Cation Dependence of Opioid Receptor Binding Supports Theory on Membrane-Mediated Receptor Selectivity

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ABSTRACT: A quantitative analysis of the binding of dynorphin A-(1–9)-nonapeptide to the opioid κ-receptors of the guinea pig cerebellum [Paterson et al. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6216–6220] shows that changes in electrostatic surface accumulation of the ligand fully account for the observed suppression of binding by a series of univalent and divalent salts. Binding to μ- and δ-receptors, on the other hand, is subject to additional ion-specific effects. These observations support the membrane locations for the receptor sites proposed by the "membrane compartments" theory for opioid receptor selection.

In spite of intensive research activity since the discovery of the opiate receptors (Pert & Snyder, 1973a,b; Simon et al., 1973; Terenius, 1973), many details of the opiate response remain to be elucidated. At least four receptor subtypes have been proposed [e.g., Kosterlitz and Paterson (1985) and Demoliou-Mason and Barnard (1986b)]. The mechanism by which stimulation leads to response, the relationships between receptor subtypes [e.g., interconvertibility (Bowen et al., 1981); heterologous complexes (Demoliou-Mason & Barnard, 1986b)], the functional differences of the receptor subtypes, the method by which receptor activity is modulated, and the mechanism(s) of receptor subtype selection have yet to be firmly established. A particular aspect of receptor modulation or regulation, the effect of cations on ligand binding, is a controversial field (Demoliou-Mason & Barnard, 1986a,b; Frances et al., 1985; Jouzac et al., 1984; Kuoakou et al., 1982; Chang et al., 1983; Puttfarken et al., 1986), yet provides support for a proposed molecular mechanism of opioid receptor selection (Schwyzer, 1986b).

Published work on the effects of cations on the binding of ligands to opiate receptors has often given seemingly contradictory results. Much of the uncertainty probably stems from a combination of the use of relatively nonselective ligands and the presence of several receptor subtypes in different proportions in the various assay systems. Problems may also arise in comparing chemically distinct classes of ligands, such as the opiate alkaloids and the opioid peptides. As the basic physical chemistry of these two classes of compounds (structure, flexibility, hydrophobicity, etc.) differs greatly, details of the interaction leading to receptor binding may be expected to differ also. Finally, the apparent *intracellular* location of a regulatory sodium-binding site (Puttfarken et al., 1986) could result in disparities between assays using whole cells and those using cell homogenates or extracts.

Advances in the problems of receptor heterogeneity have been made recently by using tissues having predominantly one type of receptor, such as rabbit cerebellum or guinea pig cerebellum, containing mainly μ- and mainly κ-sites, respectively (Meunier et al., 1983; Robson et al., 1984). In this paper we will simplify the problem of differing ligand chemistry by limiting ourselves to consideration of opioid peptide ligands. Furthermore, the data we analyze were obtained using fragmented cells, so that a potential influence of membrane transport to intracellular locations can be excluded.

The "membrane compartments" hypothesis for receptor subtype selection (Schwyzer, 1986b), which developed from studies on the interaction of peptide hormones with lipid bilayers (Schoch et al., 1979; Gremlich et al., 1983; Erne et al., 1985; Schwyzer, 1986a), posits a *membrane requirement* as well as a *receptor requirement* for peptide ligand–receptor interactions. Biologic and binding potencies and selectivities of opioid peptides for the opioid receptor κ-, μ-, and δ-sites (and of neurokinin peptides for NK-1, NK-2, and NK-3 sites) have been shown to correlate well with physical–chemical parameters describing the interaction of such peptides with pure lipid membranes (Schwyzer, 1986b, 1987). The membrane compartments hypothesis proposes that productive interaction with the κ-site is based on a spontaneous association of an amphiphilic peptide with the hydrophobic phase of the lipid membrane as a perpendicularly inserted helix, the binding taking place in an electrically negative environment (Schwyzer, 1986b; Sargent & Schwyzer, 1986). Typical opioid peptide–κ-site binding geometry has been observed for dynorphin A-(1–13)-tridecapeptide in pure lipid membranes (Erne et al., 1985). The N-terminal "message" segment of the peptide, which is necessary for triggering the receptor response, is located in a hydrophobic environment, implying that the κ-receptor binding site itself is found in such a hydrophobic compartment. The C-terminal "address" segment, which is responsible for both receptor subtype selection *and* this particular type of membrane interaction, but cannot trigger the receptor response, remains in contact with the aqueous phase. In this view the κ-triggering message segment is sequestered from the aqueous phase and the possibility of interaction with hydrophilic species is thus drastically reduced, while the charged address segment remains exposed. In contrast, both μ- and δ-receptors are proposed to be accessible to the aqueous phase and to react with peptides lacking strongly amphiphilic properties. The μ-receptor site appears to be in a negatively charged environment, while the δ-receptor site is presumably in a neutral or positively charged environment and may be located in the aqueous phase next to the membrane (Schwyzer, 1986b).

Biologic and binding potencies of peptide κ- and μ-ligands were found to correlate directly, selectivities of δ-ligands in-

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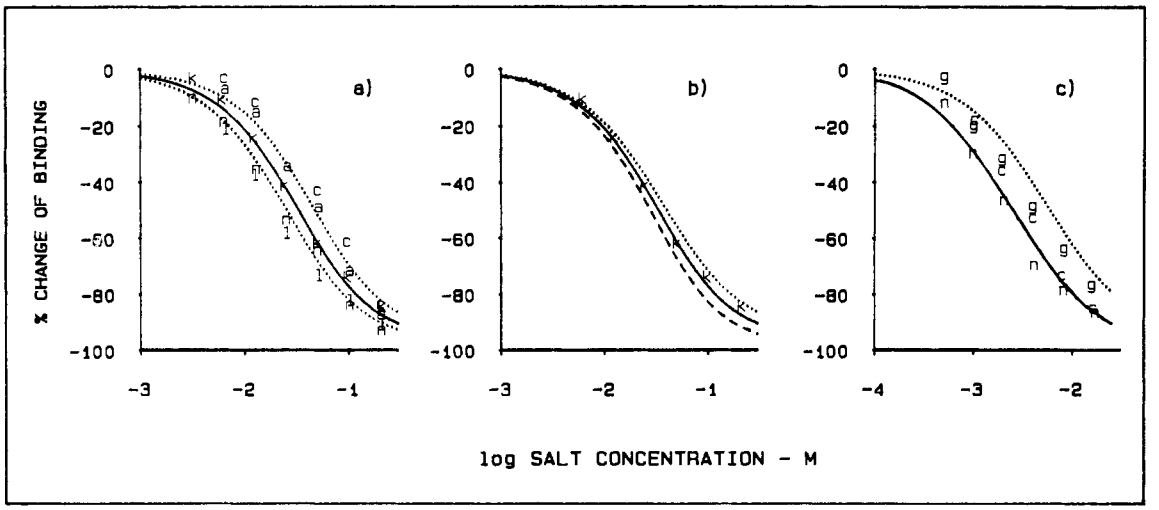

FIGURE 1: Suppression of dynorphin A-(1–9) binding to κ-sites of the guinea pig cerebellum by cations. (a) Data for the salts of five univalent cations (Paterson et al., 1986): a = NH_4Cl, c = choline chloride, k = KCl, l = LiCl, and n = NaCl. The solid line was drawn according to eq B and D by using a surface charge density σ = 0.133 charge/nm^2, a concentration of free ligand $c_f = 1 \times 10^{-10}$ M, and K_d(app) = 1×10^{-10} M. The effect of an uncertainty of a factor of 2 in the concentration of free ligand relative to the K_d is shown by dotted lines. (b) Effect of different surface charge densities on the predicted suppression by monovalent ions (k = KCl, heavy solid line as in panel a): for dotted line σ = 0.11 charge/nm^2; for dashed line σ = 0.17 charge/nm^2. (c) Data for the salts of three divalent cations: c = $CaCl_2$, g = $MgCl_2$, and n = $MnCl_2$ (Paterson et al., 1986). The dotted line was calculated by using σ = 0.133 charge/nm^2 and $c_f = 1 \times 10^{-10}$ M, as in (a). A better fit can be obtained assuming a small amount of nonspecific cation binding to the lipid phase; the heavy line was calculated assuming a 2:1 lipid:cation^{2+} complex having a K_a = 0.2 M^{-1}.

versely with the number of positive charges on the peptide (Schwyer, 1986b; Bean et al., 1988). As most biological membranes exhibit a negative surface potential due to the presence of negatively charged phospholipids or gangliosides, the simplest explanation for these correlations is that the surface concentration of κ- and μ-ligands is increased by the fixed charge surface potential of the membrane with which the receptors are associated [a general treatment of this phenomenon is given by Sargent and Schwyzer (1986)]. Influences of other possible determinants of potency and selectivity, stemming from receptor requirements for specific amino acid sequences and preferred conformations, cannot be ruled out. Definite confirmation of the role of surface accumulation would require the use of one ligand at different surface potentials. Experiments corresponding to this situation were published recently for the binding of dynorphin A-(1–9)-nonapeptide to κ-receptors of guinea pig cerebellum (Paterson et al., 1986) and analyzed here. As the binding results were obtained for various monovalent and divalent salts, the importance of specific ion effects at the binding site can also be assessed.

Theory

The relationship between bulk concentration of a charged species, c_o, the concentration of this charged species next to a charged surface, c_s, and the fixed charge surface potential, Ψ, is given by the Boltzmann equation (Aveyard & Haydon, 1973)

$$c_s = c_o \exp(-zF\Psi/RT) \qquad (A)$$

where z is the charge of the species considered, F the Faraday constant, R the gas constant, and T the absolute temperature. The fixed charge surface potential can be described by the Gouy–Chapman equation relating the surface potential to the surface charge density (electronic charges per nm^2), σ, and the ionic composition [e.g., McLaughlin et al. (1971)]:

$$\sigma = (1/2.72)[\textstyle\sum_i c_i(\exp(-z_iF\Psi/RT) - 1)]^{0.5} \qquad (B)$$

where c_i is the bulk concentration (mol/L) of the ith ion of valence z_i.

The binding of a ligand to its receptor is described by the standard binding isotherm

$$c_b = c_{b\,max}/(1 + K_d/c_f) \qquad (C)$$

where c_b is the amount bound at a free concentration c_f, $c_{b\,max}$ is the bound concentration at saturating levels of c_f, and K_d is the dissociation constant. For membrane-bound receptors located in the charged surface we must substitute c_s from eq A for c_f in eq C, yielding

$$c_b = c_{b\,max}/[1 + K_d(in)/c_o \exp(-zF\Psi/RT)] \qquad (D)$$

where instead of K_d we now write the intrinsic dissociation constant K_d(in). The exponential term of eq D may be interpreted either as a correction for the surface concentration of ligand, as derived above, or as a correction factor for the "apparent K_d" if c_o is substituted for c_f in eq C. In this interpretation the "intrinsic K_d" is defined as

$$K_d(in) = K_d \exp(-zF\Psi/RT) \qquad (E)$$

Results

The binding data of Paterson et al. (1986) were obtained for a constant concentration of the triply charged dynorphin A-(1–9)-nonapeptide of about 10^{-10} M. This corresponds to the apparent K_d they determined in their starting buffer, which had an ionic strength of about 40 mM (50 mM Tris-HCl at pH 7.4). The effect of different salt concentrations on binding can be calculated by solving eq B for Ψ at each salt concentration. Substituting this Ψ in eq d, we find the predicted value of c_b at the particular salt concentration. In line with Paterson et al. (1986) the results are expressed as a suppression of binding compared to that at the background ionic strength of 40 mM.

Figure 1a presents the experimental data and fitted curves for a range of concentrations of various monovalent salts [total ionic strength is 40 mM (buffer) plus the value given on the abscissa]. The bulk concentration of the ligand, c_o (eq B), was set to the nominal value of 1×10^{-10} M. Uncertainty in c_o is reflected in a simple shift of the suppression curve on the

X axis; the effect of a variation in c_0 by a factor of 2 is indicated by the dotted lines in Figure 1a. An uncertainty of this magnitude between experiments (e.g., between the determinations for the different ions) is inherent in the methods used. The only variable parameter in the fits shown is σ, the surface charge density. The fitted value, 0.133 charge/nm^2, is fixed by the slope of the curve and is consistent with known membrane compositions [e.g., Farahkakhsh et al. (1986)]. Examples for 20% higher and lower values of σ are shown in Figure 1b and obviously bracket the allowed range of this parameter. By use of the fitted parameters, eq E yields $K_d(\text{in}) = 1.3 \times 10^{-8}$ M.

Figure 1c reproduces the data of Paterson et al. (1986) for the influence of divalent cations on the binding of dynorphin A-(1–9)-nonapeptide. The fitted curves use the value for σ determined in the analysis of the suppression by univalent cations (Figure 1a). The dotted curve was calculated assuming pure shielding, while the solid line includes a small degree of binding of the divalent cations to the membrane phase, as is generally considered to occur (McLaughlin et al., 1971; Altenbach & Seelig, 1984). A binding constant for divalent cations of 0.2 M^{-1} was used in Figure 1c.

Discussion

If only nonspecific effects such as shielding are present, then all the salts of the same valence type should cause the same degree of suppression, as is indeed observed within experimental uncertainty. The fitted curve of Figure 1a shows that the binding data of Paterson et al. (1986) in the presence of univalent cations can be completely accounted for by electrostatic shielding effects using reasonable parameters for the system. The hypothesis that only such shielding effects are involved is strenghthened by the excellent fit, using the same membrane parameters, to the data obtained with divalent cations, which shield much more effectively than univalent cations. The amount of divalent cation binding needed to obtain complete congruence of the measured and fitted curves (0.2 M^{-1}) lies between the value of about 10 M^{-1} for phosphatidylcholine membranes determined directly by Altenbach and Seelig (1984) and that inferred from measurements of ionophore-induced conductivities of phosphatidylserine bilayer membranes, for which a binding constant of Ca^{2+} of about 0.1 M^{-1} was deduced (McLaughlin et al., 1971). In any case, the correction is minor and no other effects need be invoked to explain the observed suppression.

Thus the results for ion effects on κ-binding of dynorphin A-(1–9)-nonapeptide in fragmented cell systems can be summarized as representing predominantly shielding, with no evidence for significant specific effects of any of the ions tested. This is exactly what the membrane compartments theory of receptor selection predicts on the basis of a ligand showing electrostatic accumulation at a charged surface interacting with a binding site which is itself inaccessible to purely ionic species, such as simple salts.

Other reports in the literature on the effects of ions on κ-binding have involved nonpeptide ligands [e.g., Frances et al. (1985) and Werling et al. (1986)] or peptide derivatives of low or zero net charge [e.g., Werling et al. (1986)]. Major accumulation effects are not to be expected with these ligands, so that more subtle effects may be revealed. Nevertheless, the magnitude of the effects found is small compared to the other opioid receptors, again supporting the view that the κ-binding site is relatively inaccessible to aqueous solutes.

For the opiate μ- and δ-receptors the situation is different. Studies at constant ionic strength have revealed specific effects of, for instance, sodium (Kouakou et al., 1982; Chang et al., 1983; Puttfarken et al., 1986; Werling et al., 1986), manganese (Jauzac et al., 1984), or magnesium ions (Demoliou-Mason & Barnard, 1986b; Puttfarken et al., 1986). Thus the μ- and δ-receptors must be accessible to aqueous solutes, supporting the surface (or extra-membrane) locations embodied in the membrane compartments hypothesis for these receptors.

The implication of surface effects for pharmacological events have been pointed out by several authors [e.g., Blank (1979), Leysen and Gommeren (1981), and Sargent and Schwyzer (1986)]. Simple electrostatic accumulation and shielding as considered here are general physical phenomena and are thus applicable to interactions between any charged species. Even with neutral ligands the ionic strength of the bulk phase could play a role if there are any charges near the binding site; altered shielding of the local charges could affect charge distribution and electrostatic forces within the binding site, thus potentially influencing reaction parameters. While other mechanisms must be sought to explain ion-specific effects, the simple electrostatic effects must be considered as a backdrop in all cases and accounted for when specific effects are being investigated. Only in this way can the specific effects, such as are found with the μ- and δ-receptors, be properly defined and quantitated.

Registry No. NH_4Cl, 12125-02-9; KCl, 7447-40-7; LiCl, 7447-41-8; NaCl, 7647-14-5; dynorphin A-(1–9), 77259-54-2; choline chloride, 67-48-1.

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Articles

Murine Epidermal Growth Factor: Structure and Function

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ABSTRACT: Murine epidermal growth factor (EGF), a 53 amino acid protein, has been modified by enzymic digestion, site-specific chemical reactions, and recombinant DNA technology. After trypsin digestion the EGF derivatives EGF_{1-48} (called EGF-T) and EGF_{1-45} (called EGF-T_2) were separated from the residual EGF and the C-terminal pentapeptide by reversed-phase high-performance liquid chromatography. EGF-T competes for binding to EGF receptors with the same efficiency as EGF. The EGF-T_2 derivative had no detectable receptor binding activity even at 100 nM. The in vitro mitogenic potencies of EGF and EGF-T for Balb/c 3T3 cells were indistinguishable. Treatment of EGF-T with carboxypeptidase Y yielded two derivatives, EGF-T-(des-Arg_{48}) and EGF-T-des(Leu_{47}-Arg_{48}). There was only a 3–7-fold diminution in the binding efficiency and mitogenic potency for EGF-T-(des-Arg_{48}). However, there was more than a 100-fold decrease in the binding efficiency and mitogenic activity of EGF-T-des(Leu_{47}-Arg_{48}). These results indicated that Leu_{47} is intimately involved in the formation of the ligand–receptor complex. Studies with a number of proteases indicated that the C-terminus of EGF was susceptible to enzymic digestion; however, the N-terminus appears to be folded into a conformation which prevents access to proteolytic digestion. Consequently, the N-terminus was modified by preparing an analogue with recombinant DNA technology. Oligonucleotides corresponding to $EGF_{(3-48)}$.Met_3.Lys_{21} residues were ligated in frame to a β-galactosidase expression vector. The β-Gal–EGF fusion protein was cleaved with cyanogen bromide and $EGF_{(4-48)}$.Lys_{21} purified. This derivative was equipotent with EGF in the mitogenesis assay and bound to the EGF receptor with the same affinities as EGF. Disruption of the central antiparallel β-sheet structure of EGF at Met_{21} by treatment of EGF with cyanogen bromide reduced both the binding efficiency and the mitogenic activity of EGF more than 100-fold.

Murine epidermal growth factor (EGF) is a protein of 53 amino acids capable of stimulating the proliferation of both mesenchymal and epithelial cells (Savage et al., 1972; Taylor et al., 1972). Considerable progress is being made toward the determination of the three-dimensional structure of EGF (Mayo, 1985; Mayo et al., 1986; Montelione et al., 1986, 1987; Carver et al., 1986; Cooke et al., 1987), but there has been little progress toward an understanding of the function of particular residues or even specific regions of the EGF molecule. It is important to distinguish between the residues required for the folding and assembly of the three-dimensional structure, as well as those residues required for the binding to and activation of the EGF receptor. Preparation of EGF's and their analogues by complete chemical synthesis (Akaji et al., 1985; Heath & Merrifield, 1986; Tam et al., 1986; Scanlon et al., 1987) and by bacterial synthesis (Sumi et al., 1985) has shown that EGF mitogen can fold to its biologically active form. However, attempts to produce subdomains with full biological or potent antagonist activity have yielded derivatives with very low potency (Komoriya et al., 1984; Nestor et al., 1985; Heath & Merrifield, 1986). It is still not clear whether these analogues are missing specific residues important for the binding of EGF to its receptor or whether the short segments fail to fold into the conformation required for receptor binding. Disruption of the three-dimensional structure of EGF e.g., by reduction and carboxymethylation (Savage et al., 1972), destroys both its receptor binding and its biological activity. However, disruption of the polypeptide backbone by cyanogen

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