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Journal of Chromatography B, 724 (1999) 65–72

JOURNAL OF CHROMATOGRAPHY B

Liquid chromatographic studies with immobilized neuronal nicotinic acetylcholine receptor stationary phases: effects of receptor subtypes, pH and ionic strength on drug–receptor interactions

Irving W. Wainer*, Yanxiao Zhang, Yingxian Xiao, Kenneth J. Kellar

Department of Pharmacology, Georgetown University School of Medicine, Medical–Dental Building, Washington, DC 20007, USA

Received 21 September 1998; received in revised form 7 December 1998; accepted 7 December 1998

Abstract

Nicotinic acetylcholine receptor (nAChR) α3-subunits, β4-subunits, α3/β4-subunit combination and α4/β2-subunit combination were immobilized on chromatographic stationary phases and the binding affinities of the different nAChR subtypes were chromatographically evaluated. The observed relative binding affinities of epibatidine were α4/β2>α3/β4 and epibatidine did not bind at α3-subunits and β4-subunits. No significant difference in binding affinities was observed on the α4/β2 nAChRs immobilized in immobilized artificial membrane (IAM) particles and those sterically immobilized on Superdex 200 beads. The effects of mobile phase pH and ionic strength on the binding affinities of the α3/β4 nAChRs support were also investigated. The results are consistent with the proposed ligand–nAChR binding model in which a cationic center exists at the binding site. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Binding affinity; Stationary phases, LC; Nicotinic acetylcholine receptor

1. Introduction

Identification of new drug candidates that bind specifically to a biological target is a critical step in the drug screening process. Therefore, it is important to develop techniques to rapidly reduce the number of possible candidates. Since the fundamental processes of drug action are dynamic in nature and have much in common with the basic mechanisms involved in liquid chromatography (LC), chromatographic techniques can be used for drug affinity studies by including active biological components in chromatographic systems [1–8].

Membrane receptors mediate the initial steps in drug activity. One family of receptors is nicotinic acetylcholine receptors (nAChRs). nAChRs are the predominant excitatory neurotransmitter receptors on muscles and autonomic ganglia**.** These receptors are composed of 5 subunits arranged around a central ion channel (Fig. 1). A variety of nAChR subtypes can be formed from specific combinations of nAChR subunits identified as α1–α9, β1–β4, γ, δ and ϵ [9,10].

Recently a stably transfected cell line has been established which expresses a high density of rat α3/β4 nAChR and can be labeled by [^{3}H]epibatidine ([^{3}H]EB) [11]. In a previous study, the α3/β4 nAChRs from this cell line were embedded in the phospholipid monolayer of the immobilized artificial membrane (IAM) HPLC stationary

*Corresponding author.

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PII: S0378-4347(98)00579-9

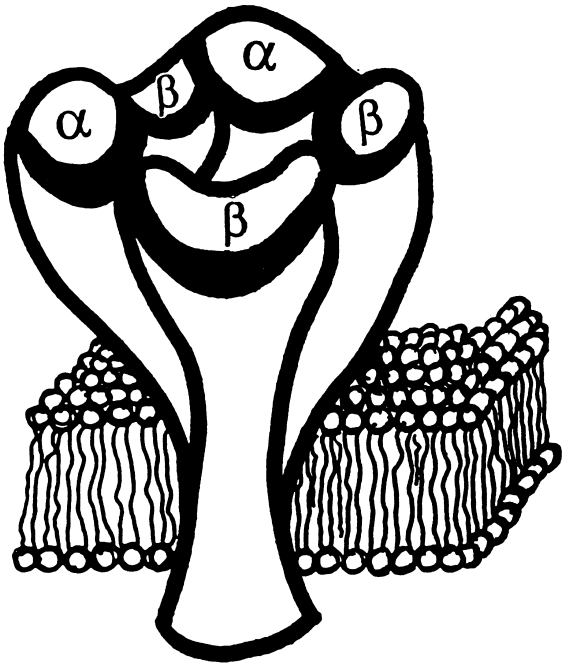


Fig. 1. Schematic illustration of structure of α3/β4 nAChRs.

phase [12]. In the IAM phase, the phosphatidylcholine headgroups form the hydrophilic surface of the support and the hydrocarbon side chains produce a hydrophobic interphase that extends from the charged headgroup to the surface of the silica [13], in which the nAChRs were entrapped without apparent loss of binding affinity. The binding affinities of five nAChR ligands, (±)-epibatidine, A85380, (−)-nicotine, carbachol and atropine, at α3/β4 nAChR were determined with the α3/β4 nAChR-IAM column [12].

In the present work, recombinant nAChR α3-subunits, β4-subunits and the α3/β4 subunit combination (referred to as the α3/β4 receptor subtype) were immobilized on an IAM stationary phase. The LC columns containing the immobilized receptor stationary phase were used to evaluate the drug binding affinities of the individual subunits and the combination. Rat forebrain nAChRs, which represent predominantly the α4/β2 receptor subtype [14,15], were also immobilized in the IAM stationary phases. The effects of mobile phase pH and ionic strength on the chromatographically determined binding affinities were investigated with the obtained nAChR columns. α4/β2 receptor subtype was also reconstituted into phospholipid bilayer of liposomes that were immobilized sterically in Superdex 200 gel beads. The methods were previously developed by Per Lundahl et al. for the immobilization of liposomes or liposomes containing membrane proteins (human red cell glucose transporter) in chromatographic stationary phase [16,17]. The chromatographic result obtained with the immobilized nAChR-liposome Superdex 200 was compared with the nAChR-IAM column.

2. Experimental

2.1. Materials

The nAChR α3 subunits, β4 subunits and the α3/β4 receptor subtype composed of α3+β4 subunits (α3/β4-subtype) were obtained from transfected human embryonic kidney cell lines that stably express each of these subunits individually or the combination [11]. The α4/β2 receptor subtype was obtained from rat brain membrane. IAM particles were obtained from Regis Chemical Co. (Morton Grove, IL, USA). Superdex 200® prep grade and glass column (HR5/5 and HR 5/10) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). L-α-Lecithin (20% phosphatidylcholine) and L-α-phosphatidylserine were purchased from Avanti Polar Lipids (Alabaster, AL, USA). (±)-Epibatidine [5,6-bicycloheptyl-^{3}H] (33.88 Ci/mmol) was from NEN Life Science Products (Boston, MA, USA). (±)-Epibatidine and A85380 were from Research Biochemical International (Natick, MA, USA). (−)-Nicotine, carbachol, atropine, cholic acid sodium salt (cholate) (>99%), cholesterol (>99%), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT, USA).

2.2. Preparation of nAChR-detergent solution

(a) *From rat forebrain tissue*: Rat whole forebrain was suspended in 50 m*M* Tris–HCl, pH 7.4 (buffer A), homogenized for 30 s with a Polytron homogenizer (Brinkmann Instrument, Westbury, NY) and centrifuged at 40 000 *g* for 10 min at 4°C. The pellet

was resuspended in 6 ml of 2% deoxycholate in buffer A (1 g of tissue per ml) and incubated for 2 h at 37°C. The mixture was centrifuged at 40 000 *g* for 30 min, and the supernatant containing nAChR-deoxycholate solution was collected.

(b) *From cells*: The solubilization procedure of the transfected cell membranes was similar to that previously described [12]. In brief, cells ($\approx 1\times10^6$) were harvested from the transfected cell lines [11] into 20 ml buffer A and homogenized for 20 s with the Polytron homogenizer and centrifuged at 35 000 *g* for 10 min. The pellets were suspended in 6 ml of 2% cholate in buffer A, stirred for 2 h at 0°C and centrifuged at 35 000 *g*. The supernant containing the solubilized nAChR was collected.

2.3. Immobilization of nAChRs in IAM particles

Dried IAM particles (200 mg) were suspended in 4 ml of the detergent solution containing nAChR subunits or subtypes, as described previously for immobilization of α3/β4-nAChRs [12]. For the immobilization of one nAChR subtype, the mixture of IAM-detergent-receptor was stirred for 1 h at room temperature. The suspension was dialyzed against 2×1 l buffer A for 24 h at 4°C. The IAM LC support with immobilized nAChRs was then washed with buffer A and centrifuged. The solid was collected.

2.4. Reconstitution and immobilization of nAChRs on Superdex 200 gel beads

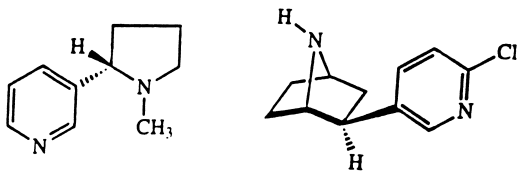
L-α-Lecithin (20% phosphatidylcholine) (60 mg), L-α-phosphatidylserine (90%)(10 mg), and cholesterol (20 mg) were solubilized together with 3 ml chloroform in a 20-ml vacuum evaporation flask. The chloroform was removed by vacuum evaporation on a water bath to form a lipid film. The lipid film was solubilized with 3 ml nAChR-cholate solution obtained by the solubilization of nAChR from rat whole forebrain or cell as described above. The nAChR-lipid-cholate solution was mixed with dried Superdex 200 beads (50 mg). The suspension was dialyzed against buffer A for 24 h at 4°C. Upon the deletion of the detergents, the nAChRs were reconstituted in the liposomal bilayer and the obtained liposomes containing nAChRs were immobilized sterically into the cavities of Superdex 200 gel beads. Non-immobilized liposomes were removed by centrifugation with 3×10 ml buffer A at 2000 *g*.

2.5. [^{3}H]epibatidine ([^{3}H]EB) binding assays for the suspensions of nAChR-IAM particles and nAChR-liposome-Superdex 200 beads

The binding affinities of [^{3}H]EB for the nAChRs and the matrixes were determined using a previously described filtration method [12]. The nAChR-IAM particles, IAM particles (as a blank), nAChR-liposome-Superdex 200 gel beads and Superdex 200 gel beads (as a blank), each corresponding to 30 mg of dried material, were each suspended in 1.25 ml of buffer A. A 250 μl aliquot of each suspension was incubated with 250 μl of [^{3}H]EB [1.5 n*M*] for 4 h at 24°C in a final volume of 2.5 ml. Experiments were carried out with and without addition of 100 μl of 300 μ*M* (−)-nicotine. Bound and free ligands were separated by vacuum filtration through Whatman GF/C filters (FP-200) (Brandel, Gaithersburg, MD) treated with 0.5% polyethylenimine. The filter-retained radioactivity was determined by liquid scintillation counting (2 min) after the incubation with scintillation liquid (7 ml) overnight. Specific binding was defined as the difference between total binding and nonspecific binding. The amount of the protein immobilized on 30 mg of dried IAM particles or Superdex 200 beads was determined using BCA reagent (Pierce, Rockford, IL, USA) with measurement at 570 nm.

2.6. Chromatography based on nAChR-IAM column and nAChR-liposome-Superdex 200 column

The nAChR-IAM particles or nAChR-liposome-Superdex gel beads were packed in a HR5/2 glass column (5 cm I.D.) which was connected to a P1000 isocratic HPLC pump (Thermo Separations, San Jose, CA, USA). [^{3}H]EB was used as a marker and the elution profile was monitored by an on-line flow scintillation detector (Radiomatic™ 525 TR, Packard Instruments). The binding affinities of [^{3}H]EB and (−)-nicotine (Fig. 2) were used to evaluate the activities of immobilized nAChRs. All chromatographic experiments were performed at a flow-rate of 0.4 ml/min at room temperature.



A B

Fig. 2. The chemical structures of (−)-nicotine (A) and (−)-epibatidine (B).

In zonal chromatographic experiments, a 100 μl-loop was used to apply the sample. The radioactive signal (CPM) was recorded every 6 s, the CPMs recorded over a 30 s period were added and treated as a single data point, and the resulting data were smoothed using the Microsoft Excel program (Microsoft Corp., Redmond, WA, USA) with a 5-point moving average to produce the elution profiles. In frontal chromatography a 50 ml sample Superloop (Amersham Pharmacia Biotech) was used to apply a series of [^{3}H]EB concentrations ranging from 60 p*M* to 1000 p*M* mobile phase through the nAChR column to obtain elution profiles showing front and plateau regions. The radioactive signal (CPM) in the outlet eluate were recorded in 6 s intervals, summed up in 1 min intervals and smoothed with a 10-point moving average using the Microsoft Excel program.

The different ionic strengths of the mobile phases were obtained by changing the concentration of ammonium acetate from 5 m*M* to 200 m*M* while maintaining the pH at 7.4. To obtain the different pHs of the mobile phases, the pH values of buffer A or buffer B [50 m*M* ammonium acetate, pH 7.4] were adjusted with hydrochloric acid or sodium hydroxide. The standard deviations were obtained using the retention volumes from at least two runs.

3. Results

3.1. Immobilization of nAChR subunits or subtypes

When the proteins isolated from the transfected cells were used for the immobilization on the IAM support or Superdex 200 gel**,** about 63 mg of the protein were immobilized per g of support as measured with BCA protein assay. When the proteins prepared from the brain tissues were used, 14 mg of protein was immobilized per g of IAM support.

[^{3}H]EB binding assays on nAChR-IAM or nAChR-liposome-Superdex 200 showed that the nAChR binding activities were retained after the immobilization procedure (Table 1). The α3/β4 nAChR prepared from cell membranes yielded a higher receptor density (5.1 nmol receptor per g immobilized protein) after the immobilization in IAM particles than in Superdex 200 gel beads. In parallel experiments using IAM particles or on Superdex 200 gel beads along as sample, no specific binding of [^{3}H]EB was detected on the two matrixes.

3.2. Specific binding activities of immobilized nAChRs subtypes

Binding of [^{3}H]EB was measured on columns prepared with membranes from cells that express α3 subunits only, β4 subunits only, a mixture of the two cell types, and from cells that express the α3/β4

Table 1
Concentration of neuronal nicotinic acetylcholine receptors (nAChR) expressed as nmol receptor/g protein immobilized in IAM particles and Superdex 200 gel beads

Sample	Specific binding (%)	Concentration of immobilized nAChR (nmol/g protein)
α4/β2 nAChR-detergent solution[a]	62	0.14
α4/β2 nAChR-IAM[a]	49	0.81
α3/β4 nAChR-detergent solution[b]	100	8.57
α3/β4 nAChR-IAM[b]	98	5.09
α3/β4 nAChR-liposome Superdex 200[b]	30	1.45

[a] Prepared from rat forebrain with deoxycholate (451 mg protein per l detergent solution).
[b] Prepared from cells with cholate (9.9 mg protein per l detergent solution).

nAChRs. The capacity factors (k') of [^{3}H]EB on α3-subunit-IAM (Fig. 3A, peak 1, $k'=0.03$), β4-subunit-IAM (Fig. 3A, peak 2, $k'=1.4$) and α3-subunit+β4-subunit-IAM (Fig. 3A, peak 3, $k'=2.0$) was low, and no significant change in the corresponding k' values was observed when a displacer, 1 μ*M* (−)-nicotine was included in the mobile phase, (Fig. 3B, peaks 1–3). [^{3}H]EB was retained on the IAM column containing the immobilized α3/β4 nAChR-IAM (peak 4, $k'=8.4$, Fig. 3A). The k' value was decreased to $k'=1.5$ when 1 μ*M* (−)-nicotine was included in the mobile phase, (Fig. 3B, peak 4 in dotted line).

[^{3}H]EB and (−)-nicotine were analyzed with frontal chromatography on the α3/β4 nAChR [12] and α4/β2 nAChR columns. Representative chromatographic profiles from the α4/β2 nAChR column

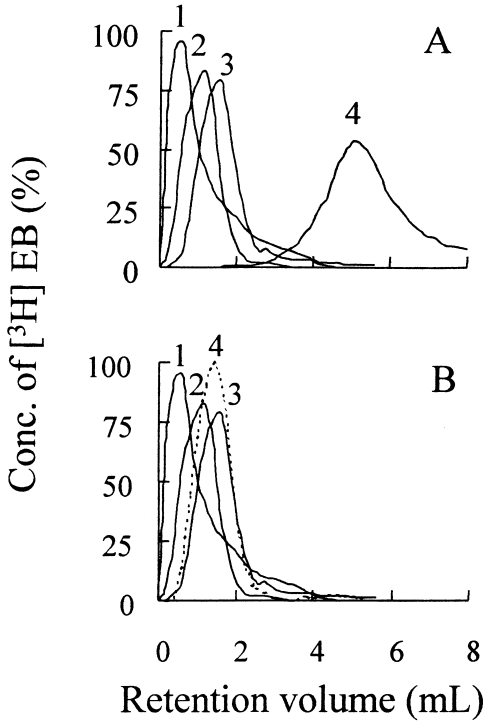


Fig. 3. Elution profiles of [^{3}H]EB (0.5 n*M*) in zonal chromatography based on the α3 nAChR column (0.5×1.2 cm) (Peak 1), the β4 nAChR column (0.5×1.1 cm) (Peak 2), the α3+β4 nAChR column (0.5×1.3 cm) (Peak 3) and the α3/β4 nAChR column (0.5×1.5cm) (Peak 4). Mobile phase: Tris–HCl buffer [50 m*M*, pH 7.4]. (A) No (−)-nicotine in the mobile phase. (B) 1 μ*M* (−)-nicotine present in the mobile phase.

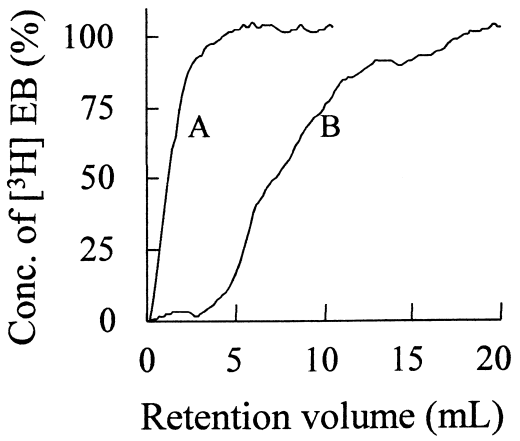


Fig. 4. Example of the elution profiles of [^{3}H]EB (60 p*M*) in frontal chromatography based on α4/β2-nAChR-IAM stationary phase (0.5×1.5 cm). Mobile phase: Tris–HCl buffer [50 m*M*, pH 7.4]. (A) 1 μ*M* (−)-nicotine present in the mobile phase. (B) No (−)-nicotine in the mobile phase.

are presented in Fig. 4. From the retention volumes of [^{3}H]EB, corresponding to the half-height of the plateau, the association constants (K_d) of [^{3}H]EB, $K_{EB}$and the K_d value of the test drug, K_{drug}, as well as the number of the active and available binding sites of immobilized receptors, *P*, were calculated using Eqs. (1) and (2) in [12]. The treatment was originally described by Winzor for analytical affinity chromatography [18] and adapted by Brekkan et al. for immobilized biomembrane chromatography [6]. The K_d values of (±)-epibatidine and (−)-nicotine were presented in Table 2. The mean and deviations of the K_d values were obtained using the retention volumes from two runs.

The results of binding to immobilized receptors showed that [^{3}H]EB and (−)-nicotine have higher binding affinities at nAChR α4/β2-subtype than at α3/β4-subtype (Table 2). These results are consistent with the results determined from ligand binding assays using membrane homogenates (Table 2). No significant binding of [^{3}H]EB was observed on the column prepared from nAChR α3-subunit, nAChR β4-subunit and α3-subunit plus nAChR β4-subunit in the cholate detergent solution as indicated by the results presented in Fig 3. The K_d values obtained from α4/β2 nAChR-liposome-Superdex 200 column were similar to those determined using the α4/β2 nAChR-IAM column (Table 2).

Table 2
Calculated K_d values of (±)-epibatidine and (−)-nicotine

Formats of nAChRs	K_d of (±)-epibatidine (n*M*)	K_d of (−)-nicotine (n*M*)
α3/β4-nAChR-IAM[a]	0.30±0.05[c]	90±30[c]
α3/β4-nAChR membrane[b]	0.40±0.07[c]	480±50[c]
α4/β2-nAChR-IAM[a]	0.04±0.005	1±2
α4/β2-nAChR membrane[b]	0.05±0.002	7±1
α4/β2-nAChR-liposome-Superdex 200[a]	0.02±0.08	8±2

[a] Frontal chromatography based on the nAChR column prepared from cells.
[b] Binding assay using membrane homogenates of rat brain tissue [11].
[c] The data also were reported in [12].

3.3. Effects of ionic strength and pH of the mobile phase on the binding of [^{3}H]EB

The effects of mobile phase ionic strength and pH on the binding affinities of [^{3}H]EB were determined with a α3/β4 nAChR column. The retention volumes increased when the pH of mobile phase was increased from pH 4 to pH 7.0 and remained constant in the pH range 7.0–9.5 (Fig. 5). The retention volumes of [^{3}H]EB were higher at low ionic strength (5 m*M* ammonium acetate) and decreased as the ionic concentration of the mobile phase increased (Fig. 6).

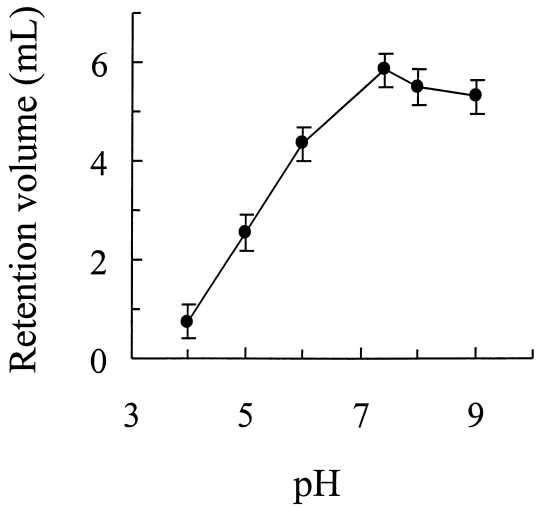


Fig. 5. Effect of mobile-phase pH on the retention volumes of [^{3}H]EB on an α3/β4-nAChR column (0.5×1.5 cm) in frontal chromatography. Ammonium acetate buffers [50 m*M*] were used for the pH range 3–7 and Tris–HCl buffers [50 m*M*] were used for the pH range 7–9. The error limits bars represent the maximum differences in retention volumes between runs (n=2) at a given pH of mobile phase.

4. Discussion

The relative binding affinities obtained on the columns containing immobilized α3/β4 and α4/β2 receptor subtypes were α4/β2>α3/β4 (Table 2). These results are consistent with the membrane-based assays and demonstrate that the immobilized receptor chromatographic technique can distinguish between the activities of nAChR subtypes.

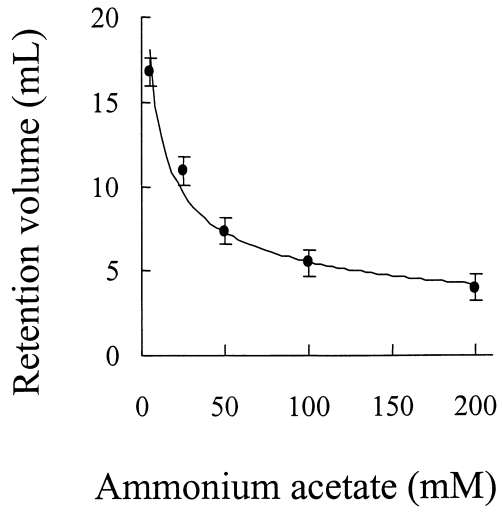


Fig. 6. Effect of the ionic strength of the mobile-phase on the retention volumes of [^{3}H]EB on an α3/β4 nAChR column (0.5× 1.5 cm) in frontal chromatography. The error limits bars represent the maximum differences in retention volumes between runs (n=2) at a given ionic strength of mobile phase.

The results of this study also demonstrate that nAChR can be reconstituted in the phospholipid bilayer of liposomes immobilized in Superdex 200 gel beads. The K_d values of [^{3}H]EB and (−)-nicotine determined using an immobilized α4/β2 nAChR-liposome column are similar to those determined using an α4/β2 nAChR-IAM column (Table 2), though the amounts of active binding sites of nAChR on the immobilized proteoliposomes are lower than those in nAChR-IAM. Since the internal volume of the liposomes is available, it is possible to study the transmembrane functions of receptors immobilized on a liposome column using 'Transport Retention Chromatography' which had been used for the study of human glucose transporters [19,20]. The number of binding site of nAChRs on the liposome surface can probably be increased by adjusting the protein–lipid–cholesterol ratios and the composition of phospholipids, which are important for the functional reconstitution of nAChRs [21,22]. However, these questions appear to be avoided when the IAM stationary phase is used.

When the mixture of independently solubilized α3-subunits and β4-subunits was immobilized, no specific binding of [^{3}H]EB was observed on the resulting column. This indicates that no self-assembly of the nAChR occurred during the immobilization and the α3-subunits and β4-subunits were independently immobilized. This also indicates that the α3-subunits and β4-subunits which form the nAChR α3/β4 subtype were not separated during solubilization with detergent and that the intact subtype complex was embedded in the lipid monolayer of the IAM stationary phase.

The stronger binding affinity of [^{3}H]EB at low ionic strength (Fig. 6) and the reduction in binding with increasing ionic strength of the mobile phase is indicative of an electrostatic-based retention mechanism. This is consistent with the pharmacophore models of the nAChR binding site [23–25]. In these models both cationic and electronegative regions are present within the nAChR binding site. The low binding affinity at low pH is also consistent with the existence of a cationic center in the binding site.

These results suggest that immobilized-receptor stationary phases may be useful in the investigation of ligand–receptor interactions. This technique can also be used to rapidly reduce the number of drug candidates in a combinatorial pool since the compounds that do not bind at the receptors will pass rapidly through the column. In addition the relative affinity of the compounds at the receptors can be readily classified according to their retention volumes or K_d values [12].

Acknowledgements

We thank Martha Dávila-Garciá of Georgetown University for assistance in the protein assays and binding experiments. This work was supported by NIH Grant No. 1R426M56591-02 (IWW).

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