Rat brain membrane-bound delta opioid receptor: Loss and reactivation of binding on dialysis and aging at low temperature

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Received 7 July 2000; revised and accepted 8 August 2000

A change in the environment of rat brain membranes by dialysis from phosphate buffered saline (PBS) to 10 mM potassium phosphate (pH 7.2) led to a 35% loss in delta opioid receptor binding, while alteration of membrane structure on freezing at −20°C for 55 days led to 85% loss of receptor binding. The dialysate, 200 mM KCl and NaCl restored receptor binding lost on dialysis. This K⁺ and Na⁺restabilization of the receptor can be through cation-π bonding, interactions that are suited to the lipid bilayer. In membranes stored at −20°C, the loss of binding is attributed to increased membrane fluidity by phospholipase A² action on membrane phospholipids, resulting in an increase of free fatty acids. K⁺ but not Na⁺restabilization of these membrane receptors may be due to the ability of K⁺ to decrease membrane fluidity.

Cell membrane receptors are governed by their environment and dynamically regulated by both extra and intracellular modulators. Thus, the G protein coupled opioid receptors that exhibit stereospecificity in ligand binding are sensitive to the composition of the membrane lipid bilayer as well as to ionic strength, pH and temperature. They are differentially regulated by monovalent and divalent cations with modulation that can be through ionic interactions as well as by cation-π bonding. Na⁺ is reported to induce a conformational change in opioid receptors from an agonist to an antagonist binding state. This may arise by an uncoupling of the receptors from their G proteins, as monovalent cations are known to bring about such uncoupling. Uncoupling which results in a loss of guanine nucleotides from the environment of the opioid receptors can be a reason that their addition stimulates receptor binding.

As with other membrane proteins, opioid receptors exhibit specific lipid interactions that are essential for high affinity opioid binding. Properties of the membrane lipid bilayer that do not allow or reduce these interactions such as an increase in membrane fluidity lead to a loss of receptor binding. In studies on the rat brain membrane-bound delta opioid receptor in which the membrane environment was changed through dialysis, and membrane structure altered upon freezing, we observed a 35% loss in binding on dialysis while aging for 55 days at low temperature (−20°C) resulted in an 85% loss of binding. In the present study, we examine factors that may be involved in this loss of binding and which contribute to a reactivation of receptor binding.

Materials and Methods

Materials
α-chymotrypsin, GTP, phospholipase A₂, chlorpromazine, bovine serum albumin fraction V and dialysis tubing that retains molecular weights of >12,000 were from Sigma Chemical Co, USA. Goat anti-rabbit IgG coupled to horseradish peroxidase (HRP) was from Banglore Genie Laboratories, India. All other chemicals were of analytical grade. Microtitre plates were maxisorb from NUNC Laboratories, Denmark.

White laboratory rats with free access to water and commercial rat feed were used in this study.

Methods

Rat brain membranes prepared from whole brains (minus cerebellum) of 60 day old rats by a modified method of Bochet et al., as given earlier served as the source of the delta opioid receptor.

Dialysed membranes were prepared by dialysing 500 µg membrane protein in 500 µl phosphate buffered saline (PBS) against 500 ml 10 mM potassium phosphate buffer pH 7.2 for 12 hr at 4°C with three changes of buffer.

For determination of membrane aging at −20°C, rat brain membranes in 0.05 mM Tris HCl pH 7.2 were
stored in 500 µl aliquots at -20°C for 5, 20, 35 and 55 days with and without the dialysate (diluted five-fold), 1 mM chlorpromazine, 200 mM KCl, NaCl or LiCl.

The dialysate was prepared by dialyzing rat brain membrane (30 mg in 3 ml PBS) against 50 ml 10 mM potassium phosphate buffer, pH 7.2 for 12 hr at 4°C with three changes of buffer, lyophilizing this buffer and reconstituting in 1.5 ml water.

To study the effect of the dialysate on the delta opioid receptor, it was pre-incubated at different dilutions (five- and ten-fold dilutions) with dialyzed and undialyzed membrane receptor for 2 hr at 4°C prior to receptor assay.

The effect of Na⁺ in presence of the dialysate on the receptor was studied by pre-incubating the dialysed receptor with the dialysate (diluted five-fold) and 0.1 to 78 mM NaCl for 2 hr at 4°C prior to receptor assay.

The effect of heat on the dialysate was studied by subjecting 20 µl aliquots to 0, 28, 37 and 56°C for 30 min and to 100°C for 5 min followed by cooling in ice prior to incubation with 10 µg of dialyzed receptor.

The effect of proteases on the dialysate was studied by incubating 200 µl dialysate with 10 µl (1 mg/ml) chymotrypsin at 37°C for 30 min, followed by 5 min in a boiling water bath to arrest the reaction. Dialyzed rat brain membranes (10 µg) were incubated with 20 µl of this dialysate for the receptor assay.

The dialysate was analyzed for Na⁺ by flame photometry and for GTP by HPLC on an ODS C18 column using 4 mM tetrabutylammonium hydrogen sulfate in 100 mM potassium phosphate buffer, pH 6.0 and this buffer with methanol (7:3) as given by Stocchi et al.¹³.

Free fatty acids in membranes and in the dialysate were analysed by the method of Bligh and Dyer¹⁴ by TLC and quantitated by gas chromatography. Ca²⁺ stimulation of fresh membranes (10 µg protein) was for 1 hr at 37°C with 1 mM CaCl₂. Phospholipase A₂ treatment of fresh membranes (10 µg protein) was for 1 hr at 37°C with 1 mM phospholipase A₂.

The delta opioid receptor was assayed by the procedure given earlier¹¹ on an ELISA measuring the binding of rabbit anti-idiotypic antibodies to antileucine enkephalin which mimic leucine enkephalin in binding to the receptor. Briefly, microtiter plates were coated with 10 µg (100 µl) membrane protein for 12 hr, blocked and incubated for 4 hr with the anti-idiotypic antibodies, followed by HRP goat anti-human IgG for 2 hr. All reactions were at 4°C. Bound peroxidase was developed with 0.04% o-phenylene diamine/0.01% H₂O₂ at 25°C for 20 min and plates scanned at 490 nm.

Protein was estimated by the method of Lowry et al.¹⁴ using bovine serum albumin as standard.

All experiments were carried out twice and run in duplicate.

**Results**

Dialysis of the rat brain membrane-bound delta opioid receptor from PBS to 10 mM potassium phosphate buffer, pH 7.2 led to 35% loss in binding of the receptor which was restored 130% by the addition of the dialysate diluted five-fold.

Neither heat treatment nor chymotrypsin digestion affected the receptor reactivating property of the dialysate.

Flame photometry of the dialysate indicated that it contains 380 µM Na⁺ while UV spectroscopy and HPLC showed the presence of GTP at 254 nm to be 20 µM.

Neither NaCl at 20-400 µM nor GTP at 50-2000 µM alone nor 20-400 µM NaCl with 50 µM GTP restored loss in binding of the dialysed receptor.

NaCl up to 78 mM did not affect the receptor reactivating property of the dialysate.

NaCl alone at 50-400 mM reactivated binding of the dialysed membrane-bound delta opioid receptor from 80 to 100%.

Increasing concentrations of K⁺ from 50 to 250 mM reactivated binding of the dialysed receptor 120 to 160%.

200 mM KCl with 20 µM GTP, the concentrations of K⁺ and GTP in the dialysate, reactivated the dialysed receptor to 135% i.e. to the same extent as the dialysate.

50 mM KCl enhanced binding of the undialysed receptor by 25% while 50 mM NaCl and LiCl inhibited the binding by 10%. More pronounced increase and inhibition of binding was observed with 200 mM K⁺, Na⁺ and Li⁺ (Fig.1).

Cu²⁺ and Zn²⁺ at 1 mM inhibited the binding of the undialysed receptor by 70 and 40% and that of the dialysed receptor 20 and 30% respectively. Inhibition by both divalent cations was by decreasing ligand affinity (kd) from 0.15 to 0.22 and 0.3 mM and the number of binding sites Bmax from 53 to 31 and 20 pmoles/mg protein, indicating a non-competitive inhibition.
Membrane aging at -20°C

Membranes stored at -20°C for 55 days in 0.05 M Tris HCl, pH 7.2 or in the dialysate (five-fold diluted) or in 200 mM KCl, NaCl or LiCl, all progressively lost up to 85% receptor binding activity over this time (Fig. 2). The loss in binding was greatest for membranes stored in the buffer, NaCl or LiCl of up to 70, 71 and 63% respectively at 35 days. For membranes stored in the dialysate or in 200 mM KCl, the loss in receptor binding after 35 days at -20°C was 25 and 44% respectively (Fig. 2).

Free fatty acid content of the membranes stored at -20°C increased with time up to 35 days from 20 to 32 nmoles/mg protein as determined by TLC (Fig. 3).

The free fatty acid content of fresh membrane stimulated with 1 mM Ca²⁺ was equivalent to that of membranes stored at -20°C for 35 days (Fig. 3).

Membranes stored at -20°C for 35 days with 1 mM chlorpromazine, a phospholipase A₂ inhibitor had less free fatty acid than membrane stored without chlorpromazine as determined by TLC and GLC (Fig. 3).

Membranes stored for 20 days at -20°C in Tris HCl, pH 7.2 exhibited 40% loss in binding of the receptor (Fig. 2) while membranes stored with 1 mM chlorpromazine for this period exhibited a 20% loss in receptor binding.

Fresh membrane-bound receptor lost 70% binding activity on treatment with 1 mM PLA₂.

Discussion

The 35% loss in binding of the rat brain membrane bound delta opioid receptor when its environment was changed through dialysis from 100 mM sodium phosphate buffer, pH 7.2 containing 140 mM sodium chloride to 10 mM potassium phosphate buffer at the same pH, was mostly studied through reconstitution. This was because of the observation that the (concentrated) dialysate restored this receptor.

Membranes stored for 55 days at -20°C in Tris HCl, pH 7.2 (1); with 1:5 diluted dialysate (2); with 200 mM KCl (3); NaCl (4) and LiCl (5) for 5, 20, 35 and 55 days and membranes stored with 1 mM chlorpromazine (6) for 20 days. Delta opioid receptor binding was assayed as given under Methods. Values represent the mean ± SD of three experiments run in duplicate.

Fig. 2—Effect of the medium on the rat brain membrane bound delta opioid receptor [Rat brain membranes (500 µg) stored at -20°C in 0.05 M Tris HCl, pH 7.2 (1); with 1:5 diluted dialysate (2); with 200 mM KCl (3); NaCl (4) and LiCl (5) for 5, 20, 35 and 55 days and membranes stored with 1 mM chlorpromazine (6) for 20 days. Delta opioid receptor binding was assayed as given under Methods. Values represent the mean ± SD of three experiments run in duplicate].

Fig. 3—Free fatty acids in rat brain membrane [Free fatty acids were extracted from rat brain membranes (2 mg protein) that had been stored at -20°C for 5, 20 and 35 days (1); from fresh membranes stimulated with 1 mM CaCl₂ (2); from membranes stored with 1 mM chlorpromazine (3) for 20 and 35 days. [Free fatty acids were separated on TLC and quantitated by GLC as given under methods. Values represent the mean ± SD of two experiments run in duplicate].
binding. Following the method of preparation the dialysate was 1 M potassium phosphate pH 7.2 with dialysable components from 3 mg of the rat brain membrane.

Opioid receptors being G protein coupled receptors, the dialysate was examined for GTP, which was found to be present at 20 &mu;M. However, the addition of up to 2 mM GTP to the dialysated receptor did not re-activate receptor binding suggesting that factors other than GTP in the dialysate are needed for restoring receptor binding. This would argue against the loss in binding on dialysis resulting from an uncoupling of the receptor from its G protein. Receptor re-activation was found to be a function of K+ in the dialysate. K+ up to 200 mM reactivated the receptor, which may indicate a role for the intracellular concentration of K+ (150 mM) in active receptor maintenance. The same may be said of Na+ from our observations that high (400 mM) but not low (400 &mu;M) concentrations restored receptor binding 100% but did not stimulate the activating property of the dialysate. K+ and Na+ re-stabilization of the membrane receptor can be through cation-π interactions that are suited to cell membrane environments as opposed to ion pairing that is not feasible in a hydrophobic medium. Cation-π bonding peaks naturally with K+ in water and is greater than with Na+ which is greater than that with Li+ (ref 4) and may underlie the greater reactivation of the dialysated receptor by K+ compared to Na+ seen in this study.

Inhibition of the undialyzed receptor by Na+ and Li+ but not by K+ would also support a stronger cation-π bonding of K+ in maintaining the stability of the receptor. Inhibition of the delta opioid receptor by the divalent cations Cu2+ and Zn2+ has been reported earlier 15 and the non-competitive nature of the inhibition eliminates their role in the activating property of the dialysate and in stabilizing the dialyzed membrane receptor conformation.

The observed rise of free fatty acids in membranes after 35 days at -20°C, resulting in an increase in membrane fluidity and consequently a loss in membrane structure, is implicated in the concomitant 70% loss in binding of the delta opioid receptor. Ca2+ stimulation of free fatty acids in the membrane, phospholipase A2 induced loss of receptor binding and chlorpromazine protection of the membrane-bound delta opioid receptor indicates phospholipase A2 in the degradation of the membrane. We noted that rat brain membranes stored in 0.05 M Tris HCl, pH 7.2 or 200 mM NaCl at -20°C lost receptor binding activity over 35 days more rapidly than those stored in the dialysate or in 200 mM KCl. This may reflect the property of K+ but not Na+ to decrease membrane fluidity, as has been observed in the rat cerebral cortex 16, thereby stabilizing the membrane. Studies measuring membrane fluidity are needed to corroborate these findings.

We conclude that K+ ions increase membrane-bound delta opioid receptor binding in fresh and aged, dialyzed and undialyzed rat brain membranes possibly by stabilizing the membrane through high cation-π interactions that may protect against membrane fluidization. Na+ may also stabilize the membrane-bound receptor through cation-π bonding but as this interaction is weaker than that with K+ it may not offer the same degree of protection against membrane destabilization due to increased fluidization.

References

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