Potency of Ligands Correlates with Affinity Measured against Agonist and Inverse Agonists but Not against Neutral Ligand in Constitutively Active Chemokine Receptor

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ABSTRACT

ORF-74, a 7TM receptor oncogene encoded by human herpes virus 8, shows 50% constitutive activity in stimulating phosphatidylinositol turnover and binds a large variety of CXC chemokines. These endogenous ligands cover a full spectrum of pharmacological properties with growth-related oncogene (GRO)-α and -γ functioning as full agonists; GROβ as a partial agonist; interleukin (IL)-8, neutrophil-activating peptide (NAP)-2, and epithelial cell-derived activating peptide (ENA)-78 as neutral ligands; granulocyte colony-stimulating factor (GCP)-2 as a partial inverse agonist; and interferon-gamma inducible protein (IP)-10 and stromal cell-derived factor (SDF)-1α as full inverse agonists. The affinity for the agonists was independent of whether it was determined in competition binding against the agonist ^{125}I-GROα, against the inverse agonist ^{125}I-IP-10, or against the neutral ligand ^{125}I-IL-8. Similarly, the affinities of the inverse agonists were within 1 order of magnitude independent of the choice of radioligand. In contrast, the neutral ligands IL-8, NAP-2, and ENA-78, which all displaced ^{125}I-IL-8 with single-digit nanomolar affinity showed up to 1000-fold lower affinity against both the radioactive agonist and against the radioactive inverse agonist. A close correlation was observed between the EC_{50} values for the ligands and their IC_{50} values measured against either radioactive agonist or radioactive inverse agonist, but a poor correlation was found to the IC_{50} value measured against the neutral ligand. It is concluded that in ORF-74, ligands compete for binding more according to pharmacological property than to structural homology and that both agonists and inverse agonists, in contrast to neutral ligands, apparently bind with high affinity either to a common conformation of the receptor or to readily interconvertible states, not available for the neutral ligands.

Chemokines constitute a large family of chemotactic cytokines that have central roles in immunological processes; they are involved in controlling leukocyte migration to the right tissue or compartment at the right time (Zlotnik et al., 1999). Other cellular processes such as angiogenesis are also influenced by chemokines (Horuk, 1998; Tachibana et al., 1998). Chemokine receptors belong to the class of rhodopsin-like, 7TM, G protein-coupled receptors (Zlotnik et al., 1999).

Chemokines as well as chemokine receptors are encoded by a number of herpesvirus and pox virus (Wells and Schwartz, 1997; Dairaghi et al., 1998). Conceivably, they have been obtained by the virus through an ancient act of molecular piracy and have subsequently been optimized structurally for a particular pharmacological phenotype of benefit to the virus. For example, the chemokine vMIP-II from human herpesvirus 8 (HHV8) functions mainly as a broad-spectrum antagonist, that prevents chemotaxis of leukocytes (Kledal et al., 1997; Damon et al., 1998). However, the function of most of the receptors encoded by herpesvirus and poxvirus is still unclear. In general, these receptors are not required for virus replication in vitro (Fields et al., 1995). However, gene-deletion experiments in, for example, cytomegalovirus of both mouse and rat, have shown that, in vivo, the virally encoded UL33 receptor is essential for targeting and/or replication of the virus in salivary glands (Davis-Poynter et al., 1997; Beisser et al., 1998). Importantly, the viral strains lacking the UL33 receptor gene are less virulent than the wild-type cytomegalovirus (Beisser et al., 1998).

ORF-74 is a CXC chemokine receptor encoded by several γ-herpesvirus, including HHV8, also called Kaposi's sarcoma-associated herpesvirus (Chang et al., 1994; Simas and Efstathiou, 1998). Initially, ORF-74 was shown to bind IL-8 with high affinity, which is in accordance with the fact that its closest relative among human chemokine receptors is the IL-8 receptor, CXCR2 (Ahuja and Murphy, 1993). Recently, ORF-74 from HHV8 was shown to be highly constitutively

ABBREVIATIONS: HHV8, human herpesvirus 8; MAP, mitogen activated protein; GRO, growth-related oncogene; IL-8, interleukin-8; ENA-78, epithelial cell-derived activating peptide-78; NAP-2, neutrophil-activating peptide-2; SDF-1α, stromal cell-derived factor-1α; IP-10, interferon-γ inducible protein; GCP-2, granulocyte colony-stimulating factor; ELR, Glu-Leu-Arg.

active; although IL-8 bound to the receptor, this binding did not affect its signaling (Cesarman et al., 1996). Human chemokine receptors preferentially signal through the Gi pathway; however, ORF-74 activates both the phospholipase C pathway, leading to high turnover of phosphatidylinositol as well as mitogen-activated protein (MAP) kinase pathways, which results in the production and secretion of vascular endothelial growth factor (Geras-Raaka et al., 1998a). Because of its high constitutive activity, ORF-74 acts as a virally encoded oncogene that causes cellular transformation and the development of highly vascularized tumors, for example, in both nude and SCID mice (Arvanitakis et al., 1997; Bais et al., 1998). Thus, it has been proposed that ORF-74 could be causally involved in the development of the highly vascularized lesions in Kaposi's sarcoma as well as the lymphomas associated with HHV8 infection (Bais et al., 1998; Geras-Raaka et al., 1998a).

In this study, we investigate the binding and signaling profiles of a number human chemokines in ORF-74 from HHV8 to probe the correlation between biological potency and affinity measured by homologous and heterologous radioligand binding in a receptor system with naturally high constitutive activity (Fig. 1). The phenomenon in which affinities can vary dramatically, depending on the employed radioligand, has been studied previously in other 7TM-receptor systems, including, for example, the tachykinin (Rosenkilde et al., 1994; Hastrup and Schwartz, 1996; Sagan et al., 1997) and opioid systems (Hjorth et al., 1996). In fact, the closest human homolog to ORF-74, CXCR2, displays a similar phenomenon with more than 1000-fold difference in measured affinity for certain ligands depending on which chemokine was used as radioligand (Ahuja and Murphy, 1996). ORF-74 offers a special opportunity to study this phenomenon, because a number of homologous agonists as well as inverse agonists and neutral ligands are available as potential radioligands. We find that in this highly constitutively active receptor, the biological potency of the various ligands in all cases is rather closely correlated to their affinity measured in competition with either the preferred agonist, growth-related oncogene (GRO)-α, or in competition with the preferred inverse agonist, IP-10. In contrast, the affinity measured in competition against the neutral ligand, interleukin-8 (IL-8), is not correlated to the potency of the ligands, especially not for IL-8 itself and the other neutral ligands.

Experimental Procedures

Materials. Some human chemokines were purchased from R&D Systems Europe Ltd. (Abingdon, UK) [GROβ, GROγ, and epithelial cell-derived activating peptide-78 (ENA-78)], and others were kindly provided by Timothy N.C. Wells (Serono, Geneva, Switzerland) [neutrophil-activating peptide (NAP)-2 and the vMIP-II]. Mikael Luther (GlaxoWellcome, Research Triangle Park, NC) provided [Met^0]stromal cell-derived factor (SDF)-1α; Kuldeep Neote (Pfizer Inc., Groton, CT) provided GROα, SDF-1α, interferon-γ inducible protein (IP-10), and granulocyte colony-stimulating factor (GCP-2). Thomas P. Boesen at this laboratory provided IL-8. The gene for ORF-74 (Genbank accession number U24275) was cloned from a biopsy taken from a Kaposi's sarcoma skin lesion from a HIV-1-infected patient (Kledal et al., 1997). The cDNA was cloned into the eukaryotic expression vector, pTEJ-8 (Johansen et al., 1990). Monoiodinated ^{125}I-IL-8, ^{125}I-GROα, [myo-^{3}H] inositol (PT6–271) and Bolton-Hunter reagent for iodination of proteins were purchased from Amersham (Little Chalfont, UK). AG 1-X8 anion exchange resin was from Bio-Rad Laboratories (Hercules, CA).

Iodination of IP-10. The Bolton-Hunter reagent was dried by a gentle stream of nitrogen for 30 to 60 min until no benzene was left. Five to ten micrograms of IP-10 was incubated on ice with 1.5 mCi of Bolton-Hunter reagent in a total volume of 50 μl of 0.1 mM Borat buffer, pH 8.5, for 1 h, and the reaction was terminated by addition of 0.5 ml of water supplemented with 0.1% v/v trifluoroacetic acid (TFA). The iodinated chemokines were purified by reversed phase HPLC.

Transfections and Tissue Culture. COS-7 cells were grown at 10% CO_2 and 37°C in Dulbecco's modified Eagle medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. Transfection of the COS-7 cells was performed by the calcium phosphate precipitation method (Rosenkilde et al., 1994).

Phosphatidylinositol Accumulation Assay. One day after transfection COS-7 cells (0.5×10^6 cells/well) were incubated for 24 h with 5 μCi of [myo-^{3}H]inositol in 0.8 ml per well of inositol-free Dulbecco's 1885 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. Cells were washed twice in 20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% (w/v) bovine serum albumin and were incubated in 1 ml of buffer supplemented with 10 mM LiCl at 37°C for 90 min in the presence of various concentrations of chemokines. Cells were extracted with 10% ice-cold perchloric acid. The resulting supernatant was neutralized with KOH in HEPES buffer, and the generated [^{3}H]inositol phosphates were purified on AG 1-X8 anion exchange resin (Berridge et al., 1983). Determinations were made in duplicates.

Competition Binding Experiments. COS-7 cells were transferred to culture plates 1 day after transfection. The number of cells
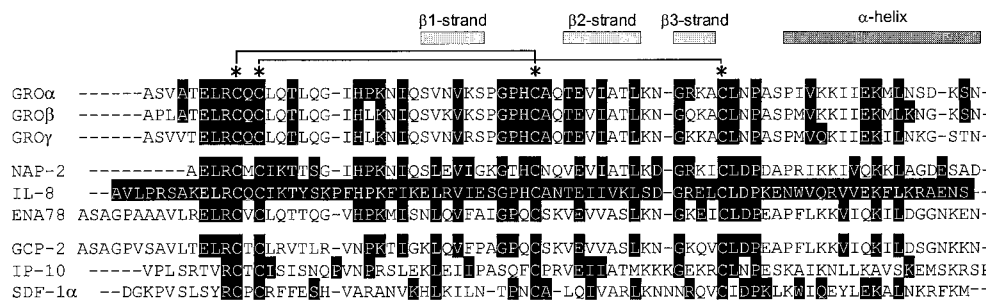
Fig. 1. Amino acid sequences of employed chemokines. Shown is the alignment of human IL-8 with various human chemokines. Upper third, GROα, GROβ, and GROγ; middle third, NAP-2, IL-8, and ENA-78; lower third, GCP-2, IP-10, and SDF-1α. Residues identical with those of IL-8 are shown as white on black. Asterisks indicate the four conserved cysteine residues; indicated between these are the disulfide bridges formed by the corresponding cysteines. Furthermore, based on structural studies of IL-8 (Clore et al., 1990), the localization of the three β-strands (β1, β2, and β3) are marked with light gray boxes above the involved residues. The localization of the α-helix is marked with a dark gray box above the involved residues (Clore et al., 1990).

seeded per well was determined by the apparent expression efficiency of the ORF-74 wild-type and was aimed at obtaining 5 to 10% specific binding of the added radioactive ligand to maintain approximately constant concentration of free radioligand (Kenakin, 1993); furthermore, the specific binding constituted >80% of total bound radioligand. Two days after transfection, cells were assayed by competition binding performed on whole cells for 3 h at 4°C using 12 pM ^{125}I-IL-8, ^{125}I-GROα, or ^{125}I-IP-10 plus variable amounts of unlabeled ligand in 0.4 ml of a 50 mM HEPES buffer, pH 7.4, supplemented with 1 mM CaCl$_2$, 5 mM MgCl$_2$, and 0.5% (w/v) bovine serum albumin. After incubation, cells were washed quickly four times in 4°C binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined as the binding in the presence of 0.1 μM unlabeled chemokine homologous to the applied radioligand. Determinations were made in duplicates.

Kinetic Binding Experiments. Association and dissociation reactions were determined in a total volume of 0.4 ml of binding buffer at 4°C using 10 to 15 pM ^{125}I-IL-8, ^{125}I-GROα, or ^{125}I-IP-10. After incubation for various periods, the cells were washed quickly four times in 4°C binding buffer supplemented with 0.5 M NaCl. The association and dissociation reactions were measured over a total period of 4 to 7 h. After an incubation period of 180 min with radioligand 10^{-7} M of either IL-8, GROα, or IP-10 was added and the dissociation of the radioactive ligand was followed. All combinations of homologous and heterologous dissociation reactions were performed; however, for the homologous dissociation of ^{125}I-GROα, 10^{-8} M (instead of 10^{-7} M) GROα was used because of the high binding affinity of this ligand to the receptor. Determinations were made in duplicate and the nonspecific binding was determined with the same concentration of homologous cold ligand as applied in the dissociation-reactions.

Calculations. IC$_{50}$ and EC$_{50}$ values were determined by nonlinear regression using GraphPad Prism 2.1 (GraphPad Software, San Diego) and B_{max} values calculated from the homologous competition binding curves using the equation $B_{max} = B_o \times (IC_{50}/[L])$ (Deblasi et al., 1989), where B$_o$ indicates the specific bound radioligand and [L] indicates the concentration of free radioligand. Data analysis of the kinetic experiments were also performed using the GraphPad Prism 2.1 program.

Results

Pharmacological Classification of Ligands by Phosphatidyl-Inositol Turnover Assay

Gene-dosage experiments confirmed the high constitutive activity of ORF-74 as well as the observation that IL-8, 10^{-7} M, had no or only minimal effect on the ability of the receptor to induce phosphatidyl-inositol production in transfected COS-7 cells (Fig. 2A) (Arvanitakis et al., 1997; Rosenkilde et al., 1999). However, as shown in Fig. 2B and as previously presented in part (Geras-Raaka et al., 1998b; Rosenkilde et al., 1999), ORF-74 in fact binds a large number of CXC

chemokines that display a surprisingly full spectrum of pharmacological properties. Thus, GROα and GROγ act as full agonists, whereas GROβ seems to be a partial agonist within the dose range that the supply of peptides allowed us to test. In contrast, IP-10 and SDF-1α are full inverse agonists, whereas GCP-2 acts as a partial inverse agonist on ORF-74 (Fig. 2B). All of these chemokines are high-potency ligands with EC$_{50}$ values ranging between 10^{-8} and 10^{-9} M (Fig. 2B). As discussed previously, the agonistic GRO peptides are known to be endogenous growth promoting or angiogenic chemokines, whereas IP-10 and SDF-1α in respect of effect on vascular growth normally are angiostaxic or angiomodulatory chemokines (Strieter et al., 1995; Moore et al., 1998; Rosenkilde et al., 1999). In contrast, a number of the more classical CXC chemokines, IL-8, NAP-2, and ENA-78, which normally function mainly as chemoattractant for especially neutrophil granulocytes during inflammation, acted as neutral ligands on the virally encoded ORF-74 receptor. In fact, it would seem that at least ENA-78 and IL-8, which could be tested at high concentrations [i.e., 10^{-6} M, should possibly be classified as low potency partial agonists (Fig. 2B)]. However, for practical reasons, these peptides are here considered to be neutral ligands.

Competition Binding Experiments with Different Classes of Radioligands

Radioligands were chosen, ^{125}I-GROα for the agonists and ^{125}I-IP-10 for the inverse agonists, because these two peptides were apparently of highest affinity in each of these classes of ligands. For the neutral ligands, ^{125}I-IL-8 was chosen because this originally was the classic ligand for ORF-74 (Ahuja and Murphy, 1993).

The Agonists. The three agonist peptides, GROα, -β, and -γ showed similar, subnanomolar affinity in competition against ^{125}I-GROα with IC$_{50}$ values ranging from 0.06 to 0.22 nM (Table 1; Fig. 3A). In competition against the neutral ligand ^{125}I-IL-8, the affinities were marginally lower [IC$_{50}$ values ranging from 0.23 to 0.37 nM (Table 1, Fig. 3B)], and in competition against the inverse agonists ^{125}I-IP-10, the affinity was again slightly lower than that [i.e., with IC$_{50}$ values ranging from 0.82 to 1.60 nM (Table 1, Fig. 3C)]. Thus, for each of the agonists, the affinity only varied within 1 order of magnitude, depending on the tracer against which it was determined.

The Inverse Agonists. Like the affinity for the agonists, the affinity for the three inverse agonists IP-10, SDF-1α, and GCP-2 was within 1 order of magnitude independent on which tracer was used for the competition binding experi-
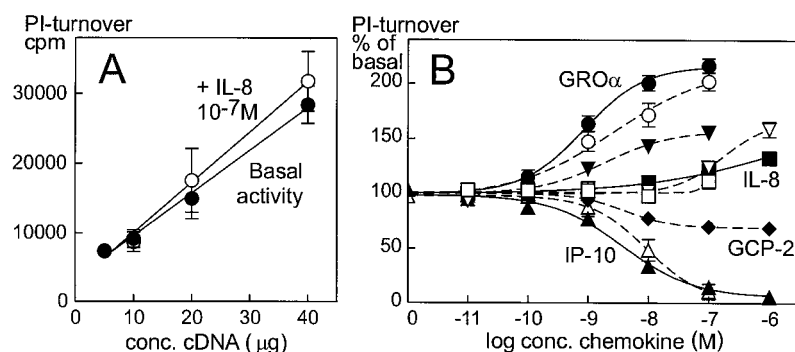
Fig. 2. Effect of selected chemokines on the basal phosphatidyl inositol turnover induced by the highly constitutively active ORF-74 receptor. Experiments were performed on transiently transfected COS-7 cells as described in detail under *Experimental Procedures*. A, gene-dosage experiment measuring the basal and IL-8 stimulated activity given in cpm accumulated in 5×10^5 cell over 90 min (cpm/5×10^5 cell/90 min) for various concentrations of cDNA. Basal activity (●) ($n = 4$) and IL-8, 10^{-7} M, stimulated activity (○) ($n = 4$). B, effect of selected chemokines with the agonists GROα (–●–) ($n = 9$), GROβ (- -▼- -) ($n = 5$), and GROγ (- -○- -) ($n = 4$); the neutral ligands IL-8 (–■–) ($n = 11$), NAP-2 (- -▽- -) ($n = 6$), and ENA-78 (- -□- -) ($n = 4$); and the inverse agonists IP-10 (–▲–) ($n = 3$), SDF-1α (- -△- -) ($n = 5$), and GCP-2 (–◆–) ($n = 3$).

TABLE 1

Binding constants of selected CXC-chemokines for the HHV8-encoded ORF74 receptor using different radiolabeled ligands. The ORF-74 receptor was transiently transfected in COS-7 cells and competition binding experiments were performed as described under *Experimental Procedures*. IC_{50} values were determined either in competition against ^{125}I-GROα ($B_{max} = 44 \pm 10$ fmol/10^5 cell), ^{125}I-IL8 ($B_{max} = 42 \pm 12$ fmol/10^5 cell), or ^{125}I-IP-10 ($B_{max} = 28 \pm 5$ fmol/10^5 cell). Shown are mean $\pm$ S.E. of the indicated number of experiments (n). The "fold difference" measured in affinity when using ^{125}I-GROα or ^{125}I-IP-10 versus ^{125}I-IL8 as radioactive ligand is listed in a separate column.

	Agonists				Neutral Ligands				Inverse Agonists			
	Ligand	IC_{50} (n)	Hill slope	Fold	Ligand	IC_{50} (n)	Hill slope	Fold	Ligand	IC_{50} (n)	Hill slope	Fold
		nM (n)				nM (n)				nM (n)		
^{125}I-GROα	GROα	0.10 ± 0.02 (11)	−1.27 ± 0.09	0.4	IL-8	1332 ± 795 (5)	−0.94 ± 0.25	888	IP-10	2.3 ± 0.60 (3)	−0.77 ± 0.06	0.55
	GROβ	0.22 ± 0.05 (5)	−0.79 ± 0.11	0.7	NAP-2	82 ± 12 (5)	−0.91 ± 0.08	23	GCP-2	0.44 ± 0.01 (3)	−0.94 ± 0.12	0.73
	GROγ	0.06 ± 0.01 (5)	−0.90 ± 0.06	0.2	ENA-78	173 ± 31 (3)	−1.20 ± 0.09	16	SDF-1α	13 ± 1.4 (4)	−0.87 ± 0.10	2
^{125}I-IL-8	GROα	0.23 ± 0.07 (5)	−1.04 ± 0.09	1	IL-8	1.5 ± 0.36 (5)	−0.91 ± 0.08	1	IP-10	4.2 ± 1.1 (3)	−1.31 ± 0.09	1
	GROβ	0.34 ± 0.10 (5)	−1.05 ± 0.10	1	NAP-2	3.6 ± 0.59 (4)	−0.92 ± 0.10	1	GCP-2	0.60 ± 0.08 (3)	−1.19 ± 0.06	1
	GROγ	0.37 ± 0.09 (5)	−1.49 ± 0.14	1	ENA-78	11 ± 2.9 (2)	−0.82 ± 0.02	1	SDF-1α	6.7 ± 1.6 (5)	−1.11 ± 0.14	1
^{125}I-IP10	GROα	0.83 ± 0.03 (5)	−0.95 ± 0.14	3.6	IL-8	1627 ± 712 (3)	−0.41 ± 0.19	1085	IP-10	0.62 ± 0.21 (7)	−0.99 ± 0.04	0.14
	GROβ	1.60 ± 0.18 (3)	−1.00 ± 0.07	4.4	NAP-2	839 ± 56 (3)	−0.58 ± 0.10	233	GCP-2	1.3 ± 0.38 (3)	−1.00 ± 0.13	2.2
	GROγ	0.82 ± 0.16 (3)	−1.35 ± 0.22	2.2	ENA-78	515 ± 251 (3)	−1.30 ± 0.14	47	SDF-1α	17 ± 9 (3)	−0.99 ± 0.38	2.5

ments (Table 1, Fig. 3G, H and I). The highest affinity for IP-10, 0.62 nM, was obtained in the homologous binding assay, whereas GCP-2 showed its highest affinity, 0.44 nM, in competition against the agonist ^{125}I-GROα, which like GCP-2 also is an ELR-chemokine (ELR refers to the sequence Glu-Leu-Arg found just before the first Cys residue in a structural family of CXC-chemokines (see Fig. 1).

The Neutral Ligands. In contrast to the agonists and inverse agonists, the affinity of the three neutral ligands, as determined in competition binding experiments, was highly dependent on the choice of radiolabeled ligand. The highest affinity for the neutral ligands, 1.5 nM for IL-8, 3.6 nM for NAP-2, and 11 nM for ENA-78, was obtained in competition against the "functionally homologous" [i.e., neutral ligand ^{125}I-IL-8 (Table 1, Fig. 3E)]. IL-8 competed for binding against both the agonist ^{125}I-GROα and against the inverse agonist ^{125}I-IP-10 with an apparent affinity that was 900- to 1100-fold lower than the affinity determined in homologous binding experiments (Table 1; Fig. 3D-F). Similarly, the affinities of the two other neutral ligands, NAP-2 and ENA-78, were 16- to 230-fold lower when determined in competition against either agonists or inverse agonists than against ^{125}I-IL-8.

The Kinetic of Receptor-Interaction for the Different Chemokines

Association and dissociation curves were performed for all three different radioligands to further elucidate the competition-binding phenomenons presented above. The association reactions showed that the binding of all three radioligands, ^{125}I-GROα, ^{125}I-IL-8, and ^{125}I-IP-10, approached equilibrium within the 180-min incubation period used for the competition-binding experiments (Fig. 4, A-C). All three radioligands could also be fully displaced from the receptor by the homologous unlabeled chemokine (Fig. 4, A-C). The heterologous dissociation reactions supported the competition binding data as ^{125}I-IL-8 could be fully displaced from the receptor by unlabeled GROα as well as IP-10 (Fig. 4E) with dissociation-curves similar to those observed with homologous dissociation by unlabeled IL-8 (Fig. 4B). ^{125}I-GROα could be fully displaced by unlabeled IP-10 and ^{125}I-IP-10 could be fully displaced by unlabeled GROα; however, IL-8 was unable to displace ^{125}I-GROα and ^{125}I-IP-10 from the ORF-74 receptor (Fig. 4, D and F), again in consistency with data obtained in the competition binding experiments.

Affinity Versus Potency of Ligands for ORF-74

For all nine ligands, there was a close correlation between their potency in either stimulating or inhibiting the constitutive activity of the ORF-74 receptor and their affinity determined for each of these ligands in competition against either the agonist ^{125}I-GROα or the inverse agonist ^{125}I-IP-10—covering a range of more than 4 orders of magnitude (Fig. 5A). In fact, the points corresponding to affinities determined in competition against the inverse agonist (Fig. 5A, closed symbols) clustered even more closely around the straight line corresponding to $EC_{50} = IC_{50}$ than the affinities determined against the agonist (Fig. 5A, open symbols), which were shifted slightly to the left. As shown in Fig. 5B, there was a positive correlation also between the affinity determined for the nine ligands in competition against the neutral ligand ^{125}I-IL-8 and their potencies determined in

signal transduction assay. However, this correlation was rather poor. Although the points corresponding to the agonists and the inverse agonists did cluster relatively closely around the line of unity, the points corresponding to the neutral ligands were located 2 to 3 orders of magnitude off this line (Fig. 5B).

Discussion

ORF-74 is a remarkable receptor that could be a showcase for certain aspects of molecular pharmacology. Not only does the receptor display an unusually high degree of constitutive activity—around 50%—it also has been optimized by the virus to recognize a large number of endogenous ligands that cover the full spectrum of pharmacological properties, from full agonism to full inverse agonism. Because these ligands are homologous peptides that can all be radioactively labeled, this receptor system offers a rather unique possibility to probe the relationship between biological potencies and affinities measured in homologous versus heterologous binding assays. This is an issue that has been difficult to address in most other receptor systems because radioactively labeled agonists and inverse agonists normally are not readily available. The main interesting observation is that in the ORF-74

receptor system, the biologically active compounds (i.e., both agonists and inverse agonists) compete for binding against each other with affinities that closely correlate to their biological potencies, whereas high-affinity neutral ligands compete with low affinity for binding against both agonists and inverse agonists.

Competition According to Pharmacological Properties, Not Structural Properties. Ligands compete for binding against each other more according to pharmacological function than according to structure in this receptor system. For example, the inverse agonists IP-10 and SDF-1α, which are non-ELR chemokines and are only distantly related to the agonistic ELR-chemokine GROα (see Fig. 1) nevertheless compete with high affinity against ^{125}I-GROα. In contrast, the ELR-chemokines IL-8, NAP-2, and ENA-78, which are more closely related structurally but are rather inactive functionally, compete with very low affinity against GROα (Fig. 3E). From these data, it seems that the radioactive agonist and the radioactive inverse agonist bind with highest affinity to highly interconvertible biologically relevant states or conformations of the receptor, which—for reasons still unknown—are nonaccessible for the neutral ligands. In contrast, the binding mode of the neutral ligand IL-8 does not
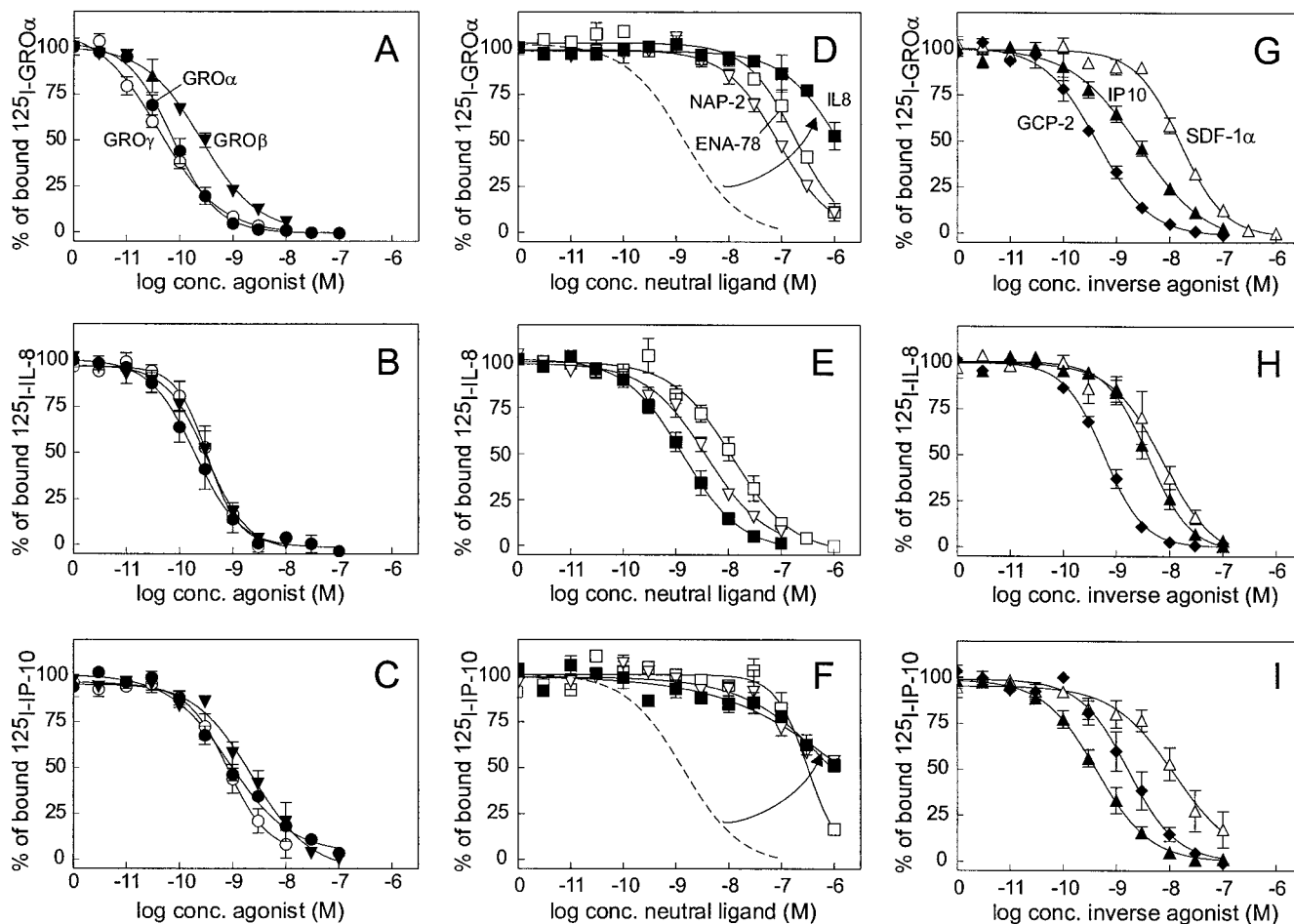

Fig. 3. Competition binding experiments with ORF-74 and three ligands with different pharmacological properties. Binding was performed on whole COS-7 cells transiently expressing ORF-74 against either the agonist ^{125}I-GROα (A, D, and G), the neutral ligand ^{125}I-IL-8 (B, E, and H), or the inverse agonist ^{125}I-IP-10 (C, F, and I). A, B, and C show competition curves for the agonists: GROα (●), GROβ (▼), and GROγ (○). D, E, and F show curves for the neutral ligands (and low potency partial agonists) IL-8 (■), NAP-2 (▽), and ENA-78 (□). G, I, and H show curves for the inverse agonists IP-10 (▲), SDF-1α (△), and GCP-2 (♦). The dotted lines in D and F correspond to the homologous competition binding curve for IL-8 shown in full in E.

correlate to any functional property of the receptor. Nevertheless, all ligands compete for IL-8 binding with high affinity relatively independent on their biological potency or property (Fig. 3).

Based on the inability of IL-8 to compete for binding against the other ligands, it could perhaps be assumed that the binding site for IL-8 would be located rather peripherally in the receptor—for example, selectively in the amino-terminal segment. However, substitutions of residues in TM-V that correspond to residues normally involved in, for example, nonpeptide antagonist binding in tachykinin receptors (Gether et al., 1993) and IL-8 binding in the homologous CXCR2 receptor (Leong et al., 1994) can eliminate IL-8 binding in ORF-74 (Rosenkilde et al., 1999). Importantly, they do so without affecting GROα binding (M.M.R. and T.W.S., unpublished observations). On the other hand, deletion of a small part of the far amino-terminal segment of ORF-74 results in a receptor that shows unaltered high constitutive activity but nevertheless binds *none* of the chemokine ligands with reasonable affinity (M.M.R. and T.W.S., unpublished observations). Thus, there is evidence both for a common binding mode for the various pharmacological classes of ligands and evidence for a rather surprising intimate binding mode for the neutral ligand IL-8 with the helical bundle of the ORF-74 receptor. This does not point to a simple structural explanation for the ability of IL-8 to compete with neither the agonists nor the inverse agonists for binding to ORF-74. Another possible explanation for the inability of IL-8 to compete for the agonist and the inverse agonist could be, for example, a very slow association-rate for IL-8 compared with GROα and IP-10 or differences in the dissociation-reactions; however, the kinetic parameters for IL-8 by itself are rather similar to those for GROα and IP-10 and a

kinetic explanation for the competition-binding phenomenons, therefore, seems unreasonable.

A High-Affinity Ligand That Does Not Affect Constitutive Receptor Activity Is Not Automatically an Antagonist. It is well known that agonists often display a low-affinity state in competition against radioactive antagonists in 7TM receptors. This phenomenon is generally interpreted as a reflection of a presumed low affinity of agonists for the G protein-uncoupled form of the receptor in the allosteric, ternary complex model (De Lean et al., 1980; Lefkowitz et al., 1993). In contrast, it is also generally assumed that (neutral-) antagonists bind to all receptor states with equal affinity, as reflected in, for example, their ability to compete against agonists in a monocomponent fashion (Williams and Lefkowitz, 1977; De Lean et al., 1980), whereas inverse agonists (also called negative antagonists) are assumed to bind preferentially, and with higher affinity, to the G protein-uncoupled form of the receptor (Barker et al., 1994; Chidiac et al., 1994; Samama et al., 1994). In the present study, the ligands have, until this point, been discussed only with reference to their primary function as either agonists, inverse agonists, or neutral ligands (i.e., the pharmacological function displayed by the ligand when binding to the receptor alone). In general, it is assumed that all ligands that show less efficacy than the full agonist (e.g., partial and inverse agonists) automatically will function also as antagonists. Through competition for occupancy of the receptor, they should diminish the signaling from the level corresponding to stimulation by the full agonist to that corresponding to stimulation/inhibition by the competing ligand. Accordingly, we have found previously that IP-10 and SDF-1α, besides being inverse agonists, as expected, could also function as antagonists on ORF-74 [i.e., they antagonized the stimulatory effect
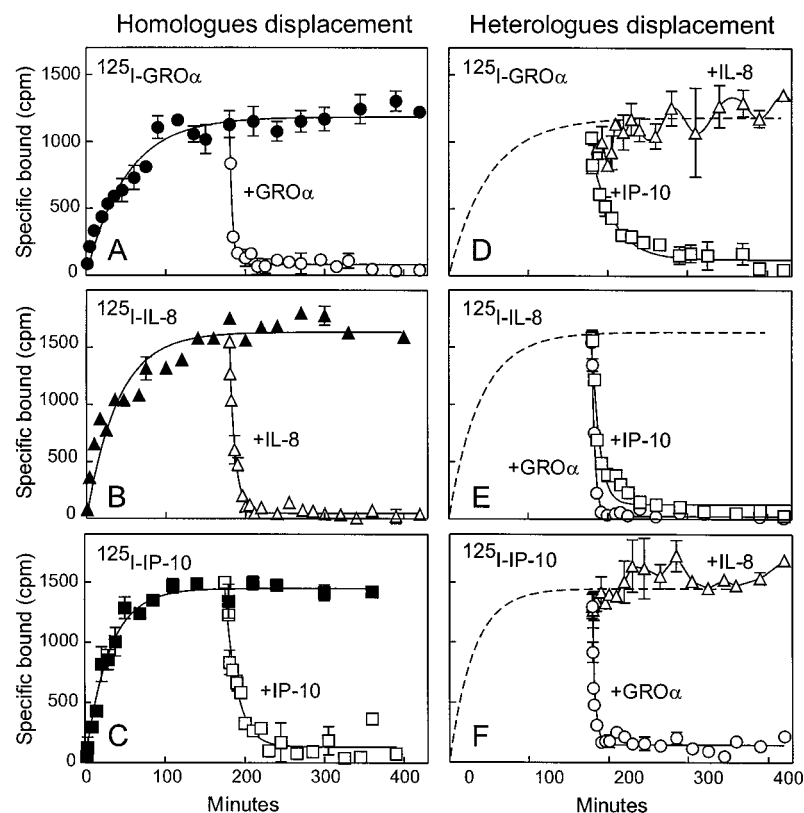

Fig. 4. Association and dissociation curves for different radioligands for ORF-74. The kinetic experiments were performed on whole COS-7 cells transiently expressing ORF-74 with the different radioligands, ^{125}I-GROα (A and D), ^{125}I-IL-8 (B and E), and ^{125}I-IP-10 (C and F). All association-reactions are shown as filled symbols, whereas all dissociation-reactions, homologous as well as heterologous displacements, are shown as open symbols. A, B, and C show the specific association and homologous dissociation reaction for ^{25}I-GROα (A, ●, association; ○, dissociation), ^{125}I-IL-8 (B, ▲, association; △, dissociation), and ^{125}I-IP-10 (C, ■, association; □, dissociation). D, E and F show the specific association, indicated as a dashed line, as well as the specific heterologous dissociation reaction for ^{25}I-GROα [D, displaced with IL-8 (△) and with IP-10 (□)], ^{125}I-IL-8 [E, displaced with GROα (○) and with IP-10 (□)], and ^{125}I-IP-10 [F, displaced with IL-8 (△) and with GROα (○)]. All association and dissociation curves have been performed three times ($n = 3$), and the curves shown are representative for the performed experiments.

of GROα (Rosenkilde et al., 1999)]. A high-affinity ligand—like IL-8 in the present study—that does not affect the constitutive activity of a receptor and is therefore neither an agonist nor an inverse agonist is generally assumed to be an antagonist—sometimes called a neutral antagonist (Costa and Herz, 1989; Kenakin, 1993). However, although IL-8 has a lower efficacy (zero or close to zero) than GROα, and although IL-8 is homologous to the agonist GROα, IL-8 is surprisingly unable to inhibit the GROα-induced signaling in the ORF-74 receptor (Rosenkilde et al., 1999). In other words, IL-8 is a true *neutral ligand*, not a neutral antagonist, because it does not function as an antagonist for the full agonist. Importantly, this lack of effect on GROα function is in good agreement with the observation that IL-8 inhibits the binding of radioactive GROα with only very low affinity (Fig. 3D).

Potency and Affinity Are Closely Correlated for the "Active" Ligands. The close correlation between potency and affinity for the "active" ligands (i.e., agonists and inverse agonists) and the fact that they compete against each other and against IL-8 in a rather monocomponent fashion may reflect the highly constitutive activity of the ORF-74 receptor. In other words, the receptor may be found mainly in one active conformation or rather in complex with a single G protein species. It will be interesting to test the interrelationship between agonists and inverse agonists in other receptor
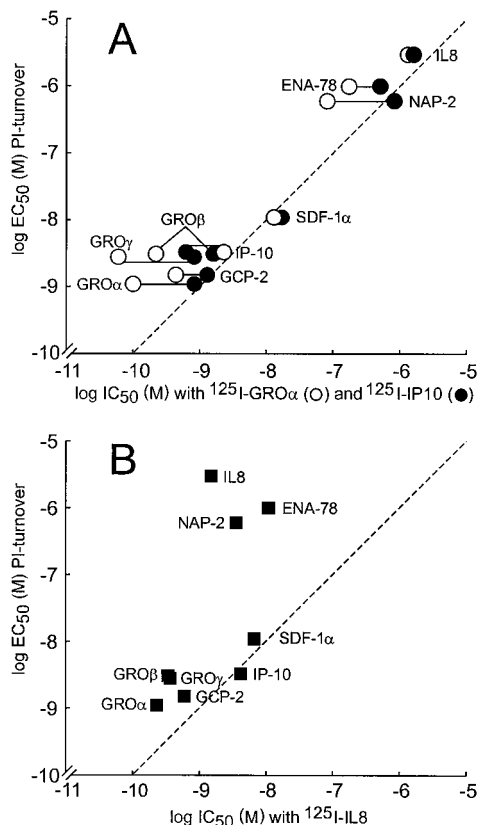
systems in which radioactive species of both types of ligands are available. It is possible, however, that it will be necessary to force more "normal" receptors into a similar situation as ORF-74, either by making them highly constitutively active by mutagenesis or by closely coupling them to a single G protein species by making suitable fusion proteins to get a picture as relatively clean as observed here in the naturally constitutively active viral oncogene.

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Fig. 5. Correlation between affinity (IC_{50}) measured in competition binding and potency (EC_{50}) determined in stimulation or inhibition of phosphatidylinositol turnover for ORF-74 chemokine ligands. A, IC_{50} values measured in competition against the agonist ^{125}I-GROα (○) and against the inverse agonist ^{125}I-IP-10 (●). B, IC_{50} values measured in competition against the neutral ligand ^{125}I-IL-8 (■). The dotted line in both panels represents a line with a slope of unity corresponding to $IC_{50} = EC_{50}$.

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