Chemical modification of epibatidine causes a switch from agonist to antagonist and modifies its selectivity for neuronal nicotinic acetylcholine receptors

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Background: Studies of ligand gated channels strongly rely on the availability of compounds that can activate or inhibit with high selectivity one set or a subset of defined receptors. The alkaloid epibatidine (EPB), originally isolated from the skin of an Ecuadorian poison frog, is a very specific agonist for the neuronal nicotinic acetylcholine receptors (nAChRs). We used EPB derivatives to investigate the pharmacophore of nAChR subtypes.

Results: Optically pure enantiomers of EPB analogues were synthesised. Analogues were obtained altered in the aromatic part: the chlorine was eliminated and the relative position of the pyridyl nitrogen changed. Voltage clamp electrophysiology was performed with these compounds on neuronal nAChRs reconstituted in *Xenopus* oocytes. The EPB derivatives show different activities towards the various nAChR subtypes.

Conclusions: Small changes in the molecular structure of EPB produce marked changes in its capacity to activate the nAChRs. Subtype specificity can be obtained by changing the position of or by eliminating the pyridyl nitrogen.

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Key words: acetylcholine receptors, epibatidine analogues, nAChR, neuronal nicotinic receptors

Received: **24 January 2000**
Revisions requested: **29 February 2000**
Revisions received: **2 May 2000**
Accepted: **8 May 2000**

Published: **27 June 2000**

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Introduction

Studies of ligand-gated channels strongly rely on the availability of compounds that can activate or inhibit with high selectivity one set or subset of receptors. For example, natural toxins, such as the snake toxin α-bungarotoxin, have largely been employed to characterise the nicotinic acetylcholine receptor (nAChR). Although very specific, these polypeptide toxins are difficult to synthesise and modify. Simpler molecules that can be made using organic chemistry methods are therefore required to study structure–activity relationships. The alkaloid epibatidine (EPB), originally isolated from the skin of the Ecuadorian poison frog *Epibetobates tricolor* [1–3], is a very specific agonist of the neuronal nAChR (reviewed in [4,5]).This opened new ways to investigate the properties of the pharmacophore of these receptors. This molecule can be synthesised (reviewed in [6–8]) and several of its derivatives and analogues have already been investigated using biochemical and electrophysiological approaches [9–17].

Both enantiomers of EPB ((+) and (–)) exhibit amongst the highest known affinities towards the central brain nAChR α4β2 (0.045 and 0.058 nM, respectively) [18] as well as towards the homomeric nAChR α7 (16 nM) [19]. Functional studies have shown that EPB is one of the most potent activators of the α4β2 receptors (EC_{50} 16 nM) but that it is a weak agonist at the homomeric α7 nAChR with an EC_{50} value in the micromolar range [20–22]. The nAChR sensitivity to EPB is influenced by small structural modifications [17,22–24]. EPB is one of the most toxic natural compounds [10,25,26]. The toxicity of EPB may arise from its capacity to activate both the central neuronal α4β2 and the ganglionic α3β4 nAChR.

The structural requirements that define an agonist versus an antagonist are poorly understood. In one of the pharmacophore models it has been proposed that a nicotinic agonist requires a charged nitrogen and a hydrogen bond acceptor at distances of 5.9 Å [27] or 4.4–5.0 Å [28]. According to a re-interpretation of this model [29], the ideal distance between these two groups would be 5.5 Å, the intramolecular nitrogen–nitrogen distance in EPB. Limitations of this model are evident when considering the different compounds acting at the nAChR. For example, tetramethyl ammonium chloride, which is a potent agonist at α4β2 and α7 receptors, does not fulfil

this 'pharmacophore model' requirement. Furthermore, tetramethyl ammonium derivatives choline and ethyltrimethyl ammonium show a strong selectivity in activating the α7 subtype [30–33].

This model is based only on affinity data obtained in binding measurements and does not take into account the efficacy of the different compounds in eliciting a functional response. We have therefore synthesised EPB derivatives and examined their functional properties on nAChRs reconstituted in *Xenopus* oocytes. Because we have recently shown a significant enantioselectivity in N-methylated derivatives of EPB [22,34], we used mainly optically pure substances.

We examined the role of the pyridine nitrogen as the hydrogen-bond acceptor in receptor activation and binding. We simplified the EPB structure by eliminating the chlorine atom to generate dechloroepibatidine (DClEPB). Then we switched the pyridine nitrogen from the *meta* to the *ortho* or *para* position to yield *exo*-2-(2-pyridyl)-7-azabicyclo[2.2.1]heptane (2PABH) and *exo*-2-(4-pyridyl)-7-azabicyclo[2.2.1]heptane (4PABH), respectively. Finally the hydrogen-bond acceptor was fully removed to give *exo*-2-phenyl-7-azabicyclo[2.2.1]heptane (PABH).

We also examined the influence of the [2.2.1]azabicyclo moiety by comparing with homoepibatidine (HEPB, *exo*-6-(2-chloro-5-pyridyl)-8-azabicyclo-[3.2.1]octane). Results obtained with this approach can be interpreted on the basis of a model in which the EPB binds at the interface between two adjacent subunits. In addition, we illustrate that the properties of EPB derivatives compared with those of EPB itself can be explained using an allosteric model of the nAChRs.

Results

Activity of DClEPB

The electrophysiological experiments with DClEPB enantiomers on the major brain nAChR α4β2 and ganglionic α3β4 are illustrated in Figure 1. Typical currents evoked by ACh and the two DClEPB enantiomers in an α4β2 responsive oocyte are illustrated in the left panel of Figure 1a. The response time course of either enantiomer showed no significant difference compared with that of ACh. Mean dose-response curves (n = 4) measured over a broad concentration range of the two enantiomers are shown in the right panel. Data are represented as a fraction of the current evoked by a saturating ACh concentration (1 mM).

The efficacy — the ratio of the current evoked at saturation for a given agonist over the current evoked by the natural agonist ACh — is different for the two DClEPB enantiomers. The (+)-form displays a higher affinity and higher efficacy. In contrast, the α4β2 nAChR exhibits a lower sensitivity to the (–)-DClEPB and also a lower efficacy of 80%.

Comparison of the EC_{50} values measured for (+)- and (–)-DClEPB with those of the (+)- and (–)-EPB shows that, in contrast to results obtained in binding experiments [9,18], removal of the chlorine atom markedly modifies the α4β2 sensitivity to EPB and causes a shift in efficiency of 40–100-fold together with a modification of the efficacy (see Table 1).

The sensitivity of the ganglionic α3β4 nAChR to the DClEPB enantiomers was analysed as described above. Both enantiomers induced strong currents, the time courses of which could not be distinguished from each other, indicating the absence of enantiomeric selectivity (see left panel Figure 1b). Dose-response measurements (n = 4) also revealed no major differences between the two DClEPB enantiomers (right panel Figure 1b). On average, the sensitivity of the ganglionic α3β4 subtype was an order of magnitude higher than that of the α4β2 nAChR (Table 1).

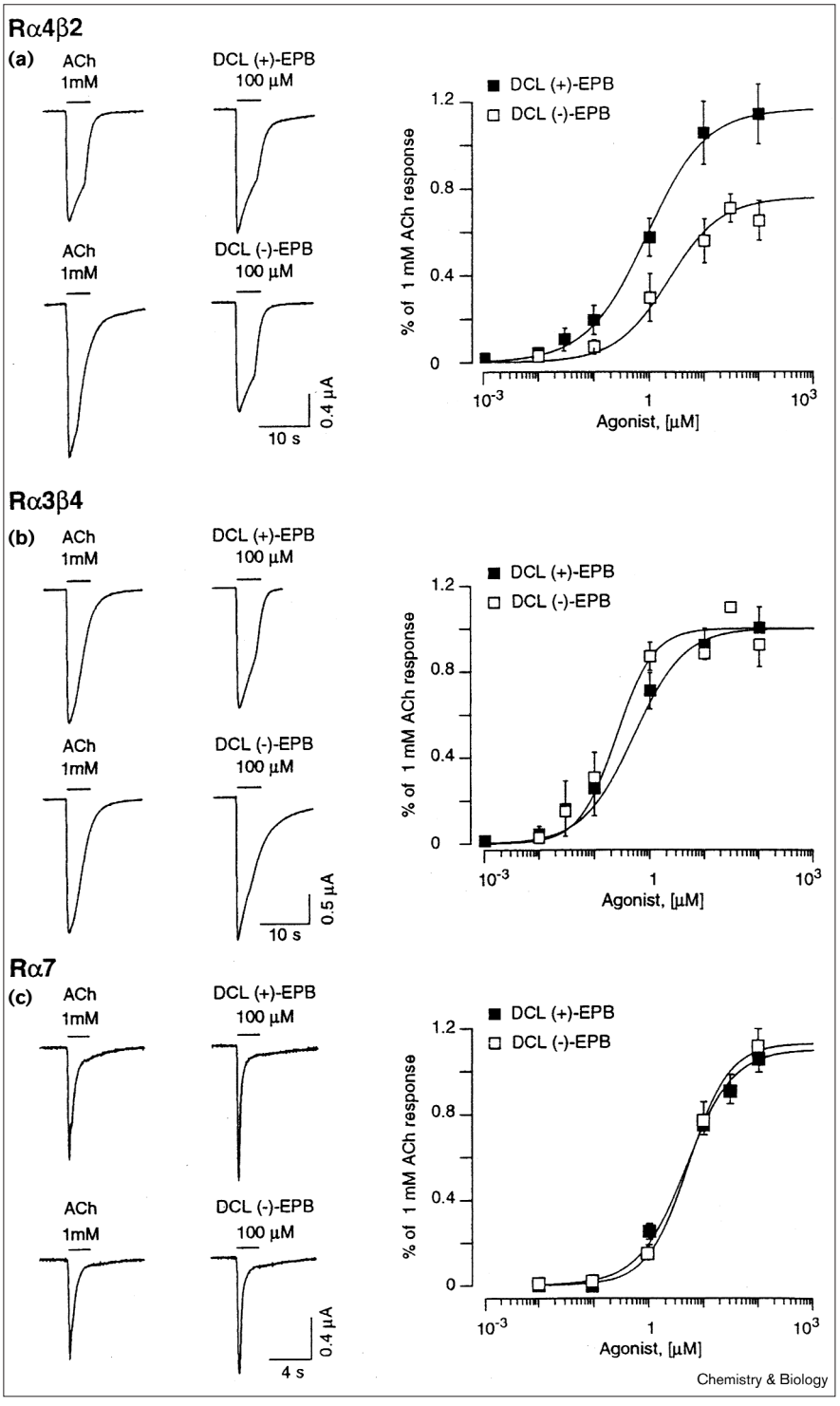
As expected from previous studies, oocytes expressing the homomeric α7 nAChR responded to ACh with very fast desensitising currents [35–38]. Typical α7 currents evoked by ACh and the two DClEPB enantiomers are shown in Figure 1c. In comparison with ACh responses, the amplitude of the currents evoked by both DClEPB enantiomers were slightly higher, suggesting a stronger efficacy (Table 1). As with the ganglionic receptor, no enantiomeric selectivity was detected in efficacy or in efficiency. Moreover, also in agreement with previous findings obtained with EPB [20] cited by [4], [21] cited by [5], [22] the α7 subtype displayed the weakest sensitivity towards DClEPB, just below that of the α4β2 nAChR (n = 4, see Table 1).

Activity of 2PABH

2PABH was the first compound in a series of modifications, which allowed us to investigate the role of the hydrogen-bond acceptor for receptor recognition and activation. The pyridine nitrogen in 2PABH was not in the *meta* position but in the *ortho* position with respect to the bicyclo moiety. This results in a decreased intramolecular N–N distance and in a changed spatial orientation of the pyridine nitrogen (Figure 2). The N–N distance calculated for EPB and DClEPB varies between 4.4 and 5.8 Å, as estimated by CS Chem3D Pro™ (CambridgeSoft) depending on the rotation around the C–C bond between the two ring systems. Accepting 5.5 Å as the optimal N–N distance for DClEPB [29] and keeping the same rotational orientation the N–N distance in 2PABH was 4.4 Å after energy minimisation.

Preliminary studies have been reported recently showing that a 30 μM concentration of either of the two 2PABH

Figure 1



Elimination of the chlorine at the 4′-position of the 3′-pyridyl group modifies the EPB affinity considerably. **(a)** Left, typical currents evoked by a saturating ACh test pulse at the α4β2 nAChR are compared to (+)-DClEPB and (–)-DClEPB. Dose-response profiles determined over a broad range of DClEPB are shown on the right. Data were normalised with respect to the ACh evoked current recorded at saturation (1 mM). Note that a 1.2 coefficient was used in the curve fitting of (–)-DClEPB whereas a scaling factor of 0.7 was applied to (–)-DClEPB. **(b,c)** Data obtained as in (a) for (+)-DClEPB and (–)-DClEPB at the ganglionic α3β4 nAChR and the homomeric α7 receptor. EC_{50} values and Hill coefficients are indicated in Table 1.

enantiomers evoked no detectable response at α4β2 or α3β4 receptors. At the α7 nAChR, only the (–)-enantiomer was a partial agonist with ~80% of the ACh response and an EC_{50} value of 32.5 ± 9.5 μM [24]. In comparison with DClEPB this is a dramatic change in the agonist profile. Dose-response curves were measured over a broad range of (+)- and (–)-2PABH concentrations (up to 300 μM) to see if 2PABH behaves as a partial

Table 1

Electrophysiological data of EPB and EPB analogues.

	μM		
	α4 β2	α3 β4	α7
(+)-EPB*	0.021 ± 0.005 1.24 ± 0.1 (100)	0.036 ± 0.013 1.52 ± 0.4 (100)	2.5 ± 0.16 1.45 ± 0.1 (60)
(–)-EPB*	0.023 ± 0.007 1.30 ± 0.1 (100)	0.019 ± 0.006 1.67 ± 0.2 (100)	2.03 ± 0.5 1.43 ± 0.1 (90)
(+)-DClEPB	0.93 ± 0.2 0.98 ± 0.06 (120)	0.51 ± 0.2 1 ± 0.025 (100)	5.25 ± 0.4 1.15 ± 0.02 (110)
(–)-DClEPB	2.8 ± 1.1 0.98 ± 0.1 (80)	0.25 ± 0.08 1.25 ± 0.14 (100)	4.6 ± 0.14 1 (110)
(+)-2PABH	32 ± 8† 1.1 ± 0.03	207 ± 37 1.2 ± 0.06	48 ± 6† 1 ± 0.06 84 ± 9 2.5 ± 0.08
(–)-2PABH	67.5 ± 16† 0.925 ± 0.08	738 ± 137 1.1 ± 0.07	32.5 ± 9.5‡ 1.40 ± 0.2 (100)
4PABH	{21 ± 6}	(100)	{0.5 ± 0.06}
PABH	{15 ± 5}	(100)	{1.6 ± 0.06}
(+)-HEPB	0.02 1.1 (100)	0.02 1.4 (100)	
(–)-HEPB	>1	0.6 / 1.6	

EC_{50} ± SEM, Hill coefficients nH ± SEM and (efficacy in % of ACh efficacy); *data taken from [15]; †IC_{50} ± SEM, Hill coefficients nH ± SEM; {response of a 300 mM solution in % ACh efficacy}; ‡data taken from [53].

agonist. Preapplication (10 s) of the compound inhibits the current evoked by a subsequent ACh test pulse (Figure 3a) showing that this compound binds to the nAChR recognition site. The small current evoked by the highest concentration of 2PABH illustrates that this compound may be a partial agonist at the α4β2 nAChRs with less than 10% efficacy. Moreover, this compound displays little enantiomeric selectivity for this receptor subtype (Figure 3a,b and Table 1).

The ganglionic α3β4 receptors did not show a significant sensitivity towards both 2PABH enantiomers [24]. Prepulse experiments similar to those described above were performed with this system (Figures 3c,d). When the 2PABH concentration was raised to 100 μM a significant inward current was observed. Furthermore, the 2PABH prepulse yielded no marked inhibition of the subsequent ACh-evoked current indicating that this compound behaves as an agonist at the ganglionic receptor subtype. However, given the low sensitivity of this receptor to 2PABH and the limited availability of the ligand, it was not possible to reach a saturating concentration. Extrapolation of the data indicates that the 2PABH EC_{50} value must be in the range of 210 to 740 μM for the (+)- and (–)-enantiomers (Figures 3c,d and Table 1) and that this receptor does not markedly distinguish between the two enantiomers.

Further investigation of 2PABH on the α7 receptor confirmed and extended the results previously obtained with this compound [24]. Exposure to (+)-2PABH evoked only very little current (less than 10% of ACh), whereas (–)-2PABH behaves as a whole agonist on this receptor subtype (Figure 3e,f). Coapplication of (+)-2PABH on top of a fixed ACh concentration provoked a decrease in the plateau current, indicating that this compound blocks α7 receptor. Determination of the dose-response inhibition on the plateau phase (Figure 3f) yielded an IC_{50} value of 48 ± 6 μM and a Hill coefficient of 1 (n = 3). Note the shortening of the peak duration concomitant to the reduction of the plateau level (Figure 3e, lower traces).

Activity of 4PABH and PABH

In 4PABH the pyridyl nitrogen was turned to the *para* position, which increased the intramolecular N–N distance

to 5.6 Å (pretty much independent of the rotational position around the C–C bond between the ring systems) and changed the orientation of the hydrogen-bond acceptor pyridine nitrogen. Elimination of the nitrogen resulted in PABH. Because of low yields and unsatisfactory enantiomer separation so far only partial (but nevertheless clear) results could be obtained with these two compounds.

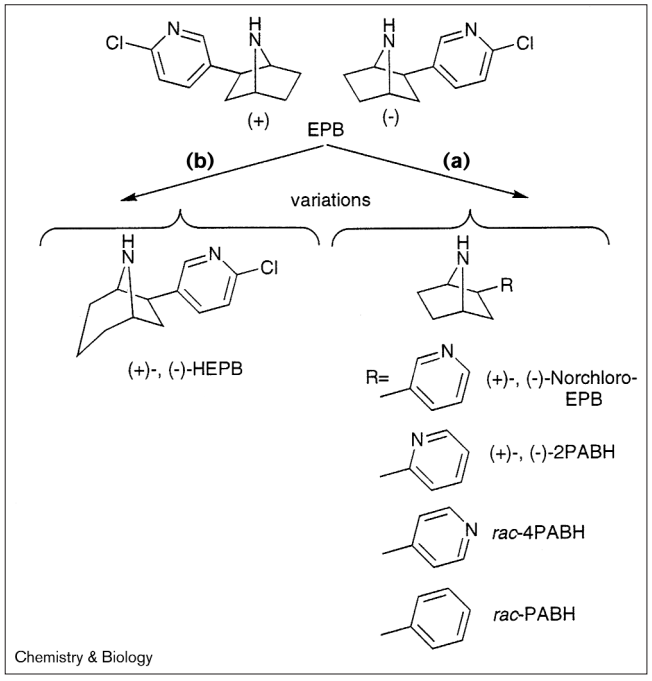
The current evoked by a 300 μM application of these ligands was compared to those evoked by saturating ACh concentrations. Data obtained for the three types of nAChRs are shown in Figure 4 and Table 1. Currents recorded in an oocyte expressing α4β2 (Figure 4a) illustrate that this receptor has little sensitivity to either 4PABH or PABH. In contrast, with the ganglionic receptor, a significant inward current resembling that evoked by ACh was recorded (Figure 4b). These results indicate that the nitrogen of the pyridine ring is not indispensable for the activation of the α3β4 receptor. Although modification of the N–N distance and the orientation of the hydrogen-bond acceptor (pyridyl nitrogen) produces little effect on the ganglionic receptor, a marked difference is observed for the homomeric α7 nAChR. As shown in Figure 4c, this receptor displays almost no detectable current even in response to high concentrations of these two compounds. Observation of the traces at higher magnification shows, however, that although 4PABH evokes only a very transient response, PABH evokes first a sharp peak of current that is followed by a second and more shallow deflection. This rebound is reminiscent of open channel blocker effects [39–42], which suggests that PABH partial agonist activity may be impaired by an additional blocking effect that could partly mask the evoked current.

Activity of HEPB

The effect of the azabicyclo moiety on receptor recognition was assessed by the synthesis of HEPB (Figure 2). The enlargement of the rigid [2.2.1]- to a [3.2.1]-cage induced some flexibility and extended the bulky bicyclo structure into the direction opposite the pyridine ring with some change in pyridine nitrogen (hydrogen-bond acceptor) orientation and N–N distance (6.2 Å) in the free molecule. This is a consequence of the change in rotational position around the 'ring-systems-connecting' C–C bond upon energy minimisation. If the C–C bond is rotated back to the EPB position, the pyridine nitrogen (hydrogen-bond acceptor) orientation and N–N distance is the same as for EPB (5.5 Å).

Typical currents evoked at the α4β2 nAChR by different concentrations of HEPB enantiomers are illustrated in Figure 5a. Responses to ACh and (+)-HEPB were comparable, showing that this compound acts as a full agonist. The efficiency is comparable to that of EPB (Table 1). A marked difference is observed, however, when comparing currents recorded in the same cell in response to (–)-HEPB. Both the amplitude and sensitivity are affected (right panel Figure 5a, Table 1). Although the α4β2 indifferently recognises the two EPB enantiomers, it strongly favours the (+)-conformation of the HEPB modification.

Figure 2



The EPB analogues. The structure of EPB was modified in two ways. **(a)** Stepwise modifications of the pyridine ring led to a series of analogues shown on the right. **(b)** The enlargement of the aza-bicyclo moiety resulted in the homologue displayed on the left. Note that the absolute configuration of the structures is only known for EPB.

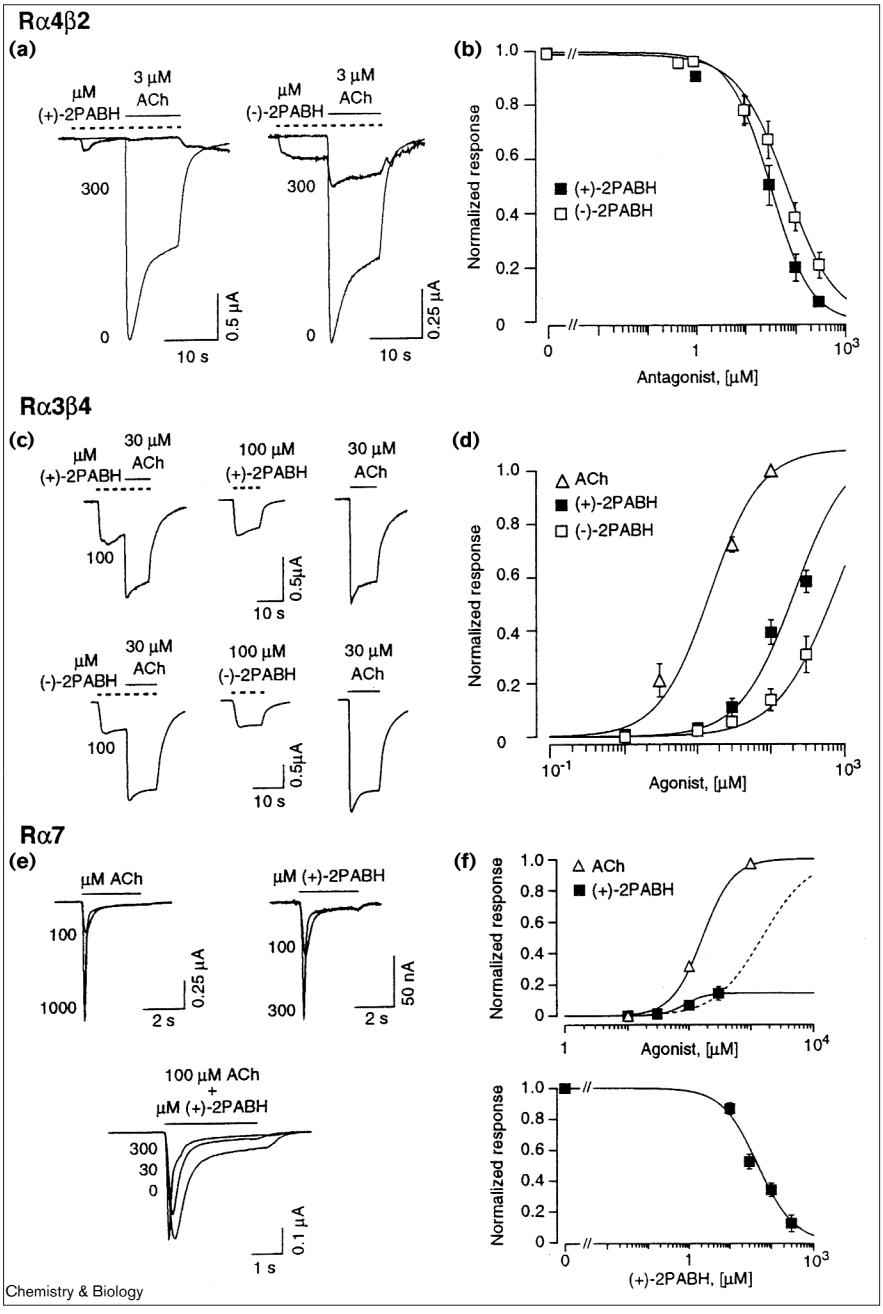
Figure 5b illustrates the responses of the α3β4 receptor to ACh and (+)-HEPB, (–)-HEPB for three increasing concentrations. ACh and (+)-HEPB evoke comparable currents but a small difference is observed for the (–)-enantiomer. Estimation of the half activation value yielded no significant difference with that of EPB itself (Table 1). (–)-HEPB exhibited a significantly lower activity but no less so than observed for α4β2.

Figure 5c illustrates the typical α7 evoked current obtained in response to ACh, (+)-HEPB and (–)-HEPB. They evoke currents of roughly the same amplitude, but (–)-HEPB is at least 100-fold less efficacious. Because of the limited amount of material available, saturation could not be assessed in any of the cells tested.

Discussion

In this work we have examined the structure–activity relationship of the agonist EPB and its non-natural derivatives on neuronal nAChRs reconstituted in *Xenopus* oocytes.

Figure 3



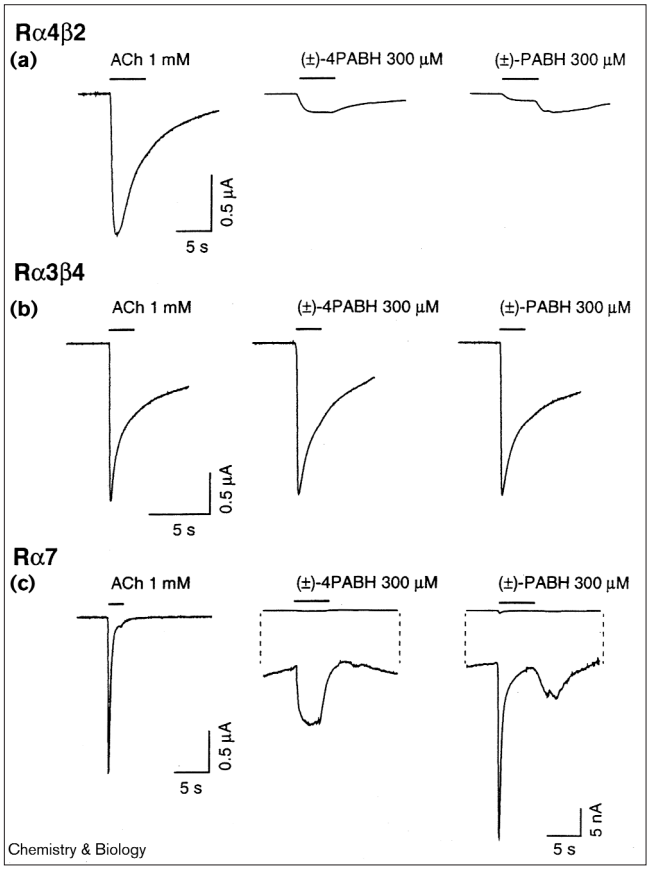
Agonist and antagonistic effects of 2PABH at the neuronal nAChRs. **(a)** Effects of pre- and coapplication of (+)- and (–)-2PABH on the ACh-evoked current (left panel). Dashed lines illustrate the timing of the 2PABH applications and bars indicate the ACh test pulses. **(b)** Percentage of inhibition caused by 2PABH pre- and coapplication are plotted as a function of the 2PABH concentration. Values were measured during the plateau phase of the ACh pulse. **(c)** Effects of 2PABH at the α3β4 nAChR. Left panel illustrates that both (+)- and (–)-2PABH are agonists at this receptor subtype and cause no inhibition of the subsequent ACh evoked current. **(d)** ACh, (+)-and (–)-2PABH dose-response relationships. Currents evoked by ACh and one of the 2PABH enantiomers were determined in the same cells (n = 3 and 5 for the (+)- and (–)-2PABH). Lines through the data points are the best fit obtained with the Hill equation. **(e)** Typical currents recorded in response to ACh, (+)-2PABH alone or during coapplication of these two drugs in an α7 expressing oocyte are illustrated in the left panel. Note the difference in scale for ACh and (+)-2PABH. **(f)** The upper panel illustrates the agonistic effect of (+)-2PABH versus ACh. The continuous line through the 2PABH data corresponds to a Hill equation with a scaling factor of 0.15. The dashed line corresponds to a Hill equation in which the EC_{50} was shifted to 1525 μM. Lower panel was obtained by plotting the fraction of plateau current (measured after 3s) as a function of the (+)-2PABH concentration. IC_{50} and EC_{50} values and Hill coefficients are summarised in Table 1.

The convergent character of the synthetic approach offered the possibility of coupling different aromatic groups to different bicycloheptenes. The EPB structure could, therefore, easily be varied and a broad range of EPB analogues (HEPB, DClEPB, 2PABH, 4PABH and PABH) were accessible (Figure 2).

The ligand-binding site of the nAChRs lies at the interface between two adjacent subunits [43–46]. The nicotinic pharmacophore for an effective agonist requires a charged nitrogen and a hydrogen-bond acceptor group [27,28,47]. The binding pocket possesses one area to recognise the positive charge and a second one to form a hydrogen bond with the pyridine nitrogen.

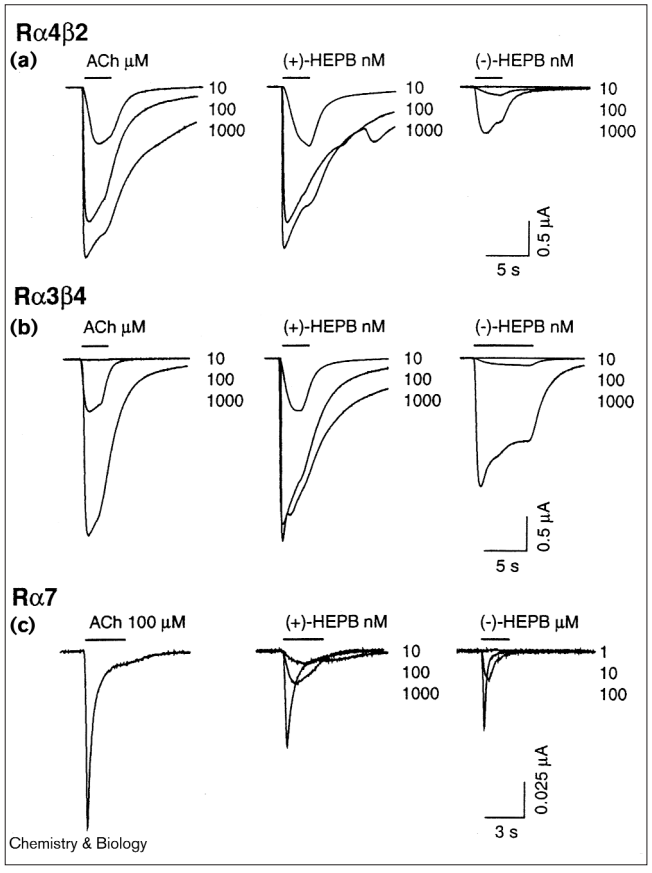
By alignment of sequences of α subunits [45,48] and by point mutations in the complementary component [49] the model of the binding site shown in Figure 6 was formulated [49,50]. The amino acids Tyr93, Trp149, Tyr151, Tyr190 and Tyr198 were shown by photoaffinity labelling

Figure 4



4PABH and PABH are weak agonists of the neuronal nAChRs. Effects of 300 μM test pulses of the racemic mixture of 4PABH and PABH are compared to currents evoked by saturating ACh (1 mM). **(a–c)** correspond to the α4β2, α3β4 and α7 receptors, respectively. To illustrate the very weak agonist effects, data obtained for the α7 receptor are plotted on two different scales. Cells were challenged once every 2 min; full recovery of the ACh evoked current was always observed after the washing period. Similar recordings were obtained in at least three cells for each of the conditions. Bars indicate the timing of the applications.

Figure 5

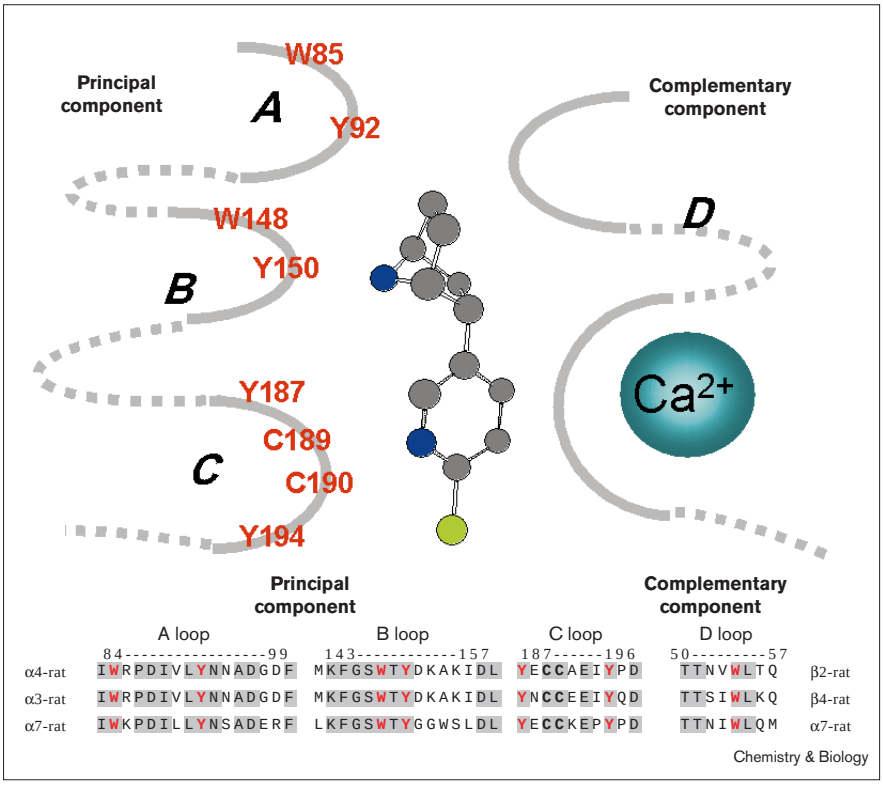


Sensitivity of neuronal nAChRs to (+)- and (–)-HEPB. **(a–c)** illustrate recordings obtained for three increasing ACh and HEPB concentrations at the α4β2, α3β4 and α7 receptors. Cells were challenged first with ACh and then either with the (+)- or (–)-enantiomers. In every case, ACh evoked currents were measured at the end of the test pulse series to verify the absence of blockade or desensitisation of the receptors.

to be part of the binding site in the α subunit in *Torpedo marmorata* [51–53]. We have identified these same amino acids (Tyr92, Trp148, Tyr150, Tyr187 and Tyr194) in the principal component of the rat α2, α3 and α7 subunits. These aromatic amino acids interact by cation–π interaction with the positive charge on the quaternary nitrogen, which is the essential feature for an nAChR-binding site [33]. The complementary component presents the ligands with a lipophilic backbone. The loops depicted in Figure 6 are also found in the ribbon diagram of a calculated molecular model of the receptor [48].

The first step in a series of modifications on the EPB structure was the elimination of the chlorine at the 4′-position of the 3′-pyridyl group (DClEPB, Figure 2). On the one hand, this structural modification specifically addresses the role of the halogen in the nAChRs activation. On the other hand, this modification simplifies the structure and allows the function of the hydrogen-bond acceptor (pyridyl nitrogen) at three different aromatic positions to be evaluated without further consideration of the relative positions of the nitrogen and the halogen. In comparison with the parent compound EPB, both DClEPB enantiomers displayed a loss in activity at the heteromeric α4β2 receptor. As summarised in Table 1 the (–)-enantiomer exhibits a 120-fold reduction in efficiency whereas the (+)-enantiomer is only reduced by about 40-fold. A similar tendency was shown at the ganglionic α3β4 nAChR. The least pronounced effects were seen with the homomeric α7 receptors which showed a reduction of about half in their sensitivity to DClEPB versus EPB. The chlorine is therefore important for the activation of the heteromeric nAChRs. In contrast, binding studies with EPB and DClEPB on rat brain homogenates have revealed that the

Figure 6



Model of the binding sites on the nAChR. Top: The ligand binding site is located at the interface between an α and the adjacent subunit. According to the multiple loop model four loops are involved in the ligand binding. The amino acids of loop ***A***–***D*** which have been localised in the binding site by affinity labelling are represented in black (for reviews see [40,45,52]). An additional loop has been identified as a calcium binding site by mutation of residues Glu161, Asp163, Ser167, Ser169 and Glu172 [49]. The ligand EPB is positioned into the gap; both nitrogen atoms are shown in blue, the chlorine in green According to the nicotinic pharmacophore a ligand possesses at least a charged nitrogen and a hydrogen bond acceptor. The aromatic amino acids at position Trp85, Tyr92, Trp148, Tyr150, Tyr187 and Tyr194 (shown in red) represent possible positions to interact by cation-π interaction with the charged azabiciyclo-nitrogen of EPB. Bottom: Sequence alignments of the principal component (rat α4, α3 and β7 nAChR sequences) in the region of loops ***A***, ***B*** and ***C*** and of the complimentary component (rat β2, β4 and α7 sequences) in the region of loop ***D*** (sequences taken from the NCBI Genbank). The loops contain conserved canonical residues which are indicated by grey background. The amino acids that are identified in the binding pocket by affinity labelling (see above) are shown in bold letters.

dechloro analogue possessed a slightly increased affinity [9,18]. These two seemingly different results can be understood because the two experimental approaches address a different state of the receptor. We looked into the capacity of a compound to activate the receptor while binding measurements cover a longer time and mostly involve a nonactive state.

The nAChR properties can be taken into account using a four-state allosteric scheme derived from the original proposition of Monod, Wyman and Changeux and co-workers [43,54–59]. According to that model the receptor is in equilibrium between conformational states. These discrete conformational states are the inactive basal (*B*), active (open) (*A*), intermediate (*I*) and inactive desensitised (*D*) states [43]. The presence of a ligand preferentially stabilises one or more states. Agonists can be stabilise the *A* (open) state as compared to the *B* state, whereas competitive inhibitors and antagonists are assumed to stabilise the *B* (closed) or *D* (closed) states. According to this model, the efficacy of a given compound reflects its capacity to preferentially stabilise the *A* state, whereas partial agonists that are less efficacious must stabilise both the active as well as a closed state. Affinity measurements reflect the binding on the receptor at equilibrium.

In the case of DClEPB, it follows that the (+)-enantiomer preferentially stabilises the *A* state of α4β2 even better than ACh, whereas the (–)-enantiomer must stabilise both the *A* and to a lesser extent the *D* and/or *B* states. The loss of agonist efficacy between these compounds and EPB reflects a reduction in stabilisation of the *A* state. The *para* chlorine in EPB obviously enhances the activation of the *A* state. As there is no difference in the N–N distance with or without chlorine, the chlorine enhances the activation of the *A* state by optimising the hydrogen bond with the receptor.

The subsequent EPB derivative was 2PABH. Switching the pyridine nitrogen from the *meta* to the *ortho* position with respect to the bicyclo ring caused a very marked difference in the ability of this compound to activate the receptors. When considering the α4β2 nAChR alone, this modification completely abolishes the ability of this derivative to activate the nAChR. The inhibition of the ACh evoked current observed in the prepulse experiments (Figure 3) reveals that this compound still binds to the receptor but now preferentially stabilises a closed state. 2PABH evokes a current at the α3β4 nAChR even if its efficacy is diminished by more than two orders of magnitude. Obviously, the hydrogen-bond acceptor in EPB and

DClEPB must be involved in binding and plays a key role in stabilising the *A* state in α3β4.

In contrast to DClEPB, not both enantiomers of 2PABH could stabilise the *A* state of the α7 nAChR but only the (–)-conformation. (+)-2PABH stabilised the *B* or *D* state and when coapplied thus inhibited the ACh response. Obviously the enantiomers of 2PABH bind to the receptor differently from those of DClEPB, due to the shorter N–N distance and the different orientation of the hydrogen bonding pyridyl nitrogen.

The small agonist effect of (+)-2PABH (Figure 3f) can be interpreted either as reflecting the low partial agonist effect of this compound or the strongly reduced sensitivity of the α7 receptor. The continuous line through the data points (filled squares) corresponds to the fit obtained with a Hill equation assuming a saturating value of 15% of the ACh current. The dashed line was drawn as an extrapolated dose response in which the EC_{50} value was shifted from 165 to 1525 μM. The fast time course of the response evoked by 300 μM (+)-2PABH (right upper traces Figure 3e) suggests that this compound behaves as a partial agonist.

The preliminary results obtained for PABH revealed that removal of the hydrogen-bond acceptor resulted in a compound discriminating for the ganglionic α3β4 nAChR subtype. Comparison with 2PABH suggests that the pyridine nitrogen of 2PABH is not involved in stabilisation of the *A* state at the α3β4 receptor. These results also indicate that the *ortho* nitrogen plays a key role in activating the α7 subtype. Comparison of the electrophysiological profile of PABH with those of EPB and DClEPB additionally underlines the importance of the pyridyl nitrogen for receptor activation. Obviously, the nitrogen as a hydrogen-bond acceptor is essential in the structures of EPB and DClEPB to make them strong agonists.

Electrophysiological data obtained with 4PABH revealed no major differences versus PABH. In contrast to an *ortho* nitrogen as in 2PABH, a *para* nitrogen seems to have no impact on activation of the receptors. Compared to DClEPB and EPB the pyridine nitrogen of 2PABH and 4PABH is moved 60° towards or away from the azabicyclo moiety. As a result, the nitrogen orbitals with the free electron pair are pointing in different directions. The data show that the spatial orientation of the *para* nitrogen, which has a nonflexible N–N distance hardly longer than EPB, does not allow an interaction with the receptor.

HEPB is EPB enlarged in the azabicyclo ring by one CH_2 unit. The differences of HEPB compared to EPB were remarkable. Although the efficacy of (+)-HEPB was similar to that of EPB, (–)-HEPB displayed a distinct loss of activity. Obviously, this structural variation induced a strong enantioselectivity. Recently published results have described a similar phenomenon with another modification of EPB. By attaching a methyl group to the 7-aza-position of EPB a reduced effectivity of the (+)-enantiomer was observed [22].

In conclusion, non-natural derivatives of EPB make it possible to investigate the structure–activity relationship of a rather simple ligand in its interaction with the neuronal nicotinic acetylcholine receptor. Small changes in the molecular structure produce marked changes in its capacity to activate or inhibit nAChR subtypes. Subtype specificity can be achieved by changing the N–N distance, by changing the orientation of the free electrons of the pyridyl nitrogen and/or by changes in the azabicyclo ring.

Significance

Epibatidine is an agonist towards the neuronal nicotinic acetylcholine receptor (nAChR) with both optical isomers equally effective, as determined by voltage clamp electrophysiology.

Small structural changes induce enantiomeric specificity, subtype specificity and dramatic changes in the effectivity to elicit a functional response. Removal of the pyridyl chlorine reduces the efficiency towards the α4β2 subtype and more so for the (–)-enantiomer. The change towards the ganglionic α3β4 subtype is smaller and more pronounced for the (+)-enantiomer. Virtually no change is observed towards the homomeric α7 subtype. Putting the pyridyl nitrogen in the *ortho* position with respect to the aza-bicyclo-ring reduces the efficiency towards α4β2 and α3β4 even further and the (–)-enantiomer is an agonist towards α7. Putting the pyridyl nitrogen in the *para* position and elimination of the pyridyl nitrogen direct the efficacy towards the α3β4 subtype. Extension of the aza-bicyclo-ring induces enantioselectivity, resulting in a 50-fold higher efficiency for one enantiomer.

A nAChR ligand contains a positively charged quaternary ammonia nitrogen and a part that interacts with the receptor by hydrogen bonding and π-interaction. The biological activity can be described as different stabilisation of the open activated or of the closed desensitised or closed resting state of the receptor by the various ligands.

Materials and methods

Chemistry

Syntheses and analysis

Unless otherwise stated reagents were obtained in analytical grade from commercial sources (Aldrich, Fluka, Merck, Sigma). NMR spectra were obtained on a VARIAN Gemini 300 spectrometer using tetramethylsilane as an internal standard. Mass spectra were recorded on a Trio 2000 spectrometer (VG ORGANIC, UK) using electrospray in positive ion mode (ES+). The optical purity is reported in % of enantiomeric excess (% ee).

The key step in the preparation of all ligands presented herein is the Heck reaction (see Figure 1). By using this highly flexible C–C-coupling synthesis, modifications in the EPB structure were easily accessible. Detailed preparations of EPB [60], HEPB [61], DClEPB [62] and 2PABH [24] were recently reported. For synthesis of 4PABH, N-methoxycarbonyl-7-azabicyclo[2.2.1]heptene (330 mg, 2.15 mmol), prepared according to Clayton *et al.* [60], was dissolved with 4-bromopyridine (849 mg, 5.38 mmol, 2.5 eq.), tetrakis(triphenyl-phosphine)palladium (124 mg, 0.11 mmol, 5 mol%), in dry N,N-dimethylformamide (3 ml), piperidine (549 mg, 6.45 mmol, 3 eq.) and formic acid (300 mg, 6.45 mmol, 3 eq.). After stirring (6.5 h, 80°C) under argon, water was added (15 ml). Ether extracts (4 × 25 ml) were washed with water, dried (sodium sulphate) and evaporated to give an oil (310 g), which was purified by flash chromatography (ethylacetate) to give N-methoxycarbonyl-4PABH (210 mg, 42%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.48 (dd, J = 4.4, 1.6 Hz, 2H), 7.22 (dd, J = 4.4, 1.6, 2H), 4.35–4.25 (m, 2H), 3.64 (s, 3H), 2.84 (dd, J = 5.0, 8.7 Hz, 1H), 2.0–1.6 (m, 6H) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 156.7, 153.9, 149.8, 122.3, 61.4, 56.5, 52.4, 47.3, 39.7, 30.9, 28.8 ppm; mass spectrum (electro spray, cone voltage +40 V) m/z 233 $[M+1]^+$.

The methyl carbamate (170 mg, 0.73 mmol) was dissolved in 33% hydrogen bromide in acetic acid (3.0 ml) and stirred at room temperature for 17 h. Water was added and the solution made basic with 40% aqueous NaOH. The mixture was extracted with dichloromethane (3 × 10 ml), dried (sodium sulfate) and evaporated to give an oil, which was purified by flash chromatography (ethylacetate/triethylamine, 90/10, v/v) to give 4PABH (81 mg, 0.46 mmol, 64%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.47 (dd, J = 4.7, 1.6 Hz, 2H), 7.14 (dd, J = 4.7, 1.6, 2H), 3.81–3.65 (m, 2H), 2.79 (dd, J = 5.0, 8.7 Hz, 1H), 2.35 (br s, 1H), 2.0–1.5 (m, 6H) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 155.0, 149.5, 122.4, 62.1, 56.3, 47.1, 39.5, 30.9, 29.6 ppm; mass spectrum (electro spray, cone voltage +40 V) m/z 175 $[M+1]^+$.

By analogy to the procedure presented above the heptene (70 mg, 0.46 mmol) was reacted with iodobenzene (235 mg, 1.15 mmol) to obtain N-methoxycarbonyl-PABH (0.20 mmol, 47 mg, 44%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.37–7.14 (m, 5H), 4.45 (br s, 1H), 3.30 (br s, 1H), 3.65 (s, 3H), 2.89 (dd, J = 8.4, 5.5 Hz, 1H), 2.0–1.5 (m, 6H) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 145.6, 129.5, 128.5, 127.2, 126.2, 62.1, 56.1, , 52.2, 48.1, 40.1, 29.9, 28.8 ppm; mass spectrum (electro spray, cone voltage +40 V) m/z 232 $[M+1]^+$.

Deprotection of the carbamate (41 mg, 0.18 mmol) in a similar way as described above, yielded PABH (17.4 mg, 0.10 mmol, 56%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.30–7.16 (m, 5H), 3.77 (dd, J = 4.2, 4.2 Hz, 1H), 3.65 (d, J = 4.1 Hz, 1H), 2.88 (dd, J = 8.8, 5.2 Hz, 1H), 1.92 (dd, J = 12.4, 8.9 Hz 1H), 1.8–1.3(m, 5H) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 146.6, 128.5, 127.2, 125.9, 62.9, 56.5, 48.0, 40.1, 30.8, 29.7 ppm; mass spectrum (electro spray, cone voltage +40 V) m/z 174 $[M+1]^+$.

Separation of the enantiomers

All enantiomers were separated on a chiral ChirobioticT column in analogy to the procedures described recently [24,34,63]. Optical purity of enantiomers was checked with the same HPLC set-up as used for the semipreparative separations. The stereoisomers of EPB, HEPB as well as of DClEPB eluted in high optical purity (> 99.8% ee). Enantiomeric (–)-2PABH and (+)-2PABH were obtained in optical purity of > 99.8% ee and > 98.0% ee, respectively.

Electrophysiology

All the cDNA constructions were cloned from rat. For the α7, α3β4 and α4β2 nAChRs reconstitution in *Xenopus* oocytes vector pcDNAIneo with RSV promoter was used. Oocytes were harvested from female *Xenopus leavis*, prepared and injected as previously described [64,65]. Oocytes were injected with rat cDNA combinations α4β2, α3β4 and α7 and the physiological properties of the different subtypes were examined 2–3 days following the injection using a dual-electrode voltage clamp. Electrophysiological recordings were performed using a dual-electrode voltage clamp apparatus (Geneclamp, Axon Instruments, Forster City, CA) [65]. During the experiments, oocytes were continuously superfused with control solution containing 82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 (NaOH). Perfusion was gravity fed at roughly 6 ml/min and drugs were applied by electromagnetic valves (general valve type III) controlled by computer. Unless indicated, cells were held throughout the experiments at –100 mV. Data are represented with their respective standard error of mean (SEM).

Acknowledgements

Rat cDNAs were kindly provided by J. Pattrick. This work was supported by the Swiss National Science Foundation 3100-046939.96 to G.W. and 3100-053638.98 and the Office Fédéral de l'Education et des Sciences to D.B.

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