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Frontal analysis of cell-membrane chromatography for determination of drug-α_{1D} adrenergic receptor affinity

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ABSTRACT

The aim of the present study was to determine drug-α_{1D} adrenergic receptor (AR) affinity by frontal analysis of cell-membrane chromatography (CMC). The cell-membrane stationary phase (CMSP) was prepared by immobilizing rat aorta cell membranes on porous silica, and the resulting CMSP was used to determine drug binding affinity to α_{1D}-AR by frontal analysis. The CMSP of rat aorta was stable and reproducible. Relative binding affinities (dissociation constant, K_d) were determined by frontal chromatography for prazosin (166.13 ± 18.36 nmol), BMY7378 (537.40 ± 30.84 nmol), phentolamine (646.92 ± 23.17 nmol), 5-methylurapidil (725.66 ± 25.48 nmol), oxymetazoline (910.56 ± 40.62 nmol) and methoxamine (1299.27 ± 51.73 nmol). These results were consistent with the affinity rank order and showed a good correlation with the affinity of the same compounds for the cloned α_{1D}-AR subtype obtained from radioligand-binding assay. The study demonstrates that frontal analysis of CMC may be used for direct determination of drug–receptor binding interactions, and that CMC is an alternative reliable method to quantitatively study ligand–receptor interactions.

1. Introduction

Cell-membrane chromatography (CMC), a novel bioaffinity chromatographic technique originated by He et al. in 1996 [1–3], can be applied to the study of drug–receptor interactions. In previous studies [4–6], human embryonic kidney (HEK) 293 cells that expressed cDNA of α_{1A}, α_{1B} or α_{1D} adrenergic receptor (AR) subtypes were cultured, and the cell-membrane stationary phase (CMSP) was prepared by immobilizing cell membranes on the surface of silica, which acted as carrier. Then, the interactions between nine ligands of the α_1-AR and α_{1A}, α_{1B} or α_{1D}-AR in the CMSP were investigated using CMC. The ligand–receptor affinity was shown by chromatographic parameters (capacity factor, k'). The results of the above studies showed that the prepared CMSP and CMC method were useful in evaluating drug–receptor affinity. In addition, the ligand-binding affinity to muscarinic acetylcholine receptor has been evaluated by CMC by immobilizing rat cerebral cell membrane and guinea pig jejunum membrane on the surface of a silica carrier [7,8]. These results have also shown that CMC can be used to evaluate drug–receptor affinity of drug candidates.

Functional assay and radioligand-binding assay undoubtedly contribute to a better understanding of the interaction between ligands and receptors, but they have some disadvantages. CMC is expected to become a reliable method to study ligand–receptor interactions. However, CMC have been not used to assay drug dissociation constant (K_d) and total receptor (B_t) exactly and directly, compared with radioligand-binding assay (RLBA) and functional assay. To improve CMC, we combined it with frontal affinity chromatography to determine K_d and B_t.

The α_1-AR, which is activated by adrenaline and noradrenaline, is a membrane protein and a member of the G-protein coupled receptor superfamily. According to recommendations from NC-IUPHAR, based on pharmacological and molecular evidence, the α_1-AR is divided into three subtypes: α_{1A}, α_{1B} and α_{1D} [9]. The α_1-AR family is of particular therapeutic interest because its constituents mediate a variety of physiological effects in many tissues, such as neurotransmission, vasoconstriction, cardiac inotropy and chronotropy, and glycogenolysis. In the past two decades, with the cloning of three α_1-AR subtypes, there was a great thrust to understand the expression of α_1-AR subtypes. The α_{1D} subtype is expressed in a variety of tissues, including vascular smooth muscle, cerebral cortex [10,11], and probably rat lung [12]. In rat aorta and iliac artery, it appears to be the predominant subtype that mediates vasoconstriction [13,14].

The aim of this research was to prepare the rat aorta cell-membrane stationary phase, determine the affinity (K_d) of drug-α_{1D} adrenergic receptor interaction and total receptor (B_t) by frontal analysis, and create an alternative reliable method to study quantitatively ligand–receptor interactions.

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2. Experimental

2.1. Chemicals

Prazosin, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspirol[4,5]decane-7,9-dionedihydrochloride (BMY7378), 5-methylurapidil, oxymetazoline, phentolamine, and methoxamine were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA); *N*-(2-(2-cyclopropylmethoxy)ethyl)-5-chloro-α-dimethyl-1*H*-indole-3-thylamine (RS17053) was provided by Tocris Bioscience (Ellisville, MO, USA). Macroporous spherical silica (7 μm, 100 Å) was purchased from the Institute of Chemistry of the Chinese Academy of Sciences (Beijing, China). The water used in the study was prepared using a Milli-Q Water Purification System (Milli-Pore, Bedford, MA, USA).

2.2. Animals

Sprague–Dawley rats (5–7 weeks old) were supplied by the Experimental Animal Centre of Xi'an Jiaotong University (Xi'an, China). All experimental protocols involving animals were reviewed and approved by the Institutional Animal Experimentation Committee of Xi'an Jiaotong University.

2.3. Membrane preparation

Rats were sedated with CO_2 and decapitated. The rat aorta was removed immediately, washed thoroughly, cut into small pieces, and added to ice-cold phosphate-buffered saline (PBS; 8.0 g L^{-1} NaCl, 1.15 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KCl, 0.2 g L^{-1} KH_2PO_4, pH 7.0). The tissue suspension was then homogenized at 4 °C (twice, each time for 1 min) with a Polytron homogenizer equipped with a PT35ST probe. The crude homogenate was centrifuged at 1000 × *g* to remove cellular debris. The supernatant was centrifuged for 20 min at 12 000 × *g* (Hermel ZK401 high-speed refrigerated centrifuge; Berthold Hermel AG, Gosheim, Germany). The buff-colored layer around the pellet was gently re-suspended in the above buffer, and centrifuged as before. All procedures were performed at 4 °C.

2.4. Preparation of CMSP

The CMSP was prepared by immobilizing the rat aorta cell membranes on the surface of silica (7 μm, 100 Å), which acted as a carrier, and was used for rapid on-line chromatography. The preparation procedure was similar to that described previously [1,2]. In brief, 600 mg macroporous spherical silica activated at 120 °C was placed in a 10-mL reaction tube, followed by a suspension of rat aorta cell membranes (generally, the concentration of membrane protein was about 2.12 mg mL^{-1}). The adsorption of the rat aorta cell membranes to the activated silica surface took place at 4 °C, until equilibrium was reached. The whole adsorption process was carried out under vacuum and ultrasonication. The reaction mixture was diluted with an equal volume of deionized water. The cell-membrane phospholipids were able to fuse spontaneously with each other (self-fusion) on the silica surface in the aqueous solution, until a resealed cell-membrane layer was obtained. The residual free cell membranes were washed with Tris–HCl buffer (pH 7.4). The prepared CMSP particles in the buffer were packed into a chromatography column (10 mm × 1.2 mm I.D.) under low pressure, by the slurry method.

2.5. Chromatographic system

The HPLC system consisted of an SCL-10Avp system controller, an LC-10AT*vp* pump, and an SPD-10A*vp* UV–vis detector (Shimadzu, Kyoto, Japan). The data were acquired by LCsolution software (version 1.0, Shimadzu, Kyoto, Japan). The mobile phase consisted of PBS (5 mM, pH 7.4) and ligand, and the flow rate was 0.2 mL min^{-1}. The ligands were detected using UV absorption at 278 nm (BMY7378, phentolamine and RS17053), 250 nm (prazosin), 282 nm (oxymetazoline), 284 nm (5-methylurapidil), or 292 nm (methoxamine). The column was placed into a column oven at a temperature of 37 °C.

2.6. Frontal chromatography studies

All ligands were dissolved in ethanol at a concentration of 10 mM as stock solutions, and further diluted with PBS to obtain a chromatographic mobile phase that contained serial concentration of ligands (oxymetazoline, 200, 400, 600, 800 and 1000 nM; 5-methylurapidil, 200, 400, 600, 800 and 1000 nM; prazosin, 80, 100, 200, 300 and 400 nM; phentolamine, 400, 600, 700, 800 and 1000 nM; BMY7378, 100, 200, 400, 600 and 800 nM; methoxamine, 200, 400, 600, 800 and 1000 nM). The chromatographic mobile phase was degassed before use. Frontal analysis was performed using the chromatographic mobile phase at a flow rate of 0.2 mL min^{-1}. A correction for the system void time was made by performing similar experiments using acetone as a non-retained solute. The retained compounds were eluted and the column regenerated between studies by passing PBS through the column. All experiments were performed in triplicate under each set of tested conditions.

2.7. Calculation of dissociation constants and binding sites

Frontal affinity chromatography can be used to characterize the binding of small molecules to an immobilized membrane-bound target and to determine binding affinities (K_d) and total receptor (B_t). K_d was calculated using a previously described approach [15–17]. In brief, K_d, as well as B_t of the immobilized cell membrane of the rat aorta can be calculated using Eq. (1):

$$[D] \times (V - V_{min}) = B_t \times [D] \times (K_d + [D])^{-1} \quad (1)$$

where [*D*] is the concentration of ligand, *V* is the retention volume of ligand measured at the midpoint of the breakthrough curve, and V_{min} is the retention volume of ligand at the highest applied concentration of the displacer ligand. *V* and V_{min} were calculated using a previously described approach [18]. From the plot of $[D] \times (V - V_{min})$ versus [*D*], ligand K_d can be obtained. The data were analyzed by non-linear regression with the sigmoidal response curve using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA).

2.8. Statistical analysis

The relation between the calculated K_d and the affinities of agents (published pK_i values obtained from RLBA at clonal a_{1D}-AR) for the receptor subtype were analyzed by correlation analysis. Correlation constants (r^2) were calculated by linear regression analysis using GraphPad Prism 4. All data were expressed as means ± SD and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Activity and specificity of the CMSP column

In the CMC experiment, because the silica surface was completely coated by cell membranes in CMSP, the ligand–CMSP interaction can reflect the ligand–receptor affinity of the cell membrane. In rat aorta, the a_{1D}-AR appears to be the predominant subtype that mediates vasoconstriction. Therefore, the selective α_{1D}-AR agents should have high affinity for the CMSP of the rat