a plateau at saturating concentrations. Scatchard plot (Fig. 8A inset) analysis of the saturation-binding curve revealed a K_d of 12.3 ± 0.8 nM and B_{max} of 9.16 ± 0.32 pmol/mg protein. The shape of the Scatchard plot (linear curve) reveals the presence of a single site. Competition binding experiments (Figure 8B) with increasing concentrations of H4-(86-100) revealed a K_i of 77 ± 7.2 nM, indicating that the binding is reversible upon the addition of the unlabelled peptide. The K_i value represents

the concentration of the competing ligand that displaces at equilibrium half of the binding of the radiolabelled peptide (Cheung and Prusoff, 1973). The difference between the K_d and K_i values may indicate that the labelled peptide possesses a higher affinity than the unlabeled peptide for the receptor, consecutive to the theoretical addition of up to 4 molecules of iodine^{125} per molecule of H4-(86-100).

FIGURE 6.

(A) Specific binding of [^{125}I]H4-(86-100) (1 nM) to membrane preparations of rat alveolar macrophages as a function of protein concentration. [^{125}I]H4-(86-100) was incubated in the presence of increasing amounts of proteins (0.01 mg to 0.08 mg protein/ml) in 0.5 ml for 45 minutes at 4°C. Binding experiments were performed as described under "Materials and Methods" and each point represents the mean ± S.E. of three experiments conducted in duplicate.

(B) Sensitivity of [^{125}I]H4-(86-100) (1 nM) binding to treatment of the rat alveolar macrophage membrane preparations with trypsin. Membranes (0.015 mg) were treated with trypsin (10 μg/mg of proteins) or heat (100°C, 45 minutes)-denatured trypsin (10 μg/mg proteins) + soybean trypsin inhibitors for 30 minutes at 37°C before the binding assay. Trypsin was eliminated by membrane centrifugation, resuspension and recentrifugation prior to binding. Results are expressed as percent binding as compared with the control (no pre-treatment). The control binding value was 1992.3 ± 103.4 fmol [^{125}I]H4-(86-100) bound per mg of proteins. Each value represents the mean ± S.E. of three experiments conducted in duplicate. * $P < 0.001$ as compared with control.

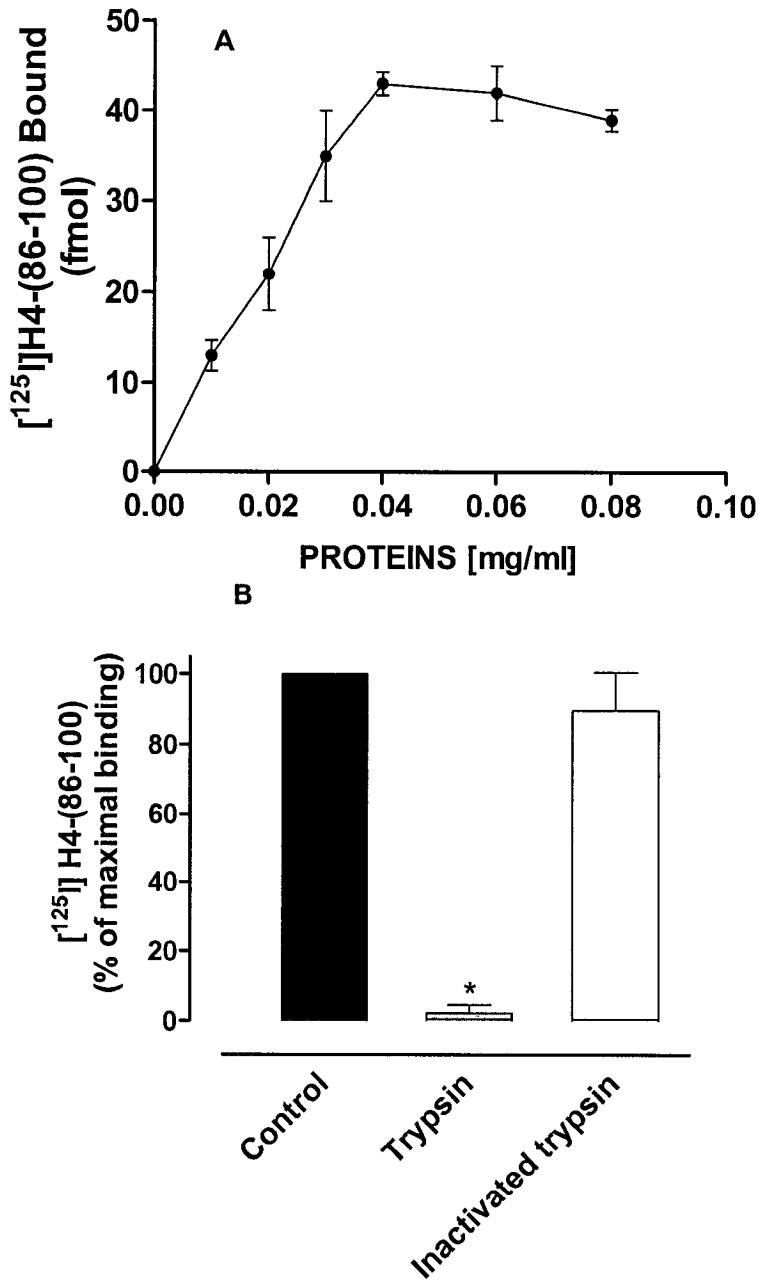

A
[125I]H4-(86-100) Bound (fmol)
50
40
30
20
10
0
0.00
0.02
0.04
0.06
0.08
0.10
PROTEINS [mg/ml]
B
[125I] H4-(86-100) (% of maximal binding)
100
80
60
40
20
0
*
Control
Trypsin
Inactivated trypsin

FIGURE 7. Time course specific association of [^{125}I]H4-(86-100) to rat alveolar macrophage membranes. [^{125}I]H4-(86-100) (1 nM) was incubated with membrane preparations (0.015 mg protein in a final volume of 0.5 ml) for the indicated time at 4°C and 22°C. Binding experiments were performed as described under "Materials and Methods" and each point represents the mean ± S.E. of three experiments conducted in duplicate.

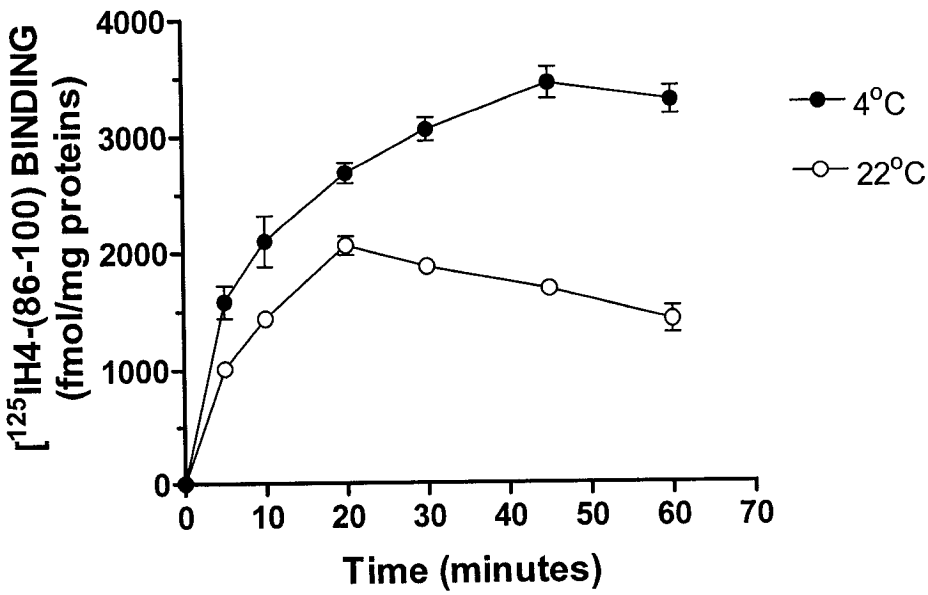
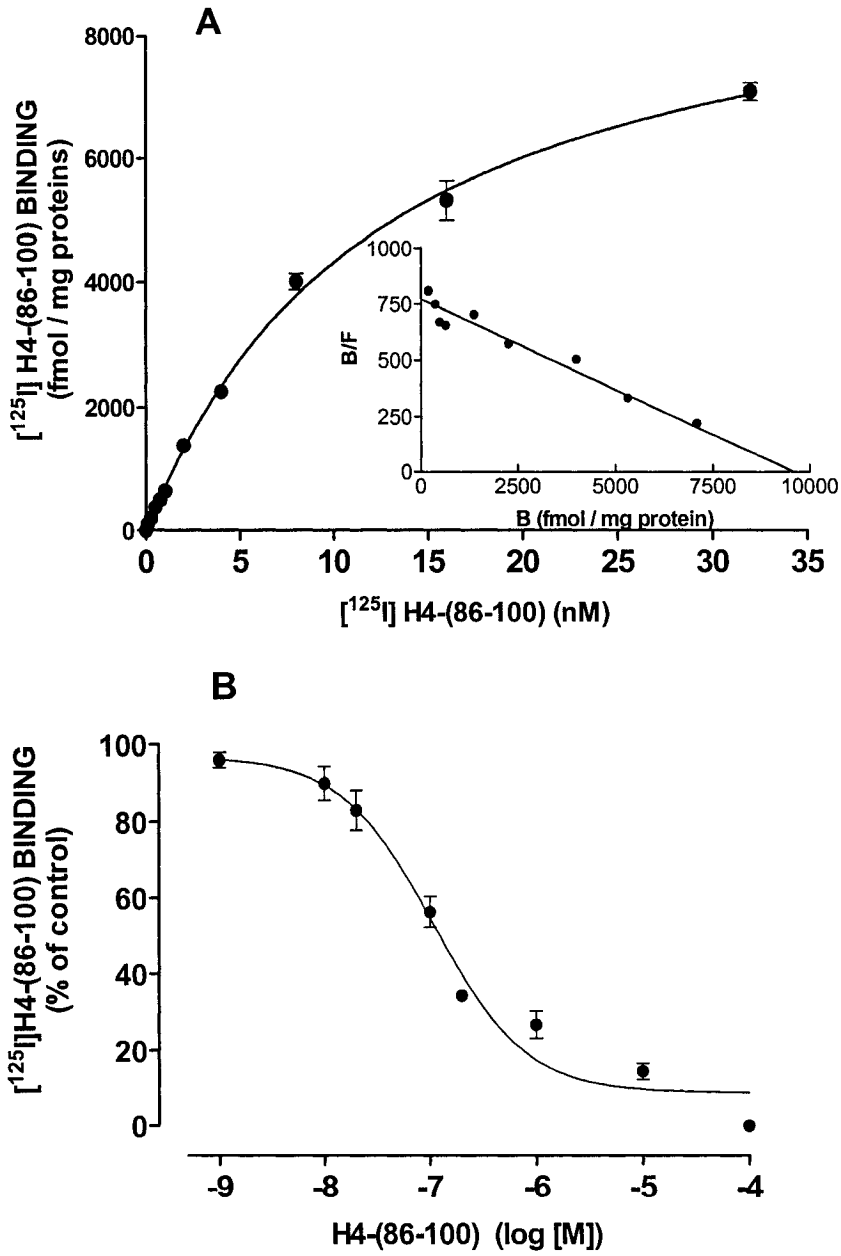

[125IH4-(86-100) BINDING
(fmol/mg proteins)
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
Time (minutes)
4°C
22°C

FIGURE 8.

(A) Saturation analysis and Scatchard plot (inset) of specific [^{125}I] H4-(86-100) binding to rat alveolar macrophage membrane preparations. Membrane preparations (0.015 mg protein in a final volume of 0.5 ml) were incubated in the presence of increasing concentration of [^{125}I] H4-(86-100) (0.1- 32.0 nM) at 4°C for 45 minutes in the presence or absence of unlabelled H4-(86-100) (20 μM).

(B) Competition of [^{125}I]H4-(86-100) binding to membranes of rat alveolar macrophages. Increasing concentrations of unlabelled H4-(86-100) were added to membrane preparations (0.015 mg protein in a final volume of 0.5 ml) for 45 minutes at 4°C to compete with the binding of [^{125}I]H4-(86-100) (1 nM). Each point represents the mean ± S.E. of three experiments conducted in duplicate.


A
8000
6000
4000
2000
0
[125I] H4-(86-100) BINDING
(fmol / mg proteins)
0
5
10
15
20
25
30
35
[125I] H4-(86-100) (nM)
1000
750
500
250
0
B/F
0
2500
5000
7500
10000
B (fmol / mg protein)
B
100
80
60
40
20
0
[125I]H4-(86-100) BINDING
(% of control)
-9
-8
-7
-6
-5
-4
H4-(86-100) (log [M])

4.4 Structure-activity relationships

H4-(86-100), HN, HN-related fragments and OGP (100 nM) were compared for their ability to inhibit the binding of the radiolabelled peptide to the alveolar MA membrane preparation (Table 1). The binding of [^{125}I]H4-(86-100) (1 nM) was inhibited by H4-(86-100), HN, HN-related peptides and OGP (10^{-7} M) according to the following order of potency: H4-(86-100) > [Ser^{1}]HN > HN-(7-15) > HN-(7-10) > HN-(2-15) > HN-(1-13) > HN-(1-10) > OGP. HN-(8-10) and HN-(6-9) at 10^{-5} M did not show any significant inhibition, suggesting that the minimal active core is HN-(7-10). Dynorphin A (1-13), SP, nociceptin at 10^{-7} and VIP at 10^{-6} M significantly inhibited the binding of [^{125}I]H4-(86-100). However, the opiate μ ligands morphine and naloxone, the κ ligand U-50488H and the δ ligand DSLET at 10^{-5}M did not affect the binding activity. The unrelated peptides may affect the binding by competing for the same specific site or may act on a distinct site on the receptor, therefore affecting the binding by an allosteric change in the conformation of the binding site. For instance, dynorphin has high affinity for multiple receptors, including μ, κ, δ opioid receptors as well as the non-opioid ORL_1 nociceptin receptor. The possibility that dynorphin interacts with a receptor specific for H4-(86-100) may be relevant to an intrinsic structural feature that adapts to the conformation of multiple peptide receptors. On the other hand, the possibility that H4-(86-100) interacts with the nociceptin receptor (opioid-like receptor-1; ORL_1) was also verified by measuring the ability of H4-(86-100) (10^{-5}M) to inhibit the binding of [^{3}H]nociceptin (0.75 nM) (Figure 9A). No significant binding inhibition was observed in the presence of H4-(86-100), suggesting that

at a concentration of 10^{-5}M, H4-(86-100) does not have specific effect on the binding. Finally, diethylenetriamine and spermidine, two polyamine agonists that potentiate the binding of NMDA ligands to the NMDA receptor, did not show any inhibition of [^{125}I]H4-(86-100) binding suggesting the non-involvement of the NMDA receptor.

4.5 Correlation between binding and analgesic activity

Recently, C-terminal histone H4 peptides were found to display non-opioid analgesic activity (Lemaire et al., 1997; Ruan et al., 2000). I.c.v. injection of these peptides in mice dose and structure-dependently blocked writhing induced by i.p. administration of acetic acid and tail-flick induced by radiant heat. Herein, the relative potencies of the peptides in inhibiting [^{125}I]H4-(86-100) binding to membrane preparations of rat alveolar MA were compared with their abilities to cause analgesia in the mouse writhing test (Ruan et al., 2000). A good correlation (r= 0.85; figure 10) was found between the binding and analgesic activities of the C-terminal histone H4 peptides.

4.6 Effects of guanine nucleotides and pertussis toxins

The possibility that the H4-(86-100) receptor is coupled to a G protein, more particularly G_i, was also explored by evaluating the effect of guanine nucleotides and PTX membrane treatment on [^{125}I]H4-(86-100) binding (Figure 11). This study shows that the binding of [^{125}I]H4-(86-100) (1 nM) is significantly inhibited by Gpp(NH)p (100 μM), GTP-γ-S (100 μM) and Gpp(NH)p (30 μM) (Figure 11A). However, both GDP-β-S (30 and 100 μM) and ATP (100 μM) did not

affect the binding activity, indicating that the inhibition was specific to guanine triphosphate derivatives. In order to determine if the receptor could be coupled to a G_i protein, membranes were pre-treated with the active (A) and inactive (B) forms of PTX (Figure 11B). Pre-treatment of the membranes (100 μg) with 0.25 μg PTX-A decreased the binding to 30% of control value while pre-treatment with PTX- B (1.0 μg) did not affect the binding activity.

4.7 Effects of monovalent and divalent cations

Cations are known to exert important influences on the binding of agonists to GPCRs (Webster and Souza, 1988). Mg^{2+} and Ca^{2+} enhance the binding of agonists to GPCRs by interacting on a site of the G-protein that stabilizes the receptor-ligand complex (Webster and Souza, 1988). On the other hand, Na^{+} is well known to decrease the affinity of agonists for GPCRs (Ardati et al., 1997; Makman and Dvorkin, 1997). In order to determine the influence of these salts on [^{125}I]H4-(86-100) binding to rat alveolar MA membranes, binding was achieved in the presence of various concentrations of divalent (Mg^{2+}, Ca^{2+}) and monovalent (Na^{+}) cations (Figure 12). Herein, we observed that both Mg^{2+} and Ca^{2+} (0.1-10 mM) increase the binding of [^{125}I]H4-(86-100). [^{125}I]H4-(86-100) binding was also carried out in the presence of increasing concentrations of NaCl (Figure 12). A significant concentration-dependent decrease in the binding of [^{125}I]H4-(86-100) was observed at 10 and 120 mM NaCl.

TABLE 1. Capacity of H4-(86-100), HN and related and unrelated compounds in inhibiting the binding of [^{125}I] H4-(86-100) (1 nM) to membranes of rat alveolar macrophages.

Compound[1]	[^{125}I] H4-(86-100) bound (%)[2]	Inhibition (%)
HN related peptides:		
Control	100.3 ± 1.2	0
H4-(86-100)	36.3 ± 2.7*	64
[Ser^{1}]HN	43.7 ± 0.3*	56
HN (7-15)	42.0 ± 6.6*	58
HN (7-10)	54.0 ± 9.0*	46
HN (2-15)	58.3 ± 7.8*	42
HN (1-13)	62.3 ± 3.4*	38
HN (1-10)	65.0 ± 6.2*	35
HN (8-10)	91.0 ± 2.6	9
HN (6-9)	98.0 ± 2.9	2
OGP	69.5 ± 7.5*	30
Unrelated peptides:		
Dynorphin A (1-13)[3]	43.3 ± 6.4*	57
Nociceptin[4]	52.7 ± 7.4*	47
SP[5]	60.7 ± 8.2*	39
VIP[6]	71.0 ± 3.6*	29
Neurotensin	98.3 ± 3.4	2
Opioid ligands:		
Naloxone[7]	96.3 ± 2.4	4
Morphine[8]	98.7 ± 1.2	1
U-50488H[9]	104.7 ± 2.7	0
DSLET[10]	104.0 ± 6.1	0
Polyamines:		
Diethylenetriamine[11]	101.3 ± 4.8	0
Spermidine[11]	97.7 ± 6.2	2

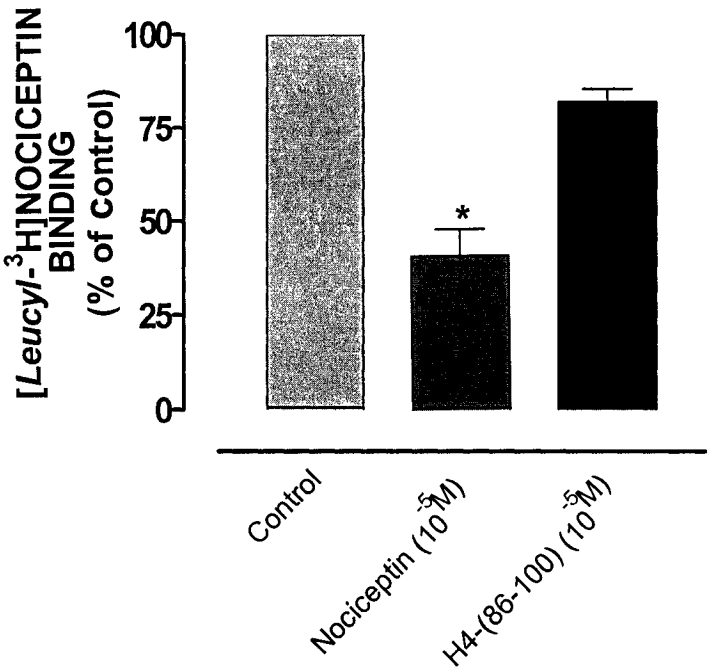
[1] All compounds were tested at a concentration of 10^{-7} M, except for HN-(8-10) (10^{-5} M), HN-(6-9) (10^{-5} M), neurotensin (10^{-5} M), VIP (10^{-6} M), opioid ligands (10^{-5} M) and polyamines (10^{-5} M).
[2] Each value represents the mean ± S.E. of three experiments conducted in duplicate. Statistical significance was determined by using one-way analysis of variance followed by Dunnet comparison test (* $P < 0.05$) as compared with control.[3] Dynorphin, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, is a high affinity ligand for opioid kappa, mu and delta receptors as well as for the non-opioid ORL_1 nociceptin receptor.[4] Nociceptin, Phe-Gly-Gly-Phe-Thr-Gly-Ala-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln, has a high affinity for the ORL_1 receptor. [5] Substance P, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met,binds to neurokinin 1 (NK1) and tachykinin receptors. [6] Vasoactive intestinal peptide (VIP), His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Met-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn, binds to VPAC and PAPAC receptors. [7] The non selective opiate antagonist naloxone has a high binding affinity for all opioid receptors.[8] Morphine has a high binding affinity for the mu opioid receptor. [9, 10 and 11] These compounds are selective ligands for opioid kappa and delta receptors and the polyamine binding site of the NMDA receptor complex, respectively.

FIGURE 9. Binding of [*Leucyl*-^{3}H]Nociceptin to membrane preparations of rat alveolar macrophages.

(A) Effects of H4-(86-100) (10 μM) and nociceptin (10 μM) on the binding of [*Leucyl*-^{3}H]Nociceptin to membrane preparations of rat alveolar macrophages (0.015 mg protein in a final volume of 0.5 ml).

(B) Effect of IFNγ on [*Leucyl*-^{3}H]Nociceptin binding to rat alveolar macrophage membrane preparations. Macrophages were incubated 24 hours at 37°C in the absence (Control) or presence of INFγ (100 U/ml). Cell membrane preparations were made from harvested cells and [*Leucyl*-^{3}H]Nociceptin binding experiments were performed as described under "Materials and Methods". The control binding value was 489 ± 36.4 fmol [*Leucyl*-^{3}H]Nociceptin bound per mg of proteins. Each value represents the mean ± S.E. of three experiments conducted in duplicate. * $P < 0.001$ as compared with control.

A)


[Leucyl-3H]NOCICEPTIN BINDING (% of Control)
100
75
50
25
0
*
Control
Nociceptin (10-5M)
H4-(86-100) (10-5M)

B)

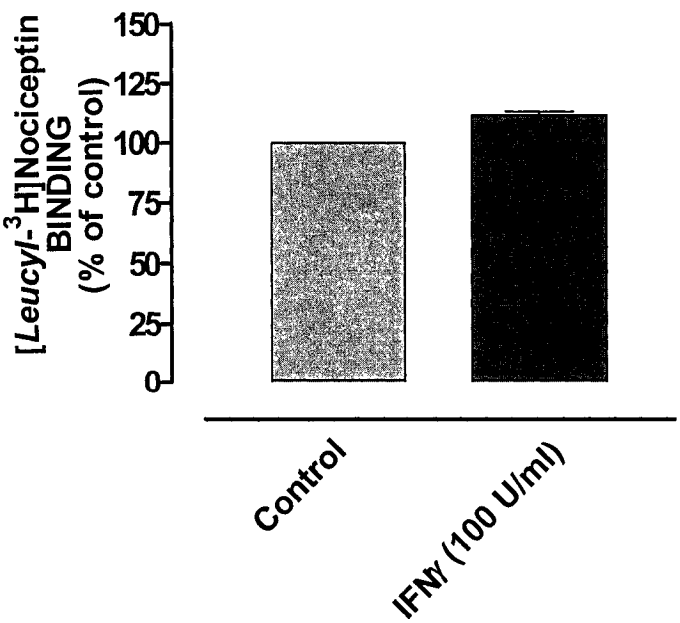

[Leucyl-3H]Nociceptin BINDING (% of control)
150
125
100
75
50
25
0
Control
IFNγ (100 U/ml)

FIGURE 10. Correlation between the binding and analgesic activities of C-terminal histone H4 peptides. The relative potencies of the peptides (10^{-7}M) in inhibiting [^{125}I]H4-(86-100) binding in membrane preparations of rat alveolar macrophages (Table 1) was compared with their abilities to cause analgesia in the mouse writhing test (10 nmol/mouse, i.c.v; Ruan et al., 2000).

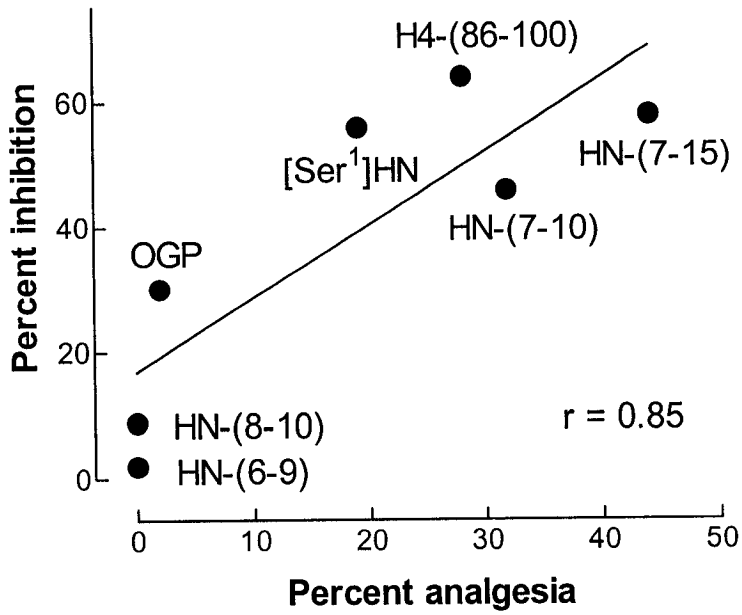

H4-(86-100)
[Ser1]HN
HN-(7-15)
HN-(7-10)
OGP
HN-(8-10)
HN-(6-9)
r = 0.85
Percent inhibition
Percent analgesia
0
20
40
60
0
10
20
30
40
50

FIGURE 11. Inhibition of [^{125}I]H4-(86-100) binding by

(A) Non-hydrolyzable GTP analogs, GTP-γ-S and Gpp(NH)p (30 and 100 μM as indicated).

(B) Effect of PTX-A pre-treatment on the binding of [^{125}I]H4-(86-100) to rat alveolar MA membranes. Binding experiments were performed with PTX-A (0.25 μg/100 μg) pre-treated (30 minutes, 30°C) membranes in the presence of 1 nM [^{125}I]H4-(86-100) in a final volume of 0.5 ml at 22°C for 20 minutes in the presence (background) or absence (total binding) of 20 μM unlabelled peptide as described under "Materials and Methods". The control binding value was 3183.6 ± 299.4 fmol [^{125}I]H4-(86-100) bound per mg of proteins. Each point represents the mean ± S.E. of three experiments conducted in duplicate. * $P < 0.001$ as compared with control.

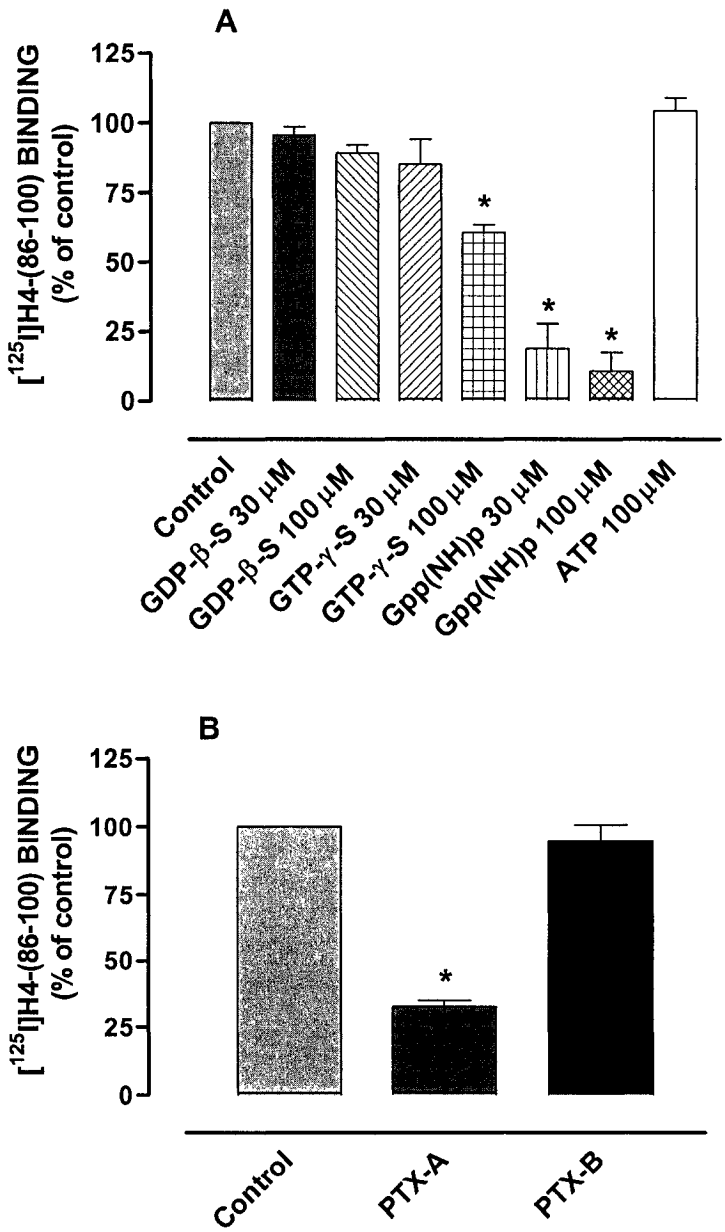
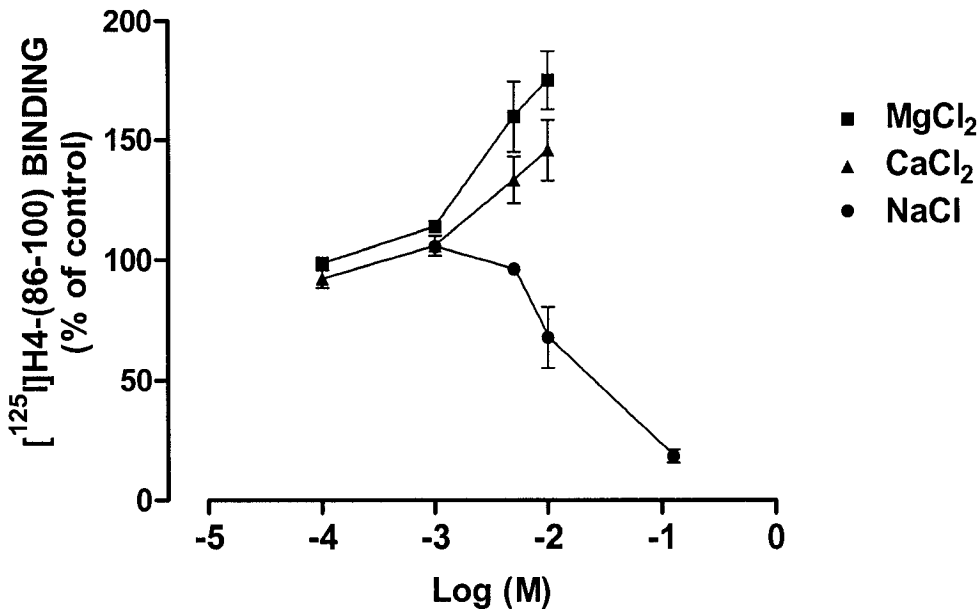

A
[125I]H4-(86-100) BINDING (% of control)
125
100
75
50
25
0
*
*
*
Control
GDP-β-S 30 μM
GDP-β-S 100 μM
GTP-γ-S 30 μM
GTP-γ-S 100 μM
Gpp(NH)p 30 μM
Gpp(NH)p 100 μM
ATP 100 μM
B
[125I]H4-(86-100) BINDING (% of control)
125
100
75
50
25
0
*
Control
PTX-A
PTX-B

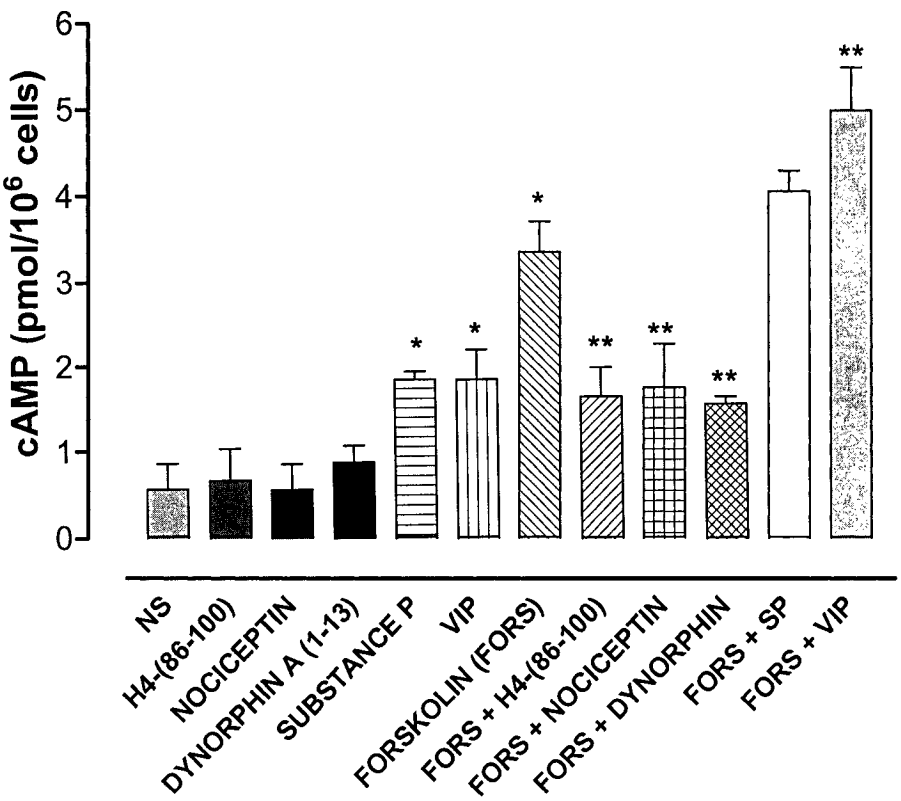
FIGURE 12. Modulation of [^{125}I]H4-(86-100) binding to rat alveolar MA membrane preparations by $MgCl_2$, $CaCl_2$ and NaCl. Binding experiments were performed on rat alveolar MA membrane preparations (0.015 mg protein in a final volume of 0.5 ml) in the presence of peptidase inhibitors at 22°C for 20 minutes with the indicated concentrations of the cations as described under "Materials and Methods". The control binding value made in the absence of salts was 2789.0 ± 187.1 fmol [^{125}I]H4-(86-100) bound per mg of protein. Each point represents the mean ± S.E. of three experiments conducted in duplicate.


[125I]H4-(86-100) BINDING
(% of control)
200
150
100
50
0
-5
-4
-3
-2
-1
0
Log (M)
MgCl2
CaCl2
NaCl

4.8 Modulatory effects of H4-(86-100) on cAMP levels: comparison with other peptides

After determining that H4-(86-100) could interact with G_iPCR in alveolar MA membranes, *in vitro* bioassays were performed to verify if the specific H4-(86-100) membrane receptor could be functionally linked to a G_i protein, thus modulating the production of a cell signal transducer such as cAMP. Therefore, H4-(86-100) was tested for its ability to modulate alveolar MA cAMP levels in the presence or absence of forskolin (10^{-8} M) and compared with nociceptin, dynorphin A (1-13), SP and VIP (Figure 13). H4-(86-100) (10^{-8} M), nociceptin (10^{-8} M) and dynorphin A-(1-13) (10^{-8} M) did not affect basal cAMP levels, whereas VIP (10^{-8} M) and SP (10^{-8} M) significantly increased the basal levels of cAMP. Forskolin alone, whose role is to activate AC without going through the signalling cascade of receptor stimulation, evoked a 6-fold increase in cAMP levels (from 0.56 to 3.36 pmol/10^6 cells). The addition of H4-(86-100) (10^{-8} M) caused a 50% inhibition in the forskolin-evoked stimulation of cAMP levels. Nociceptin and dynorphin A-(1-13) at 10^{-8} M produced similar inhibitions of the forskolin-evoked stimulation. SP (10^{-8} M) did not show any significant effect on the forskolin-evoked stimulation of cAMP whereas VIP (10^{-8} M) potentiated the effect of forskolin.

FIGURE 13. Effects of H4-(86-100), nociceptin, dynorphin A-(1-13), SP and VIP on basal and forskolin-stimulated levels of cAMP in cultured rat alveolar macrophages. Rat alveolar macrophages were stimulated for 30 minutes in the presence or absence of H4-(86-100) (10^{-8}M), nociceptin (10^{-8}M), dynorphin A-(1-13) (10^{-8}M), SP (10^{-8}M), VIP (10^{-8}M), forskolin (10^{-8}M) and forskolin coincubated with each of the peptides. After stimulation, intracellular cAMP was extracted and quantified as described under "Materials and Methods". Results are expressed as pmol cAMP/10^6 cells. Each value represents the mean ± S.E. of three experiments performed in duplicate. Statistical significance was determined by using one-way analysis of variance followed by Bonferonni comparison test. (*$P \leq 0.05$ indicates significance as compared with non-stimulated cells; ** $P \leq 0.05$ indicates significance as compared with cells stimulated with forskolin).


cAMP (pmol/10^6 cells)
0
1
2
3
4
5
6
*
*
*
**
**
**
**
NS
H4-(86-100)
NOCICEPTIN
DYNORPHIN A (1-13)
SUBSTANCE P
VIP
FORSKOLIN (FORS)
FORS + H4-(86-100)
FORS + NOCICEPTIN
FORS + DYNORPHIN
FORS + SP
FORS + VIP

4.9 Autoradiography and effect of LPS and IFNγ on cross-linked binding protein

SDS gel electrophoresis of the membrane protein cross-linked to [^{125}I]H4-(86-100) by affinity labelling in the presence of DSS revealed a major band of approximately 54 kDa and a low intensity band of approximately 30 kDa (Figure 14A1). Addition of unlabelled H4-(86-100) (20 μM) in the binding medium markedly decreased the intensity of both bands (Figure 14A2). In order to verify if the receptor was modulated by macrophage stimulating conditions, macrophages were cultured in the presence or absence of LPS (1 μg/ml) or IFNγ (100 U/ml) for 24 h and measurements of the binding activity of [^{125}I]H4-(86-100) on isolated membranes were performed by standard membrane filtration procedure (Figure 14B, lower panel) and autoradiography of cross-linked binding proteins (Figure 14B, upper panel). Pre-treatment of the cells with IFNγ, but not LPS, doubled the standard binding activity. Autoradiography of the cross-linked binding proteins revealed that the IFNγ-induced binding activity corresponded to a rise in the intensity of both 54 and 30 KDa binding proteins (Figure 14B, upper panel). Binding of [L*eucyl*-^{3}H]Nociceptin in presence and absence of IFNγ was also verified (Figure 9B). No statistical difference was found between control and INFγ stimulated assays suggesting that H4-(86-100) binding sites are not the ORL_1 receptor.

The binding capacity of different C-terminal histone H4 related peptides including H4-(86-100), [Ser^{1}]HN and OGP were tested with the cross-linking technique. As shown in figure 15A, the binding capacities of [^{125}I]H4-(86-100) to

the 54 and 30 kDa binding proteins were much higher than those of [Ser^{1}]HN and OGP. The 54 kDa protein was the major binding protein in all control and IFNγ-stimulated binding assays. Therefore, our next experiments were aimed at isolating and sequencing this binding protein. Since the binding material that could be extracted from alveolar MA membrane preparations was relatively small, we looked for a more important source of binding material. Preliminary experiments in our laboratory indicated that rat spleen showed highest [^{125}I]H4-(86-100) binding activity among various tissues (Poirier et al., 2000). The presence of the 54 kDa binding protein in a spleen membrane preparation was confirmed by cross-linking autoradiography (Figure 15B). Therefore, the spleen membranes were then used for the isolation and sequence identification of the major (54 kDa) H4-(86-100) binding protein. In order to verify if the solubilized proteins retain their binding properties, [^{125}I]H4-(86-100) was cross-linked to the bound solubilized proteins. Figure 15C shows that the autoradiography of the binding proteins in solubilized spleen membranes (major band, 54 kDa; minor band, 30 kDa) corresponds to that observed with macrophage membranes (figure 14A).

FIGURE 14. Binding and cross-linking of [^{125}I]H4-(86-100) to membrane preparations of rat alveolar macrophages.

(A) Membrane preparations were incubated with 1 nM [^{125}I]H4-(86-100) for 45 minutes at 4°C in the absence (1) or presence (2) of 20 μM of H4-(86-100). Membrane pellets were washed twice with ice cold buffer (50 mM Tris-HCl, pH 7.4) before the addition of 300 μl of 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM of DSS (final concentration). Thereafter, the membranes were rinsed and centrifuged with 1 ml of 50 mM Tris-HCl buffer, pH 7.4, the pellets were resuspended in 50 mM Tris-HCl, pH 7.4 containing 2 % SDS. The denatured proteins (80 μg) were subjected to electrophoresis on 10% SDS-PAGE gel and visualized by autoradiography. The arrows show the 54 kDa and 30 kDa binding proteins.

(B) Macrophages were incubated 20 hours at 37°C in the absence (Control) or presence of LPS (1 μg/ml) or INFγ (100 U/ml). Cell membrane preparations were made from harvested cells and binding experiments were performed as described under "Materials and Methods". Each value represents the mean ± S.E. of three experiments conducted in duplicate. * $P < 0.05$ as compared with control. The upper panel is one representative cross-linking experiment showing the autoradiography of the bound proteins, the 54 and 30 kDa bands representing the major proteins that bind to the ligands.

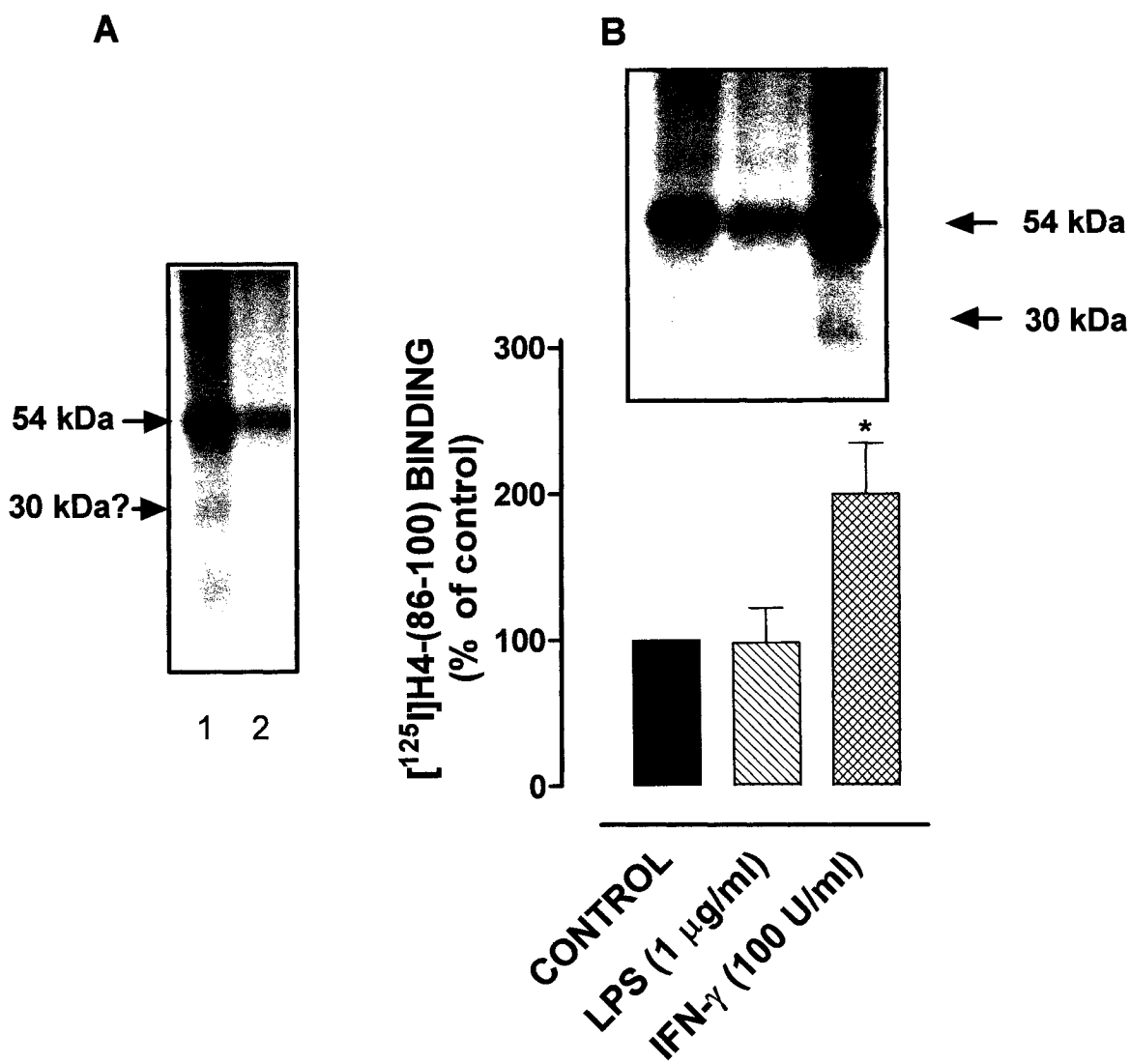
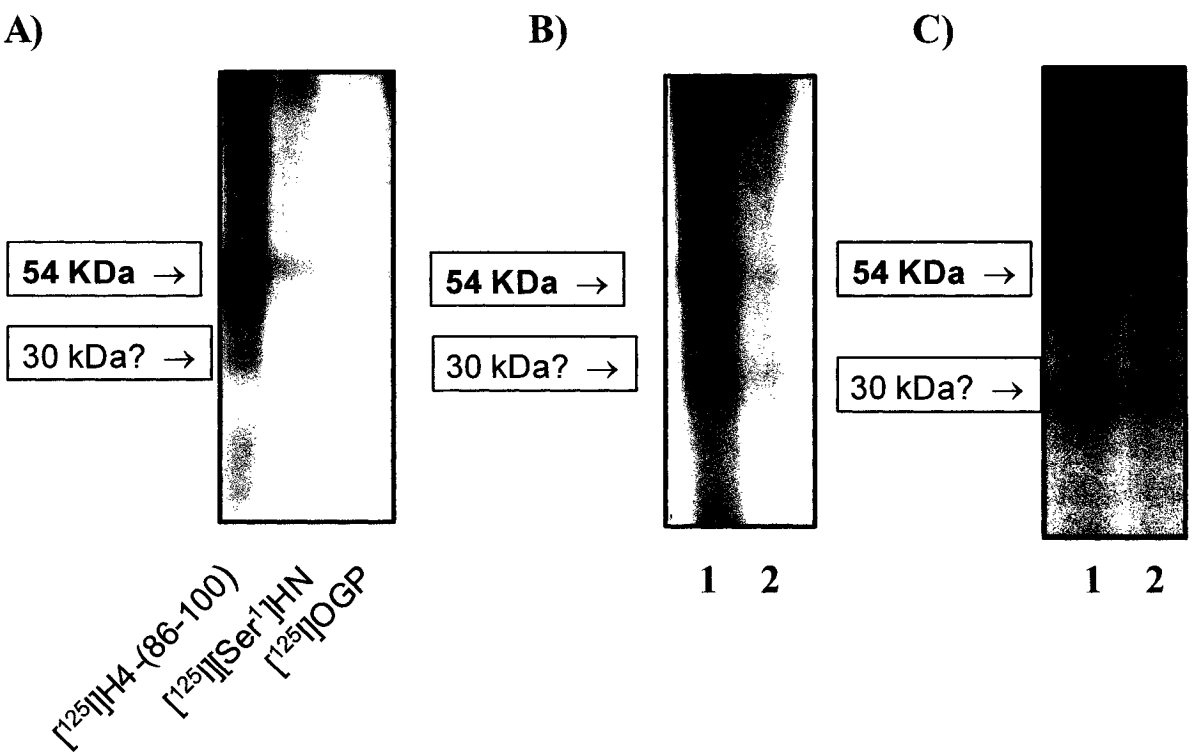

A
54 kDa
30 kDa?
1 2
B
54 kDa
30 kDa
[125I]H4-(86-100) BINDING
(% of control)
300
200
100
0
*
CONTROL
LPS (1 μg/ml)
IFN-γ (100 U/ml)

FIGURE 15. A) Cross-linking of [^{125}I]H4(86-100), [^{125}I][Ser^{1}]HN and [^{125}I]OGP to membrane preparations of rat alveolar macrophages. Membrane preparations were incubated with 1 nM [^{125}I]H4-(86-100), [^{125}I][Ser^{1}]HN or [^{125}I]OGP for 45 minutes at 4°C. Membrane pellets were washed twice with ice cold buffer (50 mM Tris-HCl, pH 7.4) before the addition of 300 μl of 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM of DSS (final concentration). Thereafter, the membranes were rinsed and centrifuged with 1 ml of 50 mM Tris-HCl buffer, pH 7.4. The pellets were resuspended in 50 mM Tris-HCl, pH 7.4, containing 2 % SDS and the denatured proteins (80 μg) were subjected to electrophoresis on 10% SDS-PAGE gel and visualized by autoradiography. The arrows show the 54 kDa and 30 kDa binding proteins.

B) C) Cross-linking of [^{125}I]H4(86-100) to membrane preparations of rat spleen (B) or solubilized receptors (C). Membrane preparations or solubilized receptors were incubated with 1 nM [^{125}I]H4-(86-100) for 45 minutes at 4°C in the absence (1) or presence (2) of 20 μM of H4-(86-100). Membrane pellets were washed twice with ice cold buffer (50 mM Tris-HCl, pH 7.4) before the addition of 300 μl of 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM of DSS (final concentration). The membranes or solubilized receptors were rinsed and centrifuged with 1 ml of 50 mM Tris-HCl buffer, pH 7.4. The pellets or solubilized receptor were resuspended in 50 mM Tris-HCl, pH 7.4, containing 2 % SDS and the denatured proteins (80 μg) were subjected to electrophoresis on 10% SDS-PAGE gel and visualized by autoradiography. The arrows show the 54 kDa and 30 kDa binding proteins. (See materials and methods for cross-linking on solubilized receptors).


A)
B)
C)
54 KDa →
30 kDa? →
54 KDa →
30 kDa? →
54 KDa →
30 kDa? →
1 2
1 2
[125I]H4-(86-100)
[125I][Ser1]HN
[125I]OGP

4.10 Purification and identification of the 54 kDa H4-(86-100) binding protein from rat spleen membranes.

Different concentrations (0.1 to 1%) of CHAPS were used to solubilized proteins from rat spleen membranes prior to the determination of [^{125}I]H4(86-100) binding activity to the solubilized material. 1% of CHAPS was shown to be the optimal concentration for the protection of binding activity. Therefore, rat spleen membrane preparations were solubilized with CHAPS 1% and the solubilized proteins were purified by affinity chromatography and electrophoresis on SDS gels. The strategy of the affinity chromatography consisted in binding H4-(86-100)-NH-CO-SS-biotin to solubilized membrane proteins prior to incubation with a Streptavidin-agarose resin. The binding proteins were eluted from the resin with 0.5 ml fractions of 0.2 M mercaptoethanol and separated by electrophoresis on SDS gels. Figure 16 illustrates the profile of elution of the proteins from the affinity column, fractions 1 to 3 containing the majority of the protein material. This material was concentrated by filtration through Microcon YM-30 filters and subjected to electrophoresis on a preparative SDS-PAGE gel as described under "Materials and Methods". After the electrophoresis, the gel was stained directly with Coomassie brilliant blue and the predominant band was cut out from the gel and sent for sequence identification to the Service Proteomique de l'Est du Quebec thinking that it was a specific H4-(86-100) binding protein. Figure 17A shows the mass spectrometry profile of a tryptic digest of the protein. The profile was subjected to a data bank of tryptic protein digests for identification and five peaks were clearly identified as tryptic digestion products of β-actin (see Figure

17A). The whole tryptic digest was thereafter subjected to chromatography on HPLC (Figure 17B). The major HPLC elution peaks had a molecular weight that corresponded to those already identified in the whole tryptic digest, demonstrating that the major band is β-actin. In separate sets of experiments, the affinity chromatography was performed on streptavidin-columns that had been preincubated with the medium in the absence (test) or presence (control) of a saturating concentrations of biotin (10 μmoles; figure 18A) or with a solubilized membrane protein preparation that had been subjected to reaction with the affinity ligand (H4-(86-100)-NH-CO-SS-biotin; 10^{-7} M) in the absence (test) or presence (control) of H4-(86-100) (10^{-4}M; figure 18B). SDS gel electrophoresis of the test assays reveal protein patterns (silver stain detection) similar to that observed in figure 16. In control experiments, the β-actin protein band suffered little or no decrease as compared with test assays, whereas protein bands of approximate M.W. of 54 and 30 kDa were markedly decreased as compared with test assays (figure 18). A decrease in the intensity of a protein band in the test assays is relevant to conditions in which the saturating concentrations of biotin (figure 18A) or H4-(86-100) (figure 18B) displace the binding of Biotyl-SS-H4-(86-100) to the affinity resin or to the receptor, respectively, resulting in the inability of the affinity column to separate the receptor. Therefore, in the affinity chromatography protein patterns, the two proteins (54 and 30 kDa) that are decreased under the control conditions are more likely to be specific binding sites for H4-(86-100).

FIGURE 16. Affinity chromatography elution profile of H4-(86-100) binding proteins solubilized from rat pleen membranes as illustrated by electrophoresis on SDS-PAGE gels. The solubilized H4-(86-100)-biotin binding proteins retained on the streptavidin-agarose resin was eluted with 0.5 ml fractions of 0.2 M mercaptoethanol as described under "Materials and Methods" and samples (5 μl out of 200 μl) were electrophoresed on analytical SDS-PAGE. The major protein bands (54 and 30 kDa; silver stain detection) were present in the first three fractions. These fractions were concentrated by filtration (filters ≤ 30 kDa, Ultracel-YM regenerated cellulose membrane, Millipore) and separated by electrophoresis on preparative SDS-PAGE gels.

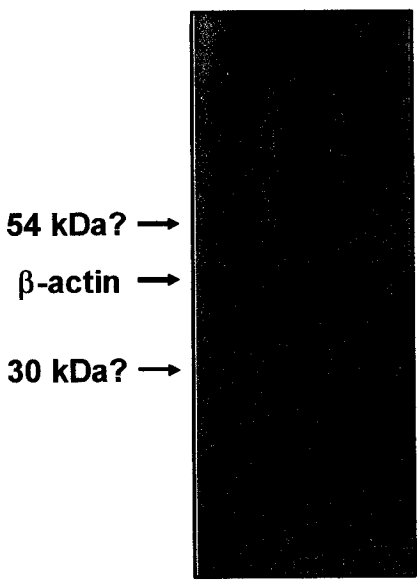
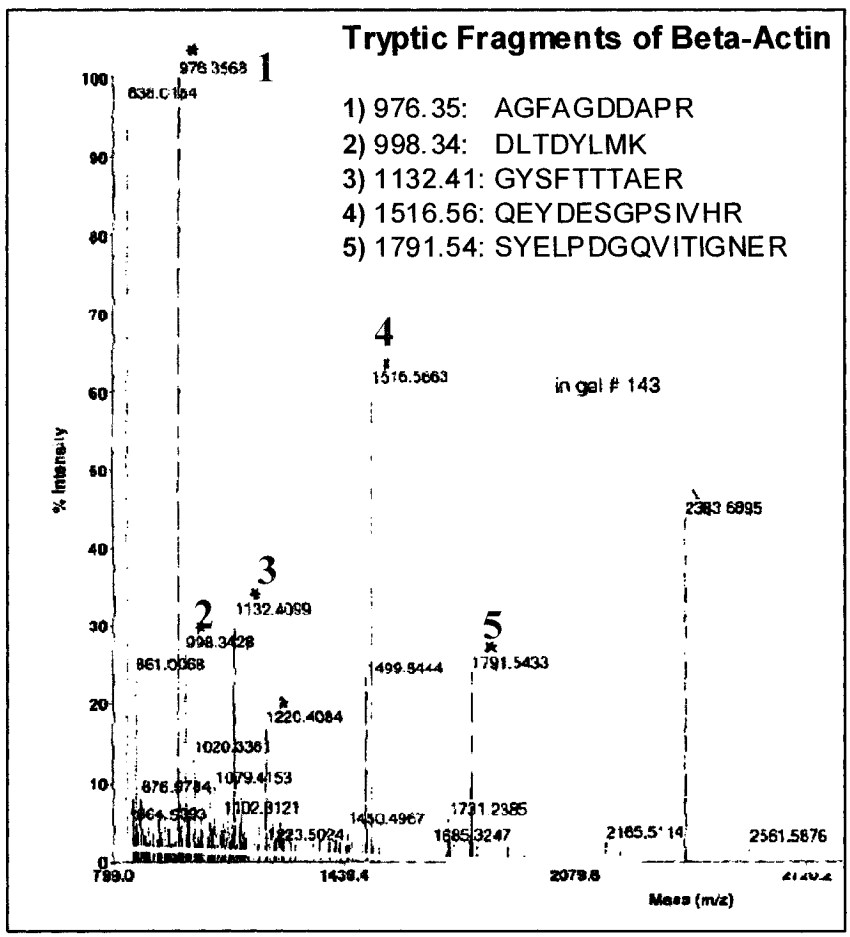

54 kDa?
β-actin
30 kDa?
1 2 3

FIGURE 17.

A) Mass spectrometry profile of a tryptic digest of the major protein band extracted from the preparative SDS-PAGE gel (figure 16). The major protein band was extracted from the SDS-PAGE gel as described under "Materials and Methods" and sent for analysis to Service Proteomique de l'Est du Quebec, Centre de recherche du CHUL, St-Foy, Quebec. The sample was subjected to tryptic digestion and then submitted to MALDI-TOF spectrometry to determine the molecular mass of resulting peptides. The indicated peaks 1 to 5 have M.W. that correspond to those of a data bank of β-Actin tryptic digests. Ten other bands were also identified as belonging to a β-Actin tryptic digest (not shown here).

B) HPLC elution profile of the tryptic digest of the major protein band extracted from the preparative SDS-PAGE gel (figure 16). The tryptic digest was also analyzed by HPLC followed by mass spectrometry on selected peptide peaks. Many of the peaks that were isolated on the HPLC were identified as peptides of β-actin digestion. Among the peaks that were identified as tryptic digest of β-actin, some of them corresponded to those listed in section A.

A)


Tryptic Fragments of Beta-Actin
1) 976.35: AGFAGDDAPR
2) 998.34: DLTDYLMK
3) 1132.41: GYSFTTTAER
4) 1516.56: QEYDESGPSIVHR
5) 1791.54: SYELPDGQVITIGNER
in gel # 143
% Intensity
Mass (m/z)
976.3568
1516.5663
1132.4099
998.3428
1791.5433
2383.6895
2561.5876

B)

1) 37.5 min = 795.0: IIAPPER
2) 43.8 min = Peak #3, Figure 15A
3) 42.0 min = Peak #4, Figure 15A
4) 49.1 min = 1161.5: EITALAPSTMK
5) 63.1 min = Peak # 5, Figure 15A
6) 65.7 min = 2215.9: DLYANTVLSGGTTMYPGIADR

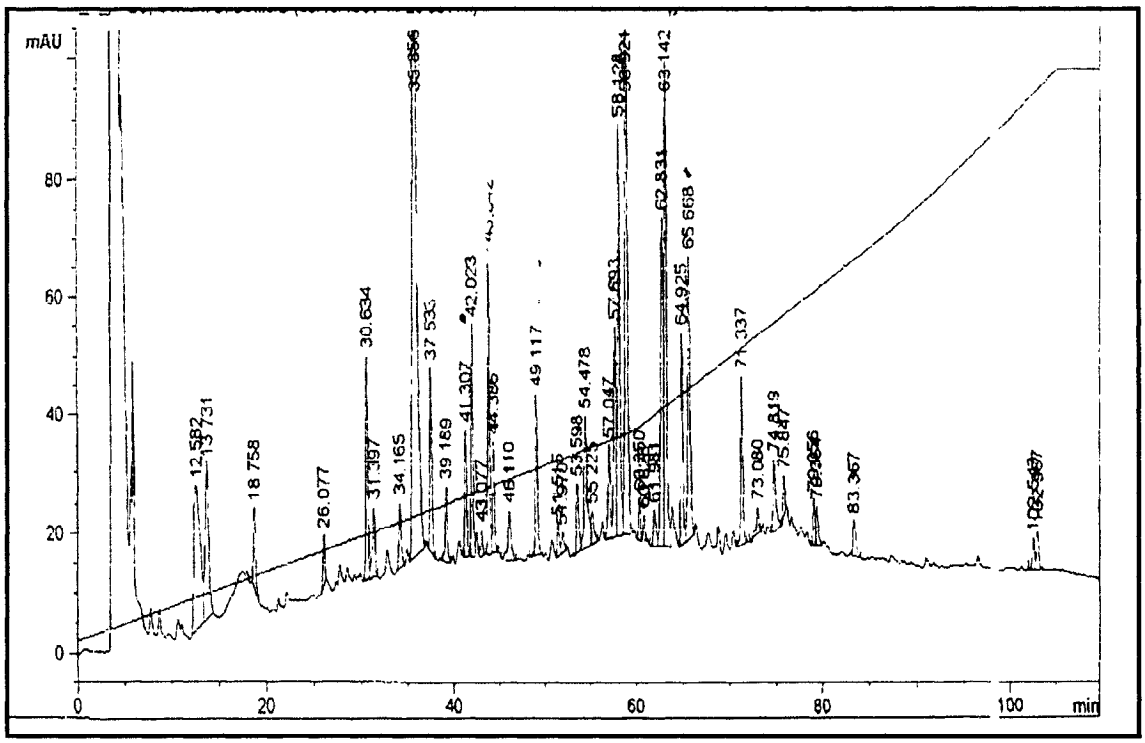
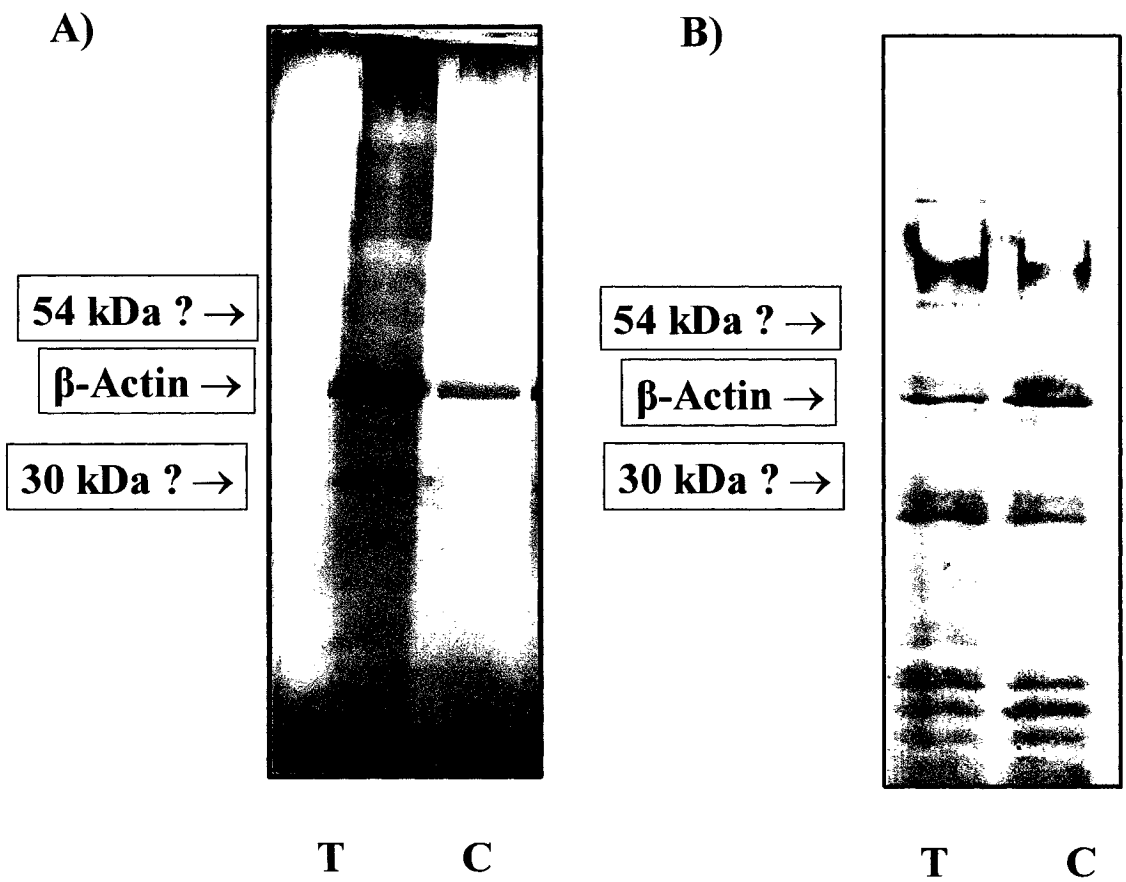


FIGURE 18. SDS polyacrylamide gel electrophoresis of CHAPS-solubilized membrane proteins isolated by affinity chromatography on streptavidin-agarose resin in the absence (T) or presence (C) of 10 μmoles of biotin (figure 18A) or with solubilized membrane proteins subjected to incubation in the absence (T) or presence (C) of 10^{-4}M of H4-(86-100) (figure 18B). The membrane protein isolation by affinity chromatography, the SDS gel separation and the detection of H4-(86-100) binding proteins were performed as described under "Materials and Methods" and as indicated in the legend of figure 16.


A)
54 kDa ? →
β-Actin →
30 kDa ? →
T
C
B)
54 kDa ? →
β-Actin →
30 kDa ? →
T
C

5. DISCUSSION

5.1 General overview

Increasing numbers of histone-derived peptides are recognized to play extranuclear and/or extracellular functions, including the stimulation of growth hormone secretion (Brown et al., 1997), the stimulation of the formation of auto-antibodies in lupus-related diseases (Salaman et al., 2001; Robert, 2001), the stimulation of microbial death (Park et al., 2000; Kim, et al., 2000), osteogenesis (Bab et al., 1992; Bab and Einhorn, 1993) and the relief of pain (Lemaire et al., 1997; Ruan et al., 2000; Siegan and Sagen 1997; Siegan et al., 1997; Hama et al., 1999). Such effects of histone derived peptides were reported to depend either upon their interaction with specific membrane receptors of the family of GPCR or with polysaccharides such as dextran-2-sulfate located inside plasma membranes. For instance, OGP was found to interact with a PTX-sensitive receptor, G_iPCR, to exert its mitogenic effects on MC3T3-E1 osteogenic cells (Gabarin et al., 2001). On the other hand, nucleosomes were found to bind to plasma membranes by an interaction between N-terminal residues of associated histone H2A and H2B and sulfated membrane polysaccharides (dextrin-2-sulfate) (Watson et al., 1999). The antimicrobial activity of H2A-derived bufforins was shown to be dependent on the positive charges of the molecules mediating their penetration through the membrane of bacteria followed by a sequence of events that comprise the formation of holes, the leakage of cell content, the disruption of cell integrity and, ultimately, cell death. It also appears that nucleosomes, which

are associated with the various types of histones, can interact with several membrane proteins. Nucleosome binding proteins of different M.W. (29, 59, 69, 94 kDa) have been isolated from plasma membranes; but their structures have not been identified and their putative receptor-like binding properties have not yet been determined (Jacob et al., 1989; Gasparro et al., 1990; Hefeneider et al., 1992; Bennet et al., 1992).

This study was aimed at determining the nature of the receptor(s) involved in the action of HN and related C-terminal histone H4 peptides. HN and C-terminal histone H4-peptides such as H4-(86-100) and OGP were shown to be analgesic in various animal models of pain, including acetic acid-induced writhing and tail-flick induced by radiant heat in mice, pain induced by the administration of formalin or complete Freund's adjuvant in rats, and hyperalgesia induced by sciatic nerve injury in rats (Ruan et al., 2000; Siegan and Sagen 1997; Siegan et al., 1997; Hama et al., 1999). Interestingly, the antinociceptive effects of H4-derived peptides and analogues were more pronounced in models of persistent and long-lasting pain (such as rat sciatic nerve injury), which is known to involve inflammatory processes than acute pain (such as tail-flick; Ruan et al., 2001), which is not accompanied by inflammation. Since MA are known to play a predominant role in inflammatory processes (Tse and Rosenthal, 1988; Henson et al., 1988; Adams and Hamilton, 1988) and since HN and its related histone H4 peptide H4-(86-100) were shown to inhibit the release of PGE_2 from isolated rat alveolar MA (Lemaire et al., in preparation), a membrane preparation from rat

alveolar MA was chosen to characterize the putative binding site(s) for [^{125}I]H4-(86-100) responsible of its modulation of inflammatory processes and pain.

5.2 High affinity saturable binding sites for [^{125}I]H4-(86-100) on MA

The binding of [^{125}I]H4-(86-100) reveals the presence of one binding site with a dissociation constant (K_d) of 12.3 ± 0.8 nM and a maximal binding capacity of 9.16 ± 0.32 pmol/mg protein. As expected, the K_i value (77 ± 7.2 nM) obtained by competitive experiments with H4-(86-100) and the K_d of the saturation curve are in the same concentration range suggesting the existence of a single reversible binding site. The relative low values of the K_d and B_{max} indicate that this single receptor possesses a high affinity and restricted number of specific binding sites for the iodinated peptide. In accordance with the value of the B_{max}, the number of binding sites/cell was estimated at 80,000 ± 11,000, suggesting that this may be a low capacity binding site. On the other hand, the characterization of [^{125}I]VIP receptors on rat alveolar macrophages revealed the presence of two binding sites: one with a high affinity (K_d= 0.20 ± 0.09 nM) and low capacity (1,190 ± 640 sites/cells) and another with a low affinity (K_d = 43.2 ± 13.8 nM) and high capacity (51,700 ± 14,000 sites/cells) (Sakakibara et al., 1994). The comparison of the binding of [^{125}I]H4-(86-100) with that of [^{125}I]VIP suggests that [^{125}I]H4-(86-100) binds to a site that is comparable to the low affinity high capacity site of VIP, even though the binding characteristics of the C-terminal histone H4 peptide are those of a G_iPCR whereas the VIP receptor is a G_sPCR.

The binding activities of [^{125}I]H4-(86-100) were markedly attenuated by pretreatment of alveolar MA membranes with trypsin, an effect that was blocked by soybean trypsin inhibitor, thus demonstrating the protein nature of H4-(86-100) binding molecule(s) (Calvo et al., 1994). The effect of temperature on the binding suggests that the binding is slightly reduced when the temperature is raised from 4°C to 22°C. Interestingly, reduction of the binding of the [^{3}H]Dihydromorphine for distinct binding domains on mu opioid G-protein coupled receptors was also observed (Zukin and Gintzler, 1980). Such decrease in binding activity at higher temperature may be due to a higher rate of receptor inactivation by proteolytic enzymes in membrane preparations.

5.3 Specificity of [^{125}I]H4-(86-100) binding

Structure-activity studies indicate that the entire length of the peptide is not necessary for binding activity, the minimal active core in HN being the fragment 7 to 10 (Figure 1) and the most potent peptide being the fragment HN-(7-15) (Table 1). The insensitivity of [^{125}I]H4-(86-100) binding to a relative high concentration (10^{-5}M) of specific ligands for μ, δ and κ opioid receptors supports the concept that the H4-(86-100) binding site is non-opioid (Ruan et al., 2000). Dynorphin A (1-13), SP, nociceptin and VIP significantly inhibited the binding of [^{125}I]H4-(86-100). These results may suggest that the allosteric conformation of H4-(86-100) receptor within the membrane is influenced by the presence those peptides. For instance, knowing that dynorphin and nociceptin can interact on the same ORL_1 receptor (Dumont et al., 1998), the inhibition of [^{125}I]H4-(86-100) binding by these

peptides may indicate that dynorphin and nociceptin can also compete with the high affinity H4-(86-100) binding site(s). This kind of interaction is competitive and normally affects the K_d without affecting the binding capacity (B_{max}). An alternative possibility to explain the effects of unrelated peptides on [^{125}I]H4-(86-100) binding may be a non-competitive interaction with the receptor complex. In a non-competitive effect, the unrelated peptides would act on a distinct binding domain of the receptor and could affect the binding of the natural ligand by an allosteric change in the conformation of the receptor. Whether the effects of the unrelated peptides on the binding of [^{125}I]H4-(86-100) is competitive or not is still unknown and remains the subject for further investigation.

On the other hand, the possibility that H4-(86-100) binds to the neurokinin 1 (NK1) receptor was suggested by the inhibitory effect of SP on [^{125}I]H4-(86-100) binding. As mentioned in the introduction, SP is a central excitatory peptide that binds to the NK1 receptor and mediates the transmission of nociceptive inputs and inflammation. In the periphery, SP can also modulate immune functions of T-cells and MA, suggesting a possible role of the NK1 receptor (for review, see Lambrecht, 2001). NK1 receptors are known to be associated with two forms of G proteins, G_q or G_s (Holst et al., 2001). In order to verify the similarity or dissimilarity of the H4-(86-100) binding site with the NK1 receptor and G-protein, the type of G protein (G_i or G_s) associated with the [^{125}I]H4-(86-100) binding site was examined.

To obtain further insight into the characteristics of H4-(86-100) binding protein, we compared the effect of mono- and divalent cations on [^{125}I]H4-(86-

100) binding. Binding of an agonist to a guanine nucleotide binding GPCR is markedly modified by mono- and divalent cations. Several divalent cations such as Mg^{2+} and Ca^{2+} increase the affinity of many GPCR for agonists at concentrations in the millimolar range. For instance, Mg^{2+} is required for receptor-mediated activation of G-protein. Mg^{2+} exerts is action by binding to a specific site on the G-protein, therefore stabilizing the formation of a high affinity ternary complex consisting of the ligand, the high affinity state of the receptor and a guanine regulatory protein (Webster and De Souza, 1988; Gierschik et al., 1988). As for the Na^{+} ion, it was demonstrated to decrease the affinity of agonists for many GPCR and modify the interaction of the G-protein with guanine nucleotide (Rishal et al., 2003; Gierschik et al., 1988). As an example, Na^{+} was shown to decrease the binding of μ and δ opioid agonists (Simon et al., 1973; Pert and Snyder, 1974; Paterson et al., 1986). In contrast, the presence of divalent ions such as Mg^{2+} reduced agonist dissociation rate even in the presence of guanine nucleotides (Chang et al., 1983). Such stimulatory and inhibitory effects of Mg^{2+} and Na^{+}, respectively, are observed on the binding of [^{125}I]H4-(86-100) to rat alveolar MA membranes, suggesting that [^{125}I]H4-(86-100) may be acting as an agonist on a putative GPCR on rat alveolar MA. The effect of other salts which would not affect the specific binding of [^{125}I]H4-(86-100) could also be investigated to emphasize the importance of GPCR affecting salts.

5.4 G_iPCR characteristics of [^{125}I]H4-(86-100) binding

Early characterization of neuropeptide and opioid receptors were basically defined by the demonstration of the pharmacological binding profile of GPCR. For instance, agonist binding activity to opioid receptors was inhibited by guanine nucleotides (Blume, 1978a, b; Childers and Snyder, 1978). The effect of guanine nucleotides resulted from an increase of the rate of dissociation of specific G-protein coupled agonists, with essentially no effect on the dissociation rate of antagonists (Childers and Snyder, 1980). It was shown in several early studies that GTP, and its non hydrolyzable analog, (Gpp(NH)p) were effective in reducing [^{3}H]dihydromorphine (DHM) binding, an agonist to the μ receptor in rat or guinea pig brain membranes, while other nucleotides including ATP, CTP, UTP, and GMP, were essentially inactive (Blume,1978a, b; Childers and Snyder, 1978, 1980; Zukin and Gintzler, 1980). Guanine nucleotide regulation was also demonstrated on VIP binding to rat peritoneal MA membranes (Segura, et al., 1992). The inhibition of [^{125}I]H4-(86-100) binding by GTP analogs, more specifically Gpp(NH)p, follows the effects of guanine nucleotides on the binding of opiates and VIP to their specific receptors, suggesting that [^{125}I]H4-(86-100) may bind to a GPCR in alveolar MA membranes.

Further more, a characteristic that distinguishes receptors that are coupled in an inhibitory fashion to a G protein, the G_iPCR, is their sensitivity to the presence of PTX. PTX inhibits the binding of a peptide ligand to G_i protein-coupled receptors or blocks the activation of the signalling pathway that includes the inhibition of AC (Gailly et al., 2000). PTX is capable by ADP-riboxylation (G_i-

ADPR) to inactivate the G_i protein, therefore uncoupling the G protein from the receptor and favoring the dissociation of bound agonists and prevention of further agonist binding activity. The inhibitory effect of the active (A) but not inactive (B) form of PTX on [^{125}I]H4-(86-100) binding suggests that the receptor is coupled to the G protein of the G_i category. The observation by Gabarin et al., (2001) that the intracellular signalling cascade elicited by OGP involves a G_i-protein-dependent activation of MAP kinases emphasizes the concept that C-terminal histone H4 peptides interact with G_i protein-coupled receptor(s). Although the role attributed to the G_i protein-coupled receptor linked to the activity of OGP involved bone marrow cell maturation and regeneration, the action of OGP was still referred to an interaction of the peptide with a prototypic G_iPCR.

It is well documented that ligands interacting with G_iPCR activate specific signalling pathways, mediated by an inhibition of AC, a decrease in the synthesis of cAMP, an activation of cGMP phophodiesterase, an activation of MAP kinases and a modulation of K^+ and Ca^{2+} channel conductance (Marinissen and Gutkind, 2001). For further insight, we have verified if the forskolin stimulation of cAMP levels in primary cultures of rat alveolar MA was modulated by H4-(86-100). The results show that the peptide does not affect the basal levels of cAMP but markedly diminishes the stimulation of cAMP levels by forskolin. Our results show that VIP induced an increase in cAMP levels with or without the presence of forskolin. The effect of VIP on the cAMP level was previously observed by different groups (Wilson, 1988; Delgado et al., 1999) and this increase resulted from the interaction of VIP receptor (VPAC) with a G_s protein. SP receptor (NK1),

which is also known to act on a G_sPCR (Holst et al., 2001), increased the basal levels of cAMP. Stimulation of G_sPCR with these peptides is known to evoke the dissociation of the G_s protein from the receptor and activate AC responsible for the conversion of ATP into cAMP. The inhibitory effect of H4-(86-100) on forskolin stimulated cAMP levels corroborate with the binding data, indicating that the peptide is acting as an agonist on a G_iPCR. However, there exist peptides that do not follow this mechanism of action such as mastoparan. Mastoporan binds directly to the G_i protein to affect its function on AC. Thus it is capable of passing through the cell membrane and binds to and activates the G_i-protein. Mastoporan is often used in control experiments for the activation of the Gi protein by a promotion of GDP/GTP exchange (McKillop et al., 1999; Pullan and Pennington, 1996). The relative high positive charge of H4-(86-100) suggests that this peptide, like mastoparan, may directly stimulate a G_i protein in the plasma membrane resulting in an inhibition of AC and a decrease in cAMP levels.

As shown in figure 4, the proposed mechanism of action for C-terminal histone H4 peptides in the regulation of MA inflammatory processes is based on the hypothesis that H4-(86-100) may interact with a specific membrane G_iPCR and modulate the formation of PGE_2 via a decrease in cAMP levels resulting from an inhibition of AC. Recently, Hinz and colleagues (2000) have demonstrated in LPS-stimulated MA that PGE_2 potentiates COX-2 mRNA expression via an AC/cAMP-dependent pathway. Lemaire et al., (in preparation) also observed under inflammatory state (LPS stimulation) that C-terminal histone H4 peptides have the capacity of inhibiting the production of PGE_2. The inhibition of cAMP by

H4-(86-100) via a G_iPCR could explain such mechanism of action. The decreased formation of cAMP in the presence of H4-(86-100) followed by the suppression of COX-2 mRNA expression and release of PGE_2 rely on the possibility that H4-(86-100) is interacting with a G_iPCR. Thus, the inhibitory effect of H4-(86-100) on cAMP production may result in an inhibition of the production of PGs and subsequent decreases in the release of pain transmitting agents such as SP, CGRP and excitatory amino acids (Ahluwalia and Perretti, 1994; Lombardo and Wilson, 1997). To emphasize the potential role of the specific MA receptor in the action of C-terminal histone H4 peptides, a close correlation (r=0.85) was established between the abilities of these peptides to inhibit [^{125}I]H4-(86-100) binding and block pain in the mouse writhing test (Ruan et al., 2000). This correlation also suggests that the characterized receptor is implicated in the modulation of pain.

Finally, cross-linking experiments with [^{125}I]H4-(86-100) demonstrated the presence of two binding proteins of M.W. of 54 and 30 kDa, respectively. The membrane density of these two binding proteins was increased when the MA were pretreated with IFN_γ during 24 hours. These data suggest that inflammatory conditions may be accompanied by an up-regulation of the number of binding sites for H4-(86-100) and an increased ability of the peptide to inhibit AC and diminish the formation and release of PGE_2. Interestingly, the exposure of $IFN\gamma$ to human leukocytes (HL-60 cells) was shown to enhance both the expression of formyl peptides receptors and guanine nucleotide binding protein, more

specifically the G_i subunit, therefore enhancing FMLP-stimulated transmembrane signalling (Klein et al., 1992).

5.5 Possible interaction of H4-(86-100) with membrane β-actin

The cross-linking and purification of the membrane binding sites suggest that H4-(86-100) may interact with two proteins with M.W. of 54 and 30 kDa, respectively. Among various C-terminal histone H4 peptides, H4-(86-100) was chosen for purification by affinity chromatography of membrane binding proteins since affinity cross-linking experiments (Figure 14) revealed higher density of cross-linked proteins when bound to [^{125}I]H4-(86-100) as compared to [^{125}I][Ser^{1}]HN and [^{125}I]OGP. The loss in binding capacity of [^{125}I]OGP may result from particular conformational change of the peptide after the iodination of the single Tyr in position 10 of the molecule. Chen et al., (2000) have shown that modification of Tyr^{10} in OGP results in substantial loss of mitogenic activity. Therefore, the cross-linking results suggest that H4-(86-100) may be a better choice to isolate the receptor from a solubilized membrane extract by affinity chromatography. The predominant associated membrane protein was purified and identified as β-actin. The sequence identification was based on the high number of tryptic peptides that had the M.W. of peptides contained in the β-actin tryptic digest databank. The possibility that β-actin extracted from alveolar MA membranes is a membrane protein rather than a contaminating cytosol protein is supported by the observation, which indicated that β-actin, can be strongly associated to plasma membranes (Lin et al., 2002). If β-actin is a specific

binding protein for H4-(86-100), there is a possibility that it is indirectly involved in its inhibitory effect on a G_iPCR. Previous studies have demonstrated that filamin A, which is composed of β-actin, has an important role in the association of GPCRs to transfected cell membranes as well as on the activation the GPCRs. For instance, Lin and colleagues (2000) demonstrated that filamin A might be required for the proper cell surface expression of D2 receptors and that functionally linked downstream signalling components were regulated by the actin cytoskeleton. Recently, the same group provided evidence that filamin A/D2 receptor interaction is required for the proper targeting or stabilization of D2 receptor at the plasma membrane (Lin et al., 2002).

On the other hand, the multiple washes of the membrane preparation prior to its solubilization and extraction of protein content decrease the possibility that one of its major associated protein is a cytosol contaminant. β-actin may represent a protein that has a great affinity for the agarose-streptavidin resin since presaturation of the streptavidin binding sites with biotin prior to the chromatography of solubilized membrane proteins did not prevent β-actin to be retained by the resin while the 54 and 30 kDa H4-(86-100) binding proteins were eliminated by this procedure (Figure 18). The second control assay strongly emphasises that β-actin is not the H4-(86-100) binding protein knowing that in presence of an excess of H4-(86-100), the β-actin band is not reduced whereas the 54 kDa binding protein is markedly reduced. Furthermore, the observations that the molecular weight of β-actin is approximately 45 kDa and that the visualisation of the receptor by cross-linking autoradiography reveals binding

proteins of 54 and 30 kDa, contradict the possibility that β-actin is a specific receptor for H4-(86-100). Therefore, is it quite possible that β-actin is a membrane protein that displays a high non-specific binding avidity for the agarose-streptavidin resin used in the affinity chromatography procedure.

Although the major H4-(86-100) binding protein was identified as β-actin, the receptor responsible for its biological activity may still be the binding proteins identified at the apparent M.W. of 54 or 30 kDa. These two putative specific binding proteins for H4-(86-100) merit further investigation, at least one of them being possibly the G_iPCR responsible of the inhibition by H4-(86-100) of the formation of cAMP and secretion of PGE_2 in rat alveolar MA in response to stimulatory conditions.

5.6 Summary and conclusions

Much research has showed that histone derived peptides play important extranuclear and/or extracellular functions. Herein, rat alveolar MA were shown to express specific binding sites for the C-terminal histone H4 fragment H4-(86-100) with characteristics of a high affinity low capacity, reversible and saturable receptor. The sensitivity of the binding activity to cations, guanine nucleotides and PTX suggests that the receptor is bound to a G_i protein. The inhibitory effect of the peptide on the forskolin-evoked increase in cAMP in cultured MA suggests that the peptide may be interacting with a membrane G_iPCR to inhibit membrane AC and modulate the formation of cAMP as a transducer of the peptide modulation of PGE_2 formation. The induction of the expression of the binding

activity of the two binding proteins by preincubation of MA with IFN_γ is somehow specific to the H4-(86-100) receptor, not being observed with many G_iPCR e.g. the ORL1 receptor for nociceptin, and reveals a correlation between the expression of the receptor and the modulation by the peptide of the formation of PGE_2 in response to inflammation-like conditions. Finally, the good correlation existing between the binding activity and the analgesic effects of C-terminal histone H4 peptides suggests that MA and/or microglial cells may be involved in the antinociceptive effects of the peptides.

Although this research provides some insights on the binding characteristics of C-terminal histone H4 peptides to MA membranes, further studies are needed to verify if the isolated 54 or 30 kDa proteins are specific binding sites for H4-(86-100) of the receptor category of G_iPCR, or otherwise if the apparent association of H4-(86-100) with membrane β-actin may participate to the regulation of an unknown G_iPCR involved in the modulatory effects of the peptide on cAMP levels and PGE_2 secretion.

5.7 Future work

An extension of this study can be proposed in regard to 2 main directions:

1) Isolation and sequence identification of the 54 and 30 kDa membrane proteins would possibly allow the identification of a specific G_iPCR. In order to verify if the affinity chromatography is capable of isolating a specific binding protein for H4-(86-100), a detection technique for the purified samples need to be explored. If the receptor retains its binding

properties after its isolation by affinity chromatography [using H4-(86-100) for elution instead of mercaptoethanol], the binding protein could be transferred on a nitrocellulose in non-denaturated conditions followed by binding with H4-(86-100) and detection by antibody raised against the C-terminal histone H4 peptide (RH-28). This result would help to identify in the SDS-PAGE protein pattern, the protein that should be selected for sequence analysis of the specific H4-(86-100) binding protein.

2) Identification of the receptor would allow its transfection in cells not expressing the receptor (e.g. Cos-1 or Cos-7) and thereafter develop a models to monitor the binding and functional activities of H4-(86-100) and derived synthetic peptide fragments and analogues for the design of potent non-opioid analgesics and anti-inflammatory agents.

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