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Anticonvulsants Based on the α-Substituted Amide Group Pharmacophore Bind to and Inhibit Function of Neuronal Nicotinic Acetylcholine Receptors

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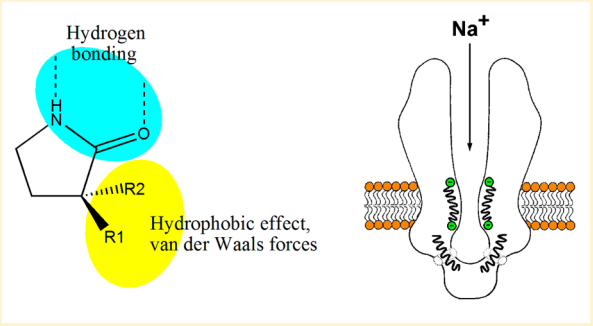
Supporting Information

ABSTRACT: Although the antiepileptic properties of α-substituted lactams, acetamides, and cyclic imides have been known for over 60 years, the mechanism by which they act remains unclear. I report here that these compounds bind to the nicotinic acetylcholine receptor (nAChR) and inhibit its function. Using transient kinetic measurements with functionally active, nondesensitized receptors, I have discovered that (i) α-substituted lactams and cyclic imides are noncompetitive inhibitors of heteromeric subtypes (such as α4β2 and α3β4) of neuronal nAChRs and (ii) the binding affinity of these compounds toward the nAChR correlates with their potency in preventing maximal electroshock (MES)-induced convulsions in mice. Based on the hypothesis that α-substituted amide group is the essential pharmacophore of these drugs, I found and tested a simple compound, 2-phenylbutyramide. This compound indeed inhibits nAChR and shows good anticonvulsant activity in mice. Molecular docking simulations suggest that α-substituted lactams, acetamides, and cyclic imides bind to the same sites on the extracellular domain of the receptor. These new findings indicate that inhibition of brain nAChRs may play an important role in the action of these antiepileptic drugs, a role that has not been previously recognized.



KEYWORDS: *Chemical kinetics, nicotinic acetylcholine receptor, antiepileptic drugs, noncompetitive inhibition, patch clamp, molecular docking*

The nicotinic acetylcholine receptor (nAChR) is a member of the superfamily of ligand-gated ion channels that mediate neurotransmission between 10^{11} neurons in a human brain and between nerves and muscles.[1,2] The receptor is a pentameric transmembrane protein that upon binding of the neurotransmitter, acetylcholine, undergoes a rapid conformational transition from the closed-channel state to the open-channel state. While the physiological and pathological role of muscle-type nAChRs present at the neuromuscular junction is well established, the significance of neuronal nAChRs in the brain remained, until recently, rather enigmatic. Function of the nAChR is orthosterically and allosterically modulated by various chemical compounds, ranging from snake venom neurotoxins to antidepressants and antiepileptic drugs.[3]

Antiepileptic activity of α-substituted lactams and cyclic imides (Figure 1) have been known for over 60 years.[4−6] For instance, methsuximide (which is converted in vivo into its active metabolite, 3-methyl-3-phenylpyrrolidine-2,5-dione) is a broad-spectrum anticonvulsant valuable in treatment of medically intractable (drug resistant) epilepsy.[7] Ethosuximide (3-ethyl-3-methylpyrrolidine-2,5-dione) is the drug of choice in the treatment of absence (petit mal) seizures.[8] α-Substituted lactams are promising experimental anticonvulsants with a good therapeutic index.[5,9,10] Historically, these two classes of drugs were developed as variations of the barbiturate scaffold[5,6] (Figure 1).

α-Substituted acetamides are also well established as anticonvulsants. For example, Bialer and Yagen with collaborators synthesized a number of novel α-alkyl-substituted acetamides and demonstrated their promising activity in preventing epileptic and soman-induced seizures in animals.[11,12] Some of those compounds are currently in clinical trials.

Many studies have been conducted to determine the physiological processes that are affected by α-substituted lactams and cyclic imides, and a number of molecular targets have been considered. The previously proposed targets include the $GABA_A$ receptor,[9,10,13] T-type (low-threshold) Ca^{2+} channels,[14] and voltage-gated Na^+ channels.[15,16] However, lack of a correlation between the anticonvulsant potency of α-substituted lactams in vivo and their effect on the proposed target in vitro disqualifies the $GABA_A$ receptor, Na^+ channel, and T-type Ca^{2+} channel from being the primary, shared targets

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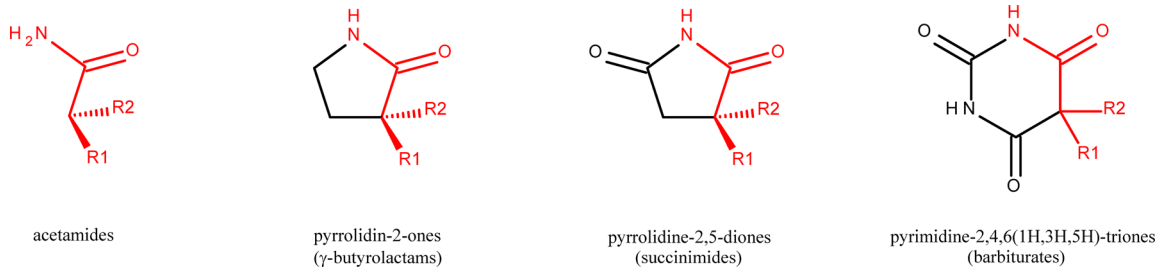

acetamides | pyrrolidin-2-ones (γ-butyrolactams) | pyrrolidine-2,5-diones (succinimides) | pyrimidine-2,4,6(1H,3H,5H)-triones (barbiturates)

Figure 1. α-Substituted amide group is the common structural motif of anticonvulsant acetamides, lactams, cyclic imides, and barbiturates.

of α-substituted lactams and cyclic imides and suggests that other targets are expected to be involved in the anticonvulsant activity of these compounds. As for α-substituted acetamides, almost nothing is known about their targets of mechanism or their action.

It was therefore of interest when I serendipitously observed that 3,3-diethylpyrrolidin-2-one (DEP; Figure 2, $R_1 = R_2 = Et$) inhibits function of the nAChR.[17] The effects of several α-substituted lactams, cyclic imides, and carboxamides on neuronal nAChRs activated by carbamoylcholine (a hydrolytically stable analog of acetylcholine) are reported here. In these experiments, I used a rapid reaction cell-flow technique with a millisecond time resolution in a combination with whole-cell patch-clamp recordings to determine the effects of these compounds on activation of the nAChR. I also used molecular docking to find the putative binding sites of these compounds on the acetylcholine binding protein (AChBP), a model for the extracellular domain of the receptor.

RESULTS AND DISCUSSION

Inhibition by 3,3-Diethylpyrrolidin-2-one (DEP) of Neuronal nAChRs Recombinantly Expressed in HEK 293 Cells. This study was triggered by my observation that DEP, while having a low potency in potentiating function of $GABA_A$ receptor (apparent K_d ca. 10 mM[18]), is actually more potent as an inhibitor of α3β4 neuronal nAChR (apparent K_d 1.9−4.7 mM, Figure 2A and B). Figure 2A shows representative current traces obtained using a rapid reaction cell-flow technique[19] with the rat α3β4 nAChR in the presence and absence of DEP. The observed increase in current elicited by carbamoylcholine is followed by a decrease due to receptor desensitization. All the current amplitudes reported in this paper are peak current amplitudes, computationally corrected for desensitization that occur during the current rise. Thus, these current amplitudes represent the concentration of the open-channel conformation, $[\overline{RL}_2]$, of fully functionally active, nondesensitized receptor.

DEP inhibits the receptor more profoundly at a high (3 mM) concentration of carbamoylcholine than at a low (0.3 mM) concentration of carbamoylcholine (Figure 2A). When applied alone at a concentration as high as 10 mM, DEP elicits no current (not shown).

The apparent dissociation constants, K_I, of DEP were determined from the concentration dependence of DEP inhibition of the nAChR (Figure 2B) by using the following equation:[20]

$$\frac{A_0}{A_I} = 1 + \frac{[I]}{K_I} \quad (1)$$

where A_0 and A_I are peak current amplitudes (corrected for desensitization that occur during the current rise) in the absence and presence of the inhibitor (DEP), respectively, and [I] is the molar concentration of the inhibitor.

At a low agonist concentration (0.3 mM carbamoylcholine), when the receptors are predominantly in the closed-channel conformation, the apparent dissociation constant, K_I, of DEP from the receptor is 4.7 ± 0.3 mM (Figure 2B). At a high agonist concentration (3 mM carbamoylcholine), when the receptors are predominantly in the open-channel conformation, the value of K_I for DEP is 1.90 ± 0.04 mM (Figure 2B).

We have previously shown[21] that even in the presence of near-saturating concentrations of carbamoylcholine only about half the α3β4 nAChRs are in the open-channel conformation. Under these conditions, the dissociation constant of DEP from the closed-channel conformation (4.7 mM) is expected to contribute to the value of the apparent dissociation constant of DEP from the open-channel conformation, $\overline{K}_I$. Therefore, the actual value of $\overline{K}_I$ for DEP is expected to be lower than the observed value of 1.9 mM.

When coapplied with near-saturating concentrations of carbamoylcholine, DEP also inhibits function of other heteromeric (α4β2) and homomeric (α7) neuronal nAChRs (Figure 2C). DEP inhibits function of the muscle-type (α1β1γδ) nAChR only at very high concentrations (Figure 2C).

The inhibitory effect of DEP does not seem to extend to other excitatory neurotransmitter receptors - it is without effect on either a recombinantly expressed *N*-methyl-D-aspartate (NMDA) receptor (data not shown) or glutamatergic excitatory postsynaptic potentials in neurons.[15]

Because of its robust functional activity (resulting in excellent signal-to-noise ratio even at low concentrations of the activating ligand, carbamoylcholine), I have chosen to use rat α3β4 nAChR stably expressed in HEK 293 cells[22] for all the further experiments described in this paper. This neuronal nAChR subtype is expressed in autonomic ganglia and in certain areas of the brain, such as the substantia nigra, hippocampus, locus coeruleus, habenulo-interpeduncular tract, and cerebellum.[2] Recent studies indicated that α3-containing nAChRs in the brain are functionally significant[2] and are involved in modulation of seizure threshold.[23] About 20 other nAChR subunit combinations exist in the brain, with α4β2 nAChR being predominant.[2] While it is not known which nAChR subtypes are most important in epileptic seizure generation, further studies should thoroughly examine nAChR subtype specificity of α-substituted lactams, acetamides, and cyclic imides.

Effect of DEP on Carbamoylcholine Activation of the α3β4 nAChR. The cell-flow technique was used to determine the dependence of the current corrected for receptor desensitization on carbamoylcholine concentration in the

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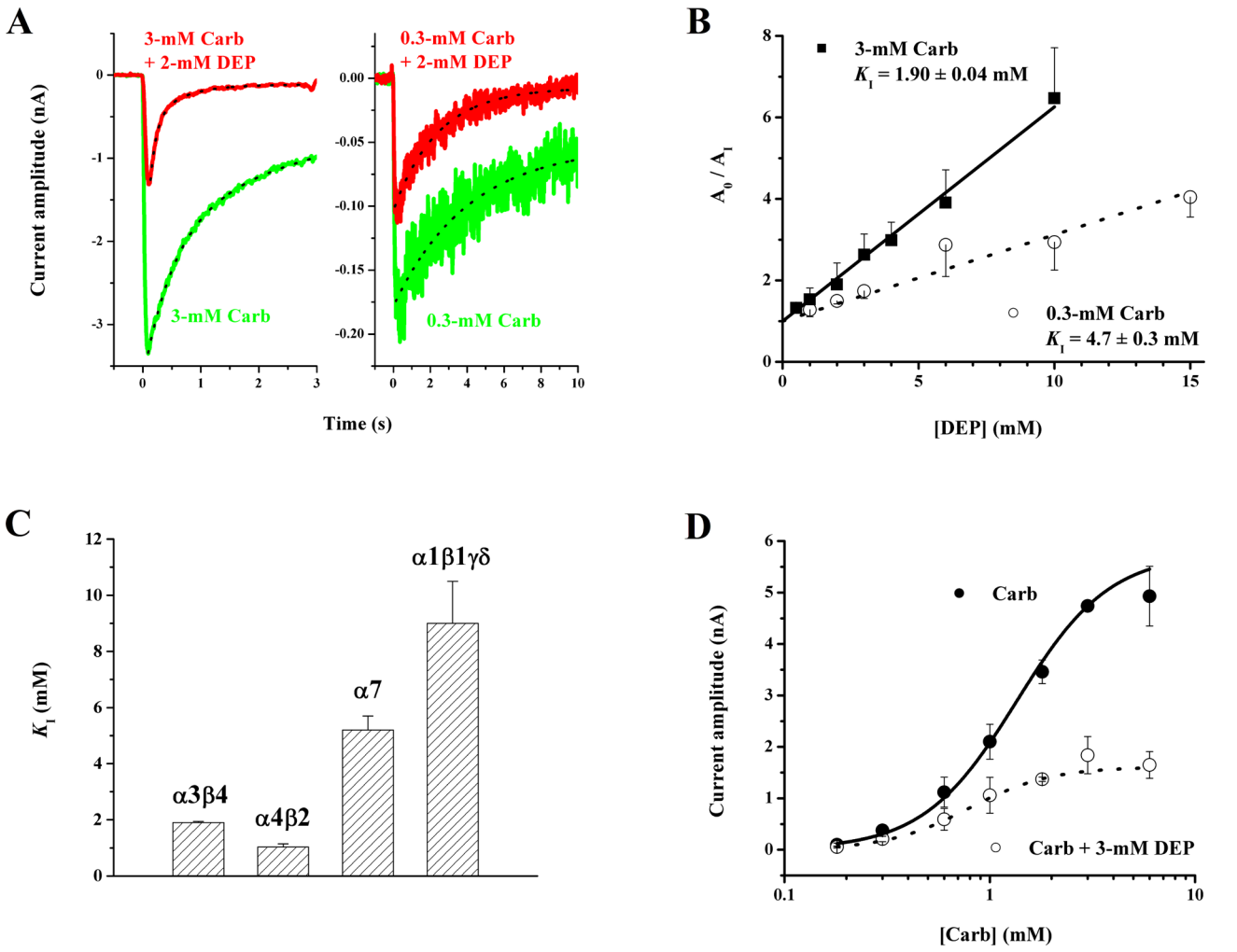

Figure 2. Inhibition of the nAChR by 3,3-diethylpyrrolidin-2-one (DEP). Experiments were conducted at room temperature (22−24 °C), pH 7.4, and −60 mV. The cell-flow technique with a 20 ms time resolution[19] was used to record the current traces and to correct the current amplitudes for receptor desensitization. DEP was coapplied with carbamoylcholine (Carb) in the extracellular buffer. The compositions of the buffers are given in the Methods section. Each data point represents 3−5 independent measurements, and the error bars represent the standard deviation. (A) Representative cell-flow experiments with the $\alpha3\beta4$ nAChR. Fitting of the time course of current decay due to desensitization with one (0.3 mM Carb) or two (3 mM Carb) exponentials is shown as black dotted lines. Left panel: 3 s application of 3 mM Carb in the absence and presence of 2 mM DEP. Right panel: 10 s application of 0.3 mM Carb in the absence and presence of 2-mM DEP. (B) Determination of the binding affinity of DEP toward the $\alpha3\beta4$ nAChR in the presence of high (3 mM, filled squares) and low (0.3 mM, hollow circles) concentrations of Carb. The ratio of the current amplitudes (corrected for receptor desensitization) in the absence, A_0, or presence, A_I, of DEP, is plotted as a function of DEP concentration. The values for the apparent DEP dissociation constant, K_I, were determined from the slopes of the lines according to eq 1. (C) Determination of the binding affinity of DEP toward neuronal and muscle-type nAChRs. The measurements were done in the presence of high (saturating) concentrations of carbamoylcholine: 3 mM for the $\alpha3\beta4$ nAChR (data from Figure 2B were used), 1 mM for the $\alpha4\beta2$ nAChR, 3 mM for the $\alpha7$ nAChR, and 1 mM for the $\alpha1\beta1\gamma\delta$ nAChR. Two-sample t test indicates that the K_I values for $\alpha4\beta2$ nAChR, $\alpha7$ nAChR, and $\alpha1\beta1\gamma\delta$ nAChR (1.03 ± 0.11, 5.2 ± 0.5, and 9.0 ± 1.5 mM, respectively) are statistically different from the K_I value for $\alpha3\beta4$ nAChR (1.90 ± 0.04 mM) at 95% confidence level. (D) Effect of DEP on activation of the $\alpha3\beta4$ nAChR by carbamoylcholine. Data were collected at the indicated concentrations of Carb in the absence (filled circles) and presence (hollow circles) of 3 mM DEP. The lines represent the fit of the data to eq 2. Two-sample t test indicates that the A_{max} and EC_{50} values in the absence of the inhibitor (5.5 ± 0.2 nA and 1.3 ± 0.2 mM, respectively) are statistically different from the corresponding values (1.6 ± 0.2 nA and 0.8 ± 0.2 mM) in the presence of the inhibitor at 95% confidence level. The n_H values in the absence (1.9 ± 0.2) and presence (2.2 ± 0.4) of the inhibitor are not statistically different at 95% confidence level.

presence and absence of 3 mM DEP (Figure 2D). The data obtained were fitted with the empirical Hill equation:

$$A = \frac{A_{max}}{1 + ([L]/EC_{50})^{n_H}} \quad (2)$$

where A is the peak current amplitude corrected for desensitization that occur during the current rise (nA), L is the molar concentration of activating ligand (carbamoylcholine), A_{max} is the maximum current amplitude (nA), EC_{50} is the half-efficient concentration of carbamoylcholine (mM), and n_H is the Hill coefficient.

In the absence of the drug, A_{max} is 5.5 ± 0.5 nA, EC_{50} is 1.3 ± 0.2 mM, and n_H is 1.9 ± 0.2. In the presence of 3 mM DEP, A_{max} decreases 3.4-fold (to 1.6 ± 0.2 nA), EC_{50} decreases 1.6-fold (to 0.8 ± 0.2), and n_H remains essentially unaffected (2.2 ± 0.4).

Since the inhibition of the receptor by DEP cannot be reversed by increasing the concentration of the activating

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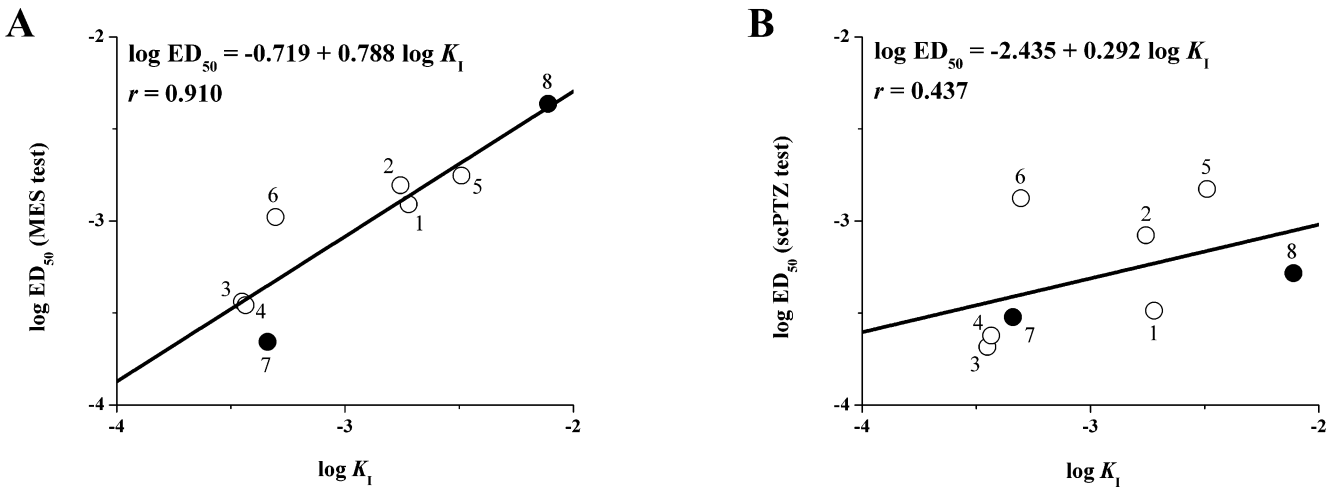

Figure 3. Structure–activity relationship among α-substituted lactams and cyclic imides. To measure the apparent K_I values, each compound at several concentrations was coapplied with 3 mM Carb (see Figure 2B). ED_{50} values (mmol/kg of body weight) for protection against MES- or scPTZ-induced seizures were measured in mice as described in the Methods section. Error bars are omitted for clarity. The hollow circles are lactams (pyrrolidin-2-ones, piperidin-2-ones, and hexahydro-2*H*-azepin-2-ones), and the filled circles are cyclic imides (pyrrolidine-2,5-diones); the symbols are numbered for easy reference to Table S1 where the K_I and ED_{50} values for each compound are given. (A) Strong correlation between anticonvulsant potency in the MES test and inhibition of the $\alpha3\beta4$ nAChR. The *P* value for the slope = 0 is 0.002. (B) Poor correlation between anticonvulsant potency in the scPTZ test and inhibition of the $\alpha3\beta4$ nAChR. The *P* value for the slope = 0 is 0.278.

ligand, carbamoylcholine (Figure 2B and D), I conclude that DEP inhibits the receptor's function noncompetitively.

In the simplest case, functioning of a ligand-gated ion channel (such as the nAChR) can be described by two sequential steps (reactions): ligand binding and channel gating:[24]

$$R \underset{k_{-1}}{\overset{2k_{+1}}{\rightleftharpoons}} RL \underset{2k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} RL_2 \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \overline{RL}_2$$

where R is the receptor, L is the activating ligand (agonist), RL_2 is the closed-channel state, $\overline{RL}_2$ is the open-channel state, $k_{+1} = k_{+2}$ is the association rate constant, $k_{-1} = k_{-2}$ is the dissociation rate constant, k_{op} is the channel-opening constant, and k_{cl} is the channel-closing constant. Thus, EC_{50} is in effect a macroscopic equilibrium constant that incorporates two microscopic equilibrium constants: the dissociation constant of the activating ligand, carbamoylcholine ($K_d = k_{+1}/k_{-1}$), and the channel-closing equilibrium constant ($\Phi = k_{cl}/k_{op}$).[25]

DEP seems to be a rather unusual inhibitor of the nAChR; it has higher affinity for the open-channel state of the receptor, and in its presence the EC_{50} value for carbamoylcholine actually decreases (Figure 2D). Many other inhibitors of the nAChR have either higher affinity toward the closed-channel state of the receptor or about equal affinity toward the closed- and open-channel states of the receptor.[20] Only a few examples of decreased EC_{50} in the presence of inhibitors can be found in the literature. With $\alpha2\beta4$ nAChR, Miledi with collaborators[26] observed a 1.6-fold decrease in EC_{50} value for ACh in the presence of 10 μM La^{3+}. Since lanthanides are expected to bind to the Ca^{2+} binding sites on proteins and one of the predicted binding sites for DEP is in the vicinity of the Ca^{2+} binding site(s) on the extracellular domain, one could speculate that some commonality may exist between the mechanisms of inhibition of nAChR by La^{3+} and DEP. For $\alpha4\beta4$ nAChR, Zwart and Vijverberg[27] observed a similar decrease in EC_{50} value for ACh in the presence of 1 μM atropine, which was attributed to potentiation of the receptor function by atropine at very low ACh concentrations.

Assuming that R binds the second molecule of L with the same affinity as the first one, the unchanged n_H suggests that the decrease in EC_{50} in the presence of DEP is due to increased affinity of the receptor toward Carb, and not due to a shift of the channel-opening equilibrium to the right.[25] However, such assumption is not always valid for the nAChR and other ligand-gated ion channels. Thus, it remains to be determined whether the observed decrease in EC_{50} in the presence of DEP is due the increased affinity of Carb toward the receptor or due to the enhanced channel-opening equilibrium.

Can an open-channel block mechanism adequately account for inhibition of the nAChR by DEP and related compounds? A sequential open-channel block mechanism has been proposed,[28] in which the binding of an inhibitor to the open-channel receptor conformation gives an inactive (nonconductive) conformation. This mechanism requires that the observed affinity of the inhibitor for the receptor increases as a fraction of the receptors in the open-channel state, $[\overline{RL}_2]$. For example, a 10-fold increase in $[\overline{RL}_2]$ would result in a 10-fold decrease in the observed K_I of the inhibitor for the receptor.[24] In my experiments, as the concentration of carbamoylcholine increased 10-fold from 0.3 to 3 mM, the $[\overline{RL}_2]$ value increased 13-fold. At the same time, the observed value of K_I for DEP decreased only 2.5-fold (Figure 2B). Thus, the data are not consistent with a simple open-channel block mechanism.

In case of charged inhibitors, a profound voltage dependency of inhibition can indicate steric block ("plugging") of the channel by the inhibitor molecule situated within the transmembrane electric field. At physiological pH, all the compounds under investigation (including DEP) are uncharged, thus the fact that inhibition of the nAChR by DEP is not voltage dependent (data not shown) cannot argue against the open-channel block mechanism.

Another possible mechanism is a cyclic one in which the binding of an inhibitor to both the closed- and open-channel receptor conformations results in an inactive state.[29] The data presented here cannot unambiguously rule out such a mechanism. The mechanisms with active (conductive) and

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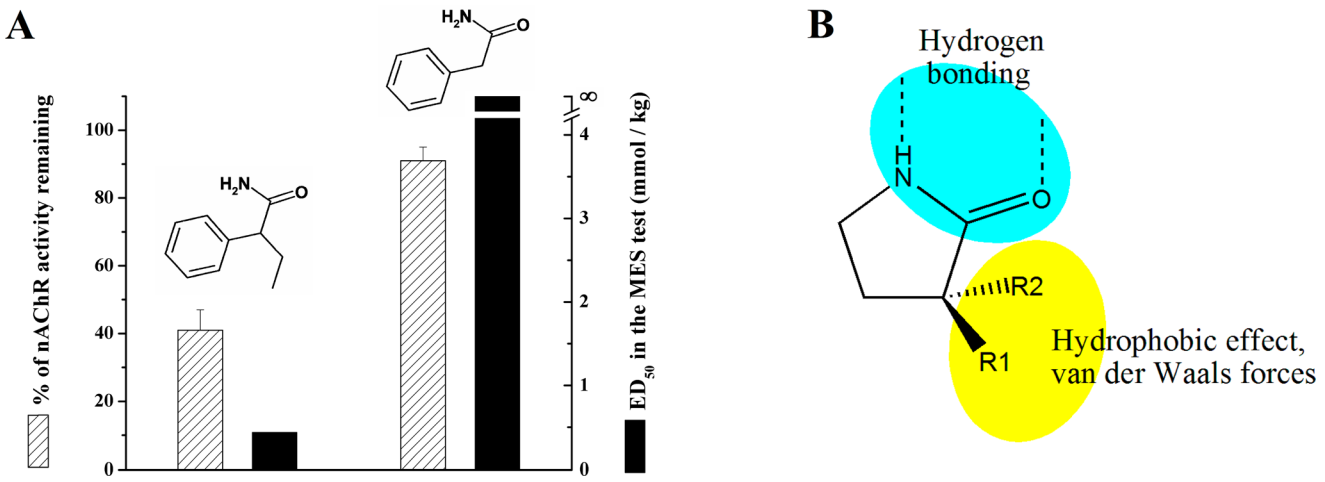

Figure 4. Amide group with at least two substituents at the α position as an antiepileptic pharmacophore of α-substituted lactams, acetamides, cyclic imides, and related compounds. (A) 2-Phenylbutyramide inhibits the nAChR and prevents MES-induced seizures, while 2-phenylacetamide is inactive. Inhibition of the $\alpha3\beta4$ nAChR was tested upon coapplication of 2-phenylbutyramide or 2-phenylacetamide (both at 2 mM concentration) with 3 mM Carb in the conditions described in Figure 2A. Protection against MES-induced seizures was tested in mice as described in the Methods section; ED_{50} for 2-phenylbutyramide was 0.44 mmol/kg (95% CI 0.33−0.55 mmol/kg), while 2-phenylacetamide was inactive at the highest dose (4.4 mmol/kg) tested. (B) Outline of the proposed pharmacophore showing the two key structural features: amide group that can form H-bonds with the receptor, and nonpolar (alkyl and/or aryl) substituents interacting with the receptor through hydrophobic effect/van der Waals forces/$\pi-\pi$ interactions.

inactive (nonconductive) $\overline{RL}_2$ states can be distinguished on the basis of the inhibitor concentration dependence of the channel-opening (k_{op}) and channel-closing (k_{cl}) rate constants.[30]

In principle, k_{cl} and k_{op} can be derived respectively from the open time and brief component of closed time distribution observed in single-channel current recordings. In reality, the assignments of dwell time components to rate constants of specific steps are frequently unreliable.[31,32] This is especially challenging for the very complex open and closed time distributions observed for neuronal nAChRs. Another technique that allows determining k_{cl} and k_{op} is laser-pulse photolysis of caged neurotransmitters in conjunction with whole-cell patch clamp recordings.[20] *N*-(α-Carboxy-2-nitrobenzyl)carbamoylcholine has been used very successfully with muscle-type nAChR.[32] However, I found that this caged compound is a potent inhibitor of $\alpha4\beta2$, $\alpha4\beta4$, and $\alpha3\beta4$ nAChRs (data not shown), which precludes its use for laser-pulse photolysis measurements with these heteromeric neuronal nAChRs.

Nature of the α-Substituent(s) Determines Binding Affinity of the Lactam Derivatives toward the nAChR. Is inhibition of the neuronal nAChRs by DEP a general property of α-substituted lactams? Will the affinity of these compounds toward the nAChR in vitro correlate with their anticonvulsant potency in vivo? Relatively large alkyl and/or aryl substituents at the α position of the lactams are necessary for potent anticonvulsant activity.[9,10] I, therefore, investigated a series of lactams with different substituents at the C-3 (α) position and their MES anticonvulsant potency in mice ranging from 0.4 to >5.3 mmol/kg of body weight (Figure 3A, Table S1).

In respect to pyrrolidin-2-ones, 3,3-dimethylpyrrolidin-2-one inhibits the receptor with very low potency (K_I 80 ± 7 mM), whereas 3-ethyl-3-benzylpyrrolidin-2-one is a substantially more potent inhibitor than 3,3-diethylpyrrolidin-2-one (DEP) or 3-isopropyl-3-methylpyrrolidin-2-one (Table S1). This is in agreement with the anticonvulsant potency of these compounds in preventing seizures induced by maximal electroshock (MES) in mice:[9] 3,3-dimethylpyrrolidin-2-one is practically inactive (the half-effective dose, ED_{50}, ≫ 5.3 mmol/kg of body weight), DEP and 3-isopropyl-3-methylpyrrolidin-2-one are active (ED_{50} = 1.23 and 1.56 mmol/kg, respectively), and 3-ethyl-3-benzylpyrrolidin-2-one is highly active (ED_{50} = 0.36 mmol/kg). The two examined hexahydro-2*H*-azepin-2-one (ε-caprolactam) derivatives and a piperidin-2-one (δ-valerolactam) derivative follow the same trend (Figure 3A, Table S1).

On the other hand, there is not much dependency between the affinity of these compounds toward the nAChR and their ability to prevent seizures induced by pentylenetetrazol (PTZ) (Figure 3B, Table S1). For example, the anticonvulsant potencies of DEP and 3-ethyl-3-benzylpyrrolidin-2-one in preventing PTZ-induced seizures are similar (ED_{50} ∼ 0.3 mmol/kg).[9] One explanation for this lack of correlation is the fact that pentylenetetrazol is an inhibitor of the $GABA_A$ receptor. However, since cholinergic and GABAergic neurotransmission systems in the brain are functionally connected, it is possible that while inhibition of $\alpha3\beta4$ nAChR cannot alleviate activation PTZ-induced seizures, inhibition of other, more abundant nAChR subtypes (e.g., $\alpha4\beta2$ or $\alpha2\beta2/\beta4$) will be able to do so. Further studies should thoroughly examine modulation by α-substituted lactams, acetamides, and cyclic imides of (i) as many of neuronal nAChR subtypes as possible (about 20 such subtypes are currently known) and (ii) other targets (such as the $GABA_A$ receptor, T-type Ca^{2+} channel, and Na^+ channel). Once enough of quantitative data are accumulated, a mathematical model describing activity of these compounds in various models of epilepsy as a function of modulation of multiple targets could be developed.

Importantly, no correlation is found between potentiation of the $GABA_A$ receptor function by α-substituted lactams and their ability to prevent either MES- or scPTZ-induced seizures[9,10] (Figure S1).

α-Substituted Succinimides Also Inhibit the nAChR. It was of interest to investigate whether compounds structurally related to lactams have a similar effect on the $\alpha3\beta4$ nAChR. Certain 3-substituted pyrrolidine-2,5-diones (succinimides) and

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piperidine-2,6-diones (glutarimides) have long been known to be efficient anticonvulsants.[6] I demonstrate here that 3-substituted pyrrolidine-2,5-diones (Figure 1) inhibit the $\alpha3\beta4$ nAChR (Figure 3A, Table S1, Figure S2). As with pyrrolidin-2-ones, the extent of the inhibition corresponds to their anticonvulsant activity. 3-Ethyl-3-methylpyrrolidine-2,5-dione (ethosuximide) has very little activity in the MES test (ED_{50} = 4.3 mmol/kg;[33] > 7.1 mmol/kg[34]) and, correspondingly, is only modestly active in inhibiting the $\alpha3\beta4$ nAChR (K_I = 7.43 mM; Table S1). 3-Methyl-3-phenylpyrrolidine-2,5-dione (an active metabolite of methsuximide) is more active in the MES test (ED_{50} = 0.22 mmol/kg) and, correspondingly, is more active in inhibiting the $\alpha3\beta4$ nAChR (K_I = 0.46 mM; Table S1).

Similarly to DEP, 3-methyl-3-phenylpyrrolidine-2,5-dione inhibits the receptor more profoundly at high carbamoylcholine concentrations (Figure S2), suggesting that cyclic imides, like lactams, are noncompetitive inhibitors.

Inhibitory Activity toward the nAChR Is a Common Property of α-Substituted Lactams, Acetamides, and Cyclic Imides. The above data indicate that the pharmacophore of the anticonvulsant lactams and cyclic imides consists of an amide group with nonpolar substituents in the α position. The carbonyl and NH groups may form hydrogen bonds with the protein, thus contributing to binding specificity, while the aryl and/or alkyl substituents at the α position are likely to play a key role in determining binding affinity.

The above observation suggested that one should investigate simple compounds that contain an α-substituted amide group. One such compound is 2-phenylbutyramide, an acetamide with two substituents (phenyl and ethyl) in the α position (Figure 1, R_1 = Et, R_2 = Ph). For comparative purposes, I also tested a compound with a single substituent at the α position (2-phenylacetamide).

Using the cell-flow technique, I found that in the presence of 2-mM phenylbutyramide the receptor-mediated current decreased by 59%, whereas 2-phenylacetamide is essentially inactive (Figure 4A). The anticonvulsant effect of these two compounds was tested in mice in the MES seizure model. The tests showed that 2-phenylbutyramide is active (ED_{50} 0.44 mmol/kg of body weight, 95% CI 0.33−0.55 mmol/kg), while 2-phenylacetamide is completely inactive (no protection against seizures at the highest dose tested, 4.44 mmol/kg of body weight). Thus, two substituents at the α position (R1 and R2 in Figure 4B) are required for activity. Our preliminary results indicate that 2-phenylbutyramide is also active in several other rodent models of epilepsy (to be published elsewhere). The demonstration that 2-phenylbutyramide is a good anticonvulsant corroborates our structure- and mechanism-based hypothesis that α-substituted amide group might represent an antiepileptic pharmacophore acting via inhibition of neuronal nAChRs. Although 2-phenylbutyramide (Hyposterol) has been used clinically in the 1950−1960s as a cholesterol-lowering drug,[35] its antiepileptic activity has not been recognized until now. This suggests that a stronger focus on structure- and mechanism-based approaches will be beneficial in finding new antiepileptic drugs.

In lactams and cyclic imides, the α carbon already has a methylene "substituent" as a part of the cyclic structure (Figure 1). Consequently, even the derivatives with a single substituent satisfy the above rule and are active (Table S1). The α-substituted amide group pharmacophore and the noncovalent interactions likely involved in its binding to the receptor are depicted in Figure 4B.

Within the same dose range of α-substituted lactams and succinimides, excellent correlation is observed between the in vivo activity in the MES test and inhibition of the nAChR (Figure 3A), but no correlation is observed between the in vivo activity in either the MES test or the scPTZ test and potentiation of the $GABA_A$ receptor (Figure S1),[9,10] inhibition of Na^+ channels,[15] or inhibition of T-type Ca^{2+} channels.[14] This might suggest that the trend in Figure 3A is primarily due to the binding of these compounds to brain nAChRs. However, one needs to keep in mind that absorption, distribution, metabolism, and excretion (ADME) can and likely do factor in to the observed correlation.

The extent of absorption of a drug can vary considerably, especially with oral administration. In our case, since the compounds are administered i.p., fast and essentially complete absorption is expected. Once absorbed, the compounds in question will distribute in tissues, and such distribution will not be uniform: for example, the plasma concentrations (mg/L) of α-substituted cyclic imides 3-ethyl-3-methylpyrrolidine-2,5-dione (ethosuximide) and 3-methyl-3-phenylpyrrolidine-2,5-dione in adult patients are 1.6−3.1 times the daily doses (mg/kg of body weight) (*Antiepileptic Drugs*,[36] pp. 662 and 683). An additional level of complexity can arise from differential binding of drugs to serum proteins. Once in the body, drugs are eliminated by metabolism and excretion. α-Substituted cyclic imides are metabolized mainly through hydroxylation of the aryl and alkyl substituents at the α position, and this mechanism is expected for α-substituted lactams and acetamides also. Additionally, α-substituted acetamides can undergo deamidation. Elimination half-lives of 3-ethyl-3-methylpyrrolidine-2,5-dione (ethosuximide) and 3-methyl-3-phenylpyrrolidine-2,5-dione are long (9−26 h in rat and 30−80 h in humans, *Antiepileptic Drugs*,[36] pp. 661 and 683). No such data are available for α-substituted lactams, but elimination half-lives of α-substituted acetamides tend to be shorter (e.g., about 1 h in rat for 2-*sec*-butyl-2-propylacetamide[12]).

There are no data on blood concentrations of the α-substituted lactams, and the available data on blood concentrations of α-substituted cyclic imides are very limited. The K_I value for inhibition of the nAChR by 3-methyl-3-phenylpyrrolidine-2,5-dione, 0.46 mM, is close to the therapeutic range of its steady-state blood plasma concentrations in humans, 0.05−0.21 mM (*Antiepileptic Drugs*,[36] p. 683)

Further studies (e.g., using affinity isolation of anticonvulsant-binding proteins from solubilized brain tissue/membranes or using mice with "knocked out" nAChR subunit genes) will be needed to unambiguously demonstrate that neuronal nAChRs are indeed the primary pharmacological targets of these compounds in the brain. Such studies would also help pinpoint specific nAChR subtypes involved in the observed antiepileptic effects.

α-Substituted lactams, Acetamides, and Cyclic Imides Are Predicted To Bind to the Same Sites on the Acetylcholine Binding Protein (AChBP). To explore possible binding sites of α-substituted lactams, acetamides, and cyclic imides on the AChR, I conducted molecular docking simulations with 3,3-diethylpyrrolidin-2-one (DEP), 2,2-diethylacetamide (2-ethylbutyramide), and 3,3-diethylpyrrolidine-2,5-dione using an experimentally determined atomic structure of the acetylcholine binding protein (AChBP) of *Lymnaea stagnalis* as a target. The AChBP is a well-established model for the extracellular domain of the nAChR.[37,38]

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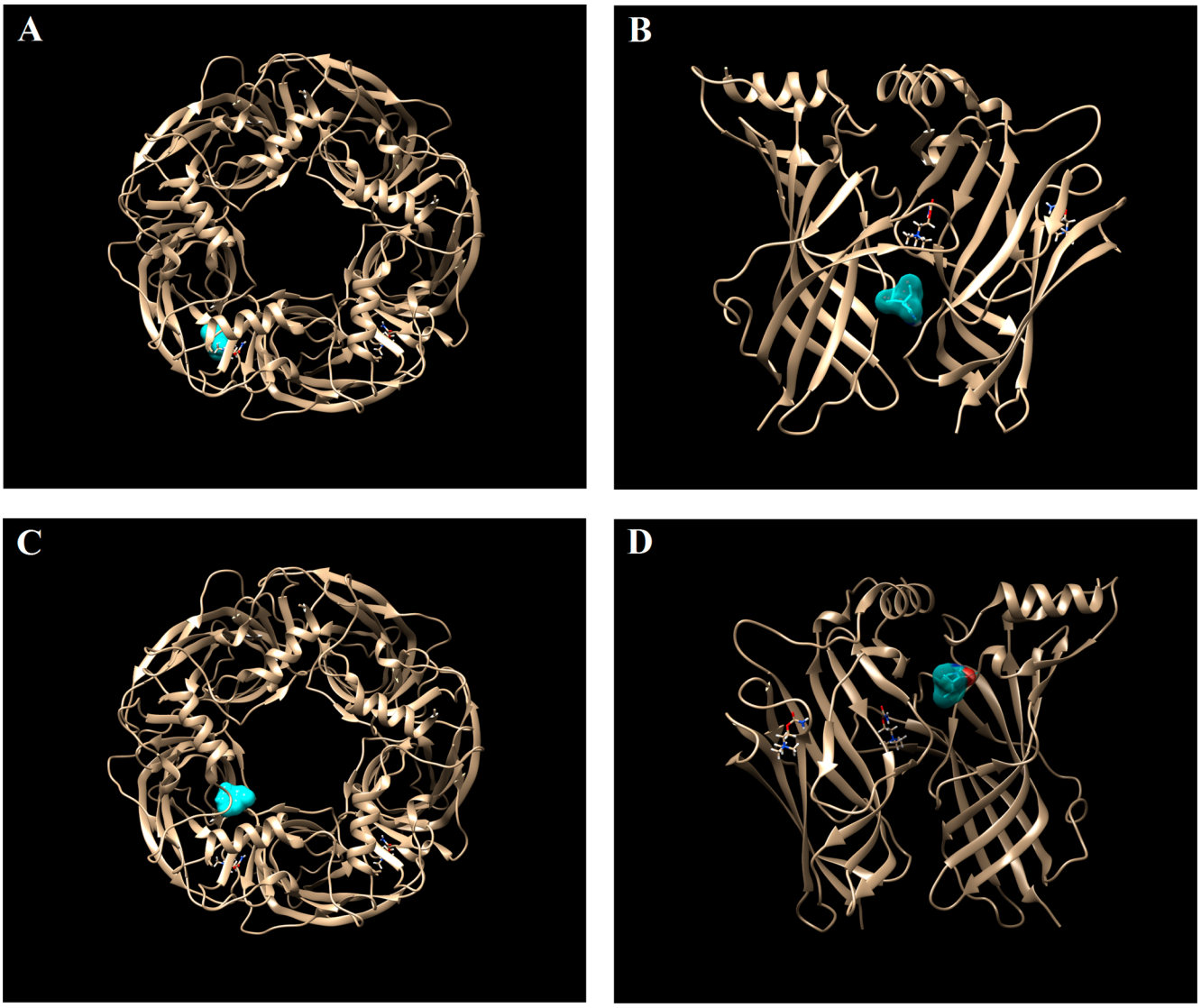

Figure 5. Molecular docking of DEP to the AChBP suggests two distinct binding sites. An energy-minimized conformation of DEP was docked to AChBP-Carb complex (PDB ID: 1UV6) as described in the Methods section. The Carb molecules are shown in stick format, while DEP is shown in solid surface format. DEP (light blue) bound to the site on the outer rim of the AChBP pentamer is shown in (A) (view from the top) and (B) (view from the outside of the protein; only subunits C and D are shown). DEP (light blue) bound to the site in the vestibule of the AChBP pentamer is shown in (C) (view from the top) and (D) (view from the inside of the protein; only subunits C and D are shown). UCSF Chimera was used to visualize docking results. Docking summaries and possible hydrogen bonds between the AChBP and 3,3-diethylpyrrolidin-2-one (DEP), 2,2-diethylacetamide (2-ethylbutyramide), and 3,3-diethylpyrrolidine-2,5-dione are given in Tables S2−S4.

The five orthosteric ("agonist") binding sites of the AChBP pentamer are known to be promiscuous in regard to various compounds and readily accommodate not only agonists and competitive (orthosteric) inhibitors of the nAChR, but also noncompetitive (allosteric) inhibitors of the receptor and even the molecules of PEG and HEPES.[39,40] Thus, it was not surprising that docking the α,α-diethyl derivatives to unliganded AChBP (PDB ID: 1UX2, with the HEPES molecule removed) resulted in many of the high-score dockings being in the orthosteric binding sites formed by loop C (data not shown).

Therefore, in subsequent computations I employed the complex of the AChBP with carbamoylcholine (PDB ID: 1UV6) as a target for the compounds in question. For consistency, docking space was limited to the single interface of subunits C and D (with carbamoylcholine molecule bound at the same interface). The highest-score dockings of all three α,α-diethyl derivatives were observed predominantly in two distinct positions (binding sites) on the protein. The results for DEP appear in Figure 5, and Tables S2−S4 give docking summaries and possible hydrogen bonds for all three derivatives.

One allosteric binding site is located on the outside of the pentamer, approximately halfway between the orthosteric binding site and where the lipid bilayer would be in intact membrane-bound receptor (Figure 5A and B). This site is within the same subunit interface area (spanning from the orthosteric site down to the membrane) as the Ca^{2+} binding sites.[41] X-ray diffraction experiments with *Aplysia californica* AChBP[42] indicate that the lower part of methyllycaconitine molecule resides in this site.

Another allosteric binding site is located in the inner part of the pentamer - in the vestibule that leads to what would be the ion channel in the intact receptor (Figure 5C and D). Frequent hydrogen bonds between Val^{83} of the subunit C and the

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compounds in question were observed (see Tables S2−S4). Binding to this site has been observed before for galanthamine, physostigmine, and codeine[43,44] and for several analogs of methyllycaconitine.[45]

Similar results were obtained with all the compounds listed in Table S1: they all dock predominantly to the same two binding sites, and all the highest-scored ligand poses are in the site located below the orthosteric binding site (data not shown).

It is instructive to compare my docking results with the recent comprehensive endeavor by Ulens with collaborators to draw a molecular blueprint of allosteric binding sites in the extracellular domain of α7 nAChR.[46] Using X-ray diffraction crystallography, these authors found three binding sites: one at the top of the domain, near the N-terminal α-helix (the "top pocket"), another in the vestibule leading to the ion channel (the "vestibule pocket"), and the third, formed by loop C of a (+) subunit and loop F of a (−) subunit, below the agonist binding site (the "sub-agonist pocket"). The two most highly scored and frequent docking sites predicted for α,α-diethyl derivatives of pyrrolidin-2-one, acetamide, and pyrrolidine-2,5-dione in this study (Figure 5) correspond to the "sub-agonist pocket" and the "vestibule pocket" in the above paper.

A recent X-ray diffraction study of the muscarinic acetylcholine receptor suggests that positive allosteric modulators increase the affinity of that receptor for ACh by binding to a site adjacent to/partially overlapped with the ACh binding site and thus slowing down the dissociation of ACh molecule.[47] Conceivably, a similar mechanism could exist in the nicotinic acetylcholine receptor. Thus, binding of DEP to the "subagonist pocket" could account for the possible increase in the receptor's affinity for Carb in the presence of DEP (Figure 2D).

CONCLUSION

Epileptic seizures are caused by an imbalance between inhibitory and excitatory neurotransmission in the brain. The imbalance can arise from dysfunctions of diverse yet functionally interconnected ion channels.[48] This suggests that (i) a dysfunction of one neurotransmission pathway can be compensated by modulating another, physiologically opposite neurotransmission pathway, and (ii) simultaneous modulation of multiple neurotransmission steps (e.g., potentiation of inhibitory $GABA_A$ receptors and inhibition of excitatory glutamate and nicotinic acetylcholine receptors) might provide for a more efficient management of seizures. Currently, neuronal nAChRs are not considered to be among the main targets of antiepileptic drugs.[48] However, as our awareness of the role of neuronal nAChRs in epilepsy began to increase in the recent years, it has been suggested that inhibition of function of these receptors in the brain may be a valuable therapeutic approach to treat seizures.[49,50] A few antiepileptic drugs, such as carbamazepine[51] and lamotrigine,[52] have been shown to inhibit neuronal nAChRs.

Although the anticonvulsant activity of α-substituted lactams, acetamides, and cyclic imides has been known for over six decades and some are used clinically, no unifying mechanism of their action has emerged so far, and the search for targets of α-substituted lactams, acetamides, and cyclic imides has not yet produced a shared and pharmacologically predictive target. Surprisingly, the structural similarity of these drugs (Figures 2 and 4B) and the consequent implication of a shared, common target have not been fully recognized until now. My data indicate that these three classes of compounds bind to the major heteromeric subtypes of neuronal nAChRs and noncompetitively inhibit their function. Interestingly, the binding affinity of α-substituted lactams and cyclic imides toward the nAChR correlates with their potency in preventing maximal electroshock (MES)-induced seizures in mice. (For α-substituted acetamides, too few compounds have been examined so far to be able to notice a trend.) However, that is not the case for PTZ-induced seizures. Thus, inhibition of the $\alpha3\beta4$ nAChR can explain antiepileptic activity of α-substituted lactams, acetamides, and cyclic imides in some, but not all animal models of epilepsy. Further work will be needed to delineate the roles of various neuronal nAChR subtypes and to clarify the contribution of pharmacokinetic factors to the antiepileptic activity of these compounds.

Many lactam, acetamide, and cyclic imide derivatives with anticonvulsant activity have been synthesized. However, lack of understanding of how they exert their anticonvulsant effect has precluded rational, mechanism-based concepts from being developed and used for efficient design and screening of new derivatives. Now we are able to predict in vivo activity of these antiepileptic drugs using simple in vitro measurements with a well-defined, recombinantly expressed target. The observed relationship between the inhibitory potency of these nAChR inhibitors and their anticonvulsant activity in the MES seizure model suggests that prospective antiepileptic drugs could be screened for their ability to inhibit neuronal nAChRs and that additional useful compounds may be found among the many existing and yet-to-be-discovered noncompetitive inhibitors of this receptor. Modulation of other targets (such as the $GABA_A$ receptor, T-type Ca^{2+} channel, and Na^+ channel), while not a mechanism of action shared by all α-substituted lactams, acetamides, and cyclic imides, may contribute to antiepileptic activity of individual compounds belonging to these three chemical classes. Therefore, lead compounds should be assayed against all the major nAChR subtypes as well as against other putative targets.

METHODS

Chemicals. 3-Substituted pyrrolidin-2-ones (γ-butyrolactams), piperidin-2-ones (δ-valerolactams), and hexahydro-2*H*-azepin-2-ones (ε-caprolactams)[9,10] were a kind gift from Douglas F. Covey (Washington University in St. Louis, St. Louis, MO). Carbamoylcholine chloride (Carb), 3-ethyl-3-methylpyrrolidine-2,5-dione (ethosuximide), and 3-methyl-3-phenylpyrrolidine-2,5-dione (α-methyl-α-phenylsuccinimide) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). 2-Phenylacetamide was obtained from TCI America (Portland, OR), and 2-phenylbutyramide from Acros Organics (Morris Plains, NJ). Other chemicals were of the highest purity available and obtained from EM Science, TCI America, Sigma-Aldrich, or Fisher Scientific.

Stock solutions of 3-ethyl-3-benzylpyrrolidin-2-one were made in DMSO. The final concentration of DMSO in the extracellular buffer was 0.5%. Under the experimental conditions employed (a single application for 10 s or less), DMSO alone at this concentration does not affect current responses. Other compounds were dissolved directly in the extracellular buffer.

Cell Culture. The KX$\alpha3\beta4R_2$ cell line (HEK 293 cells stably expressing the rat neuronal $\alpha3\beta4$ nAChR)[22] was cultured essentially as described previously,[21] except that I now use Opti-MEM I reduced serum medium (Invitrogen Corp., Carlsbad, CA) supplemented with 5% fetal bovine serum. These improved cell culture conditions (optimized nutrient composition and better pH control) result in substantially higher expression levels of the functionally active receptors than we previously reported.[21]

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The A4B2.2 cell line (HEK 293 cells stably expressing the human neuronal $\alpha4\beta2$ nAChR) was cultured as described previously,[53] except that I used Opti-MEM I medium supplemented with 5% fetal bovine serum.

For transient expression of $\alpha7$ nAChR, HEK 293T cells were cultured at 37 °C in Opti-MEM I medium supplemented with 5% fetal bovine serum. Transient transfection was performed essentially as described before,[54] but using PolyFect transfection reagent (Qiagen Inc., Valencia, CA). Typically, 1 μg of human $\alpha7$ nAChR DNA expression vector (pCI-neo), 1 μg of human RIC-3 DNA expression vector (pGen-IRES-hyg), 0.5 μg of pGreen Lantern GFP-S65T DNA expression vector, and 20 μL of PolyFect reagent were used, and transfection was carried out for 6 h. After this time, the PolyFect-containing medium was replaced with fresh medium. Cells were used for measurements 24−36 h post transfection, with receptor-expressing cells being selected based on their GFP fluorescence.

I am grateful to Kenneth J. Kellar and Yingxian Xiao (Georgetown University, Washington, DC) for the KX$\alpha3\beta4R_2$ cell line, W. Craig Moore (AstraZeneca Pharmaceuticals, Wilmington, DE) for the RIC-3 vector, Roger L. Papke (University of Florida, Gainesville, FL) for the $\alpha7$ nAChR vector, and McHardy M. Smith and Alison Rush (Merck & Co., North Wales, PA) for the A4B2.2 cell line.

The BC_3H1 cell line expressing the mouse fetal muscle-type $\alpha1\beta1\gamma\delta$ nAChR was cultured as described previously.[19]

Whole-Cell Current Recordings. The whole-cell currents were recorded as described by Hamill et al.[55] using an Axopatch 200A integrating patch clamp amplifier and Clampex 9 data acquisition software (both from Axon Instruments, Foster City, CA). The composition of the buffer in the recording electrode was (i) 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), adjusted to pH 7.4 with KOH, for recordings with the neuronal nAChRs and (ii) 145 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM HEPES, adjusted to pH 7.4 with KOH, for recordings with the muscle-type nAChR (BC_3H1 cells). The composition of the extracellular (bath) buffer was (i) 145 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 25 mM HEPES, adjusted to pH 7.4 with NaOH, for recordings with the neuronal nAChRs and (ii) 140 mM NaCl, 5 mM KCl, 1.7 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 25 mM HEPES, adjusted to pH 7.4 with NaOH, for recordings with the muscle-type nAChR (BC_3H1 cells). Recording electrodes were pulled from borosilicate glass capillaries on a PIP5 two-stage vertical pipet puller (HEKA Elektronik, Lambrecht/Pfalz, Germany), and electrode resistance was typically 2−3 MΩ. Series resistance was typically 3−5 MΩ, and was up to 70% compensated electronically. Currents were low-pass filtered at 0.2 or 0.5 kHz using a 4-pole Bessel filter and digitized at 0.5 or 1.25 kHz, respectively. All measurements were carried out at room temperature (22−24 °C) and a transmembrane voltage of −60 mV.

Rapid Application of Ligand Solutions and Computational Correction of the Observed Current for Receptor Desensitization. The cell-flow method that involves rapid ligand application with 20 ms time resolution and off-line computational correction of the observed peak current for receptor desensitization that occur during the current rise has been described in detail.[19] A computer program written in house and running under Origin 3.5 data analysis software (OriginLab Corp., Northampton, MA) was used.

Inhibitors were coapplied with carbamoylcholine in the extracellular buffer. Typically, only one inhibitor application per cell was done.

Anticonvulsant Activity Tests. The anticonvulsant activity of 2-phenylacetamide, 2-phenylbutyramide, and 3-methyl-3-phenylpyrrolidine-2,5-dione was tested at the Anticonvulsant Screening Program, a component of the National Institute of Neurological Disorders and Stroke (NINDS), Rockville, MD, under the direction of James P. Stables and Tracy Chen. The compounds were tested for their ability to protect mice against maximal electroshock (MES) seizures and against seizures produced by subcutaneous injection of PTZ (scPTZ) in as previously described.[34,56] The compounds suspended in methylcellulose were administered i.p. to adult male CF #1 mice in a volume of 0.01 mL/g of body weight. For each compound, a time of peak effect (TPE) was established in preliminary qualitative tests. In the MES test, 50 mA AC (60 Hz) was delivered for 2 s through corneal electrodes, and the animals were observed for the presence of tonic-clonic seizures. In the scPTZ test, PTZ (Metrazol) was injected s.c. at 85 mg/kg of body weight, and the animals were observed for the presence of clonic seizures. Median half-effective doses (ED_{50}s) for 2-phenylbutyramide and 3-methyl-3-phenylpyrrolidine-2,5-dione were calculated using the probit method. Since 2-phenylacetamide is completely inactive at the highest dose tested, ED_{50} could not be calculated for that compound.

2-Phenylacetamide was administered to groups of three mice 0.5 h before convulsant challenge at three doses (0.74, 2.22, and 4.44 mmol/kg of body weight). 2-Phenylbutyramide was administered to groups of eight mice 0.25 h before convulsant challenge at five doses (0.15, 0.31, 0.46, 0.58, and 0.77 mmol/kg of body weight). 3-Methyl-3-phenylpyrrolidine-2,5-dione was administered to groups of eight mice 0.5 h before convulsant challenge at six doses (0.16, 0.21, 0.22, 0.24, 0.26, and 0.32 mmol/kg of body weight) in the MES test and at seven doses (0.20, 0.25, 0.27, 0.30, 0.40, 0.58, and 0.79 mmol/kg of body weight) in the scPTZ test. These animal studies were conducted in accordance with federal and State of Utah regulations using protocols approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

Data on the anticonvulsant activity in mice of other compounds under investigation were taken from the literature.[9,10,33]

Molecular Docking. Energy-minimized ligand structures were generated using MMFF94 force field in ChemBio3D Ultra software ver. 13.0 (CambridgeSoft/PerkinElmer Informatics, Cambridge, MA). *Lymnaea stagnalis* AChBP structures were retrieved from RCSB Protein Data Bank as PDB files and prepared for docking using DockPrep tool of UCSF Chimera ver. 1.10.1 extensible molecular modeling system (http://www.cgl.ucsf.edu/chimera/). Docking of ligands to unliganded AChBP (PDB ID: 1UX2) or AChBP-carbamoylcholine complex (PDB ID: 1UV6) was performed using AutoDock Vina[57] via Opal web service implemented in the UCSF Chimera software. For the unliganded AChBP, the search space (the entire protein) was an 85 × 85 × 85 Å^3 rectangular cuboid centered at 45 × 45 × 35 Å^3 *xyz* coordinates. For the AChBP-carbamoylcholine complex, the search space (subunits C and D of the protein) was a 50 × 50 × 70 Å^3 rectangular cuboid centered at 140 × 130 × 90 Å^3 *xyz* coordinates.

Data Analysis. All linear and nonlinear least-squares fitting, statistical calculations, and plotting of the experimental results were performed using Origin 7.0 data analysis software (OriginLab Corp., Northampton, MA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00259.

Numerical values for binding affinities and anticonvulsant potencies of α-substituted lactams and cyclic imides, summaries of molecular docking, figure illustrating lack of correlation between anticonvulsant potency of α-substituted lactams and their potentiation of the $GABA_A$ receptor, and figure illustrating inhibition of the $\alpha3\beta4$ nAChR by 3-methyl-3-phenylpyrrolidine-2,5-dione (MPPD) (PDF)

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Author Contributions

A.V.K. designed and conducted experiments, analyzed data, and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

I dedicate this paper to the memory of George P. Hess (1923−2015), a brilliant scientist and a great mentor.

■ ABBREVIATIONS

ACh, acetylcholine; AChBP, acetylcholine binding protein; Carb, carbamoylcholine; DEP, 3,3-diethylpyrrolidin-2-one; ED_{50}, median half-effective dose; $GABA_A$ receptor, γ-aminobutyric acid receptor, type A; MES, maximal electroshock; nAChR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartate; PTZ, pentylenetetrazol; scPTZ, subcutaneous injection of pentylenetetrazol

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