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Block of cyclic nucleotide-gated channels by tetracaine derivatives: Role of apolar interactions at two distinct locations

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Abstract—A series of new tetracaine derivatives was synthesized to explore the effects of hydrophobic character on blockade of cyclic nucleotide-gated (CNG) channels. Increasing the hydrophobicity at either of two positions on the tetracaine scaffold, the tertiary amine or the butyl tail, yields blockers with increased potency. However, shape also plays an important role. While gradual increases in length of the butyl tail lead to increased potency, substitution of the butyl tail with branched alkyl or cyclic groups is deleterious.

Cyclic nucleotide-gated (CNG) channels are Ca^{2+}-permeable non-selective cation channels gated by the binding of cyclic nucleotides. Despite their unique mode of activation, sequence analysis places CNG channels in the voltage-gated potassium channel superfamily. Like their K^+-selective cousins, CNG channels function as tetramers with the four subunits arranged around a central ion conduction pathway. CNG channels are poised to act as integrators of two important second messenger systems: they are both cyclic nucleotide sensors and Ca^{2+} sources. Indeed, in visual and olfactory sensation, CNG channels translate light- and odorant-induced changes in cyclic nucleotide levels into electrical signals, and Ca^{2+} influx through open channels contributes to feedback regulation (reviewed in[1,2]). CNG channels are found in a wide range of tissues including brain, heart, kidney, testis, liver, lung, skeletal muscle, adrenal gland, pancreas, and colon, suggestive of a broad physiological utility.[3–5] Limited progress has been made in deducing the functions of CNG channels beyond their established roles in vision and olfaction. For example, CNG channels have been implicated in synaptic modulation[6] and enterotoxin-linked resistance to colon cancer.[7] However, such studies rely on multiple pharmacological agents, all of which modulate CNG channel activity, but none of which are highly selective for CNG channels.[8] Potent and selective agents will be invaluable pharmacological tools for uncovering the physiological roles of CNG channels and may lead to the development of novel therapeutic agents.

Tetracaine, a local anesthetic, is a non-selective blocker of CNG channels, as well as voltage-activated Na^+ and Ca^{2+} channels. However, targeted modification of the tetracaine scaffold has the potential to enhance potency and selectivity for block of CNG channels.[9] Studies of block of Na^+ channels suggest a bimodal interaction with the channel in which negatively charged residues in the selectivity filter of the channel interact with the positively charged amine, while hydrophobic pore-lining residues interact with the apolar portion of the local anesthetic.[10,11] Tetracaine likely blocks CNG channels via a similar interaction with the pore. In fact, mutagenesis implicates a key negatively charged residue in the selectivity filter of CNGA1 as an important determinant for tetracaine binding.[12] Furthermore, addition of one or two positive charges to the tertiary amine of tetracaine results in dramatic increases in the voltage dependence of block and apparent affinity for the channel.[9,13] Previous work has revealed that the apolar butyl tail of tetracaine is essential for high affinity block.[13] In this study, we further explore the importance of hydrophobic interactions by increasing the hydrophobic content at two positions on the tetracaine scaffold.

Keywords: Ion channels; Local anesthetics; Pore blockers; cGMP; Retinal rods; Vision.
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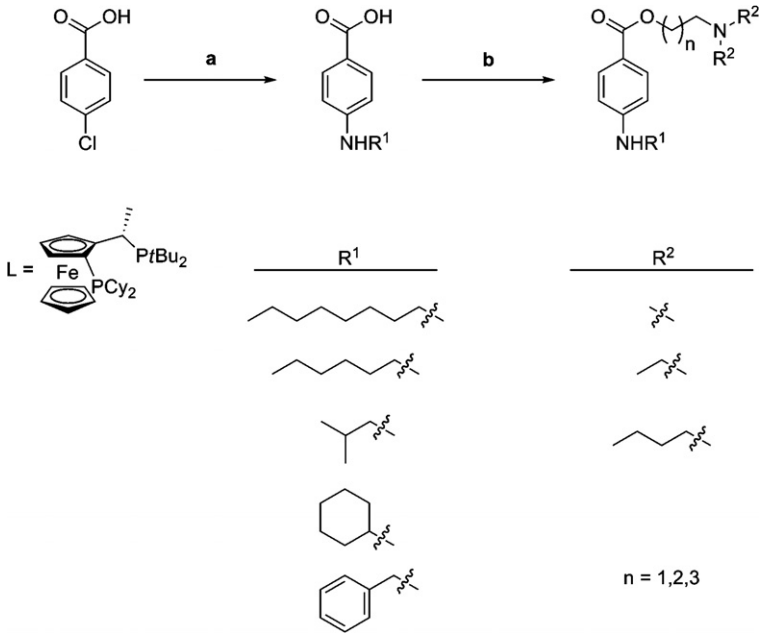


Scheme 1. Reagents and conditions: (a) 0.05 mol % $Pd(OAc)_2$, 0.05% L (Josiphos ligand), R^1NH_2 (1.2 equiv), LiHMDS (2.4 equiv), DME, 100 °C (38–88%); (b) *N*,*N*′-carbonyldiimidazole, $R^2_2NCH_2(CH_2)_nOH$, NaH, DME, 60 °C (51–93%).

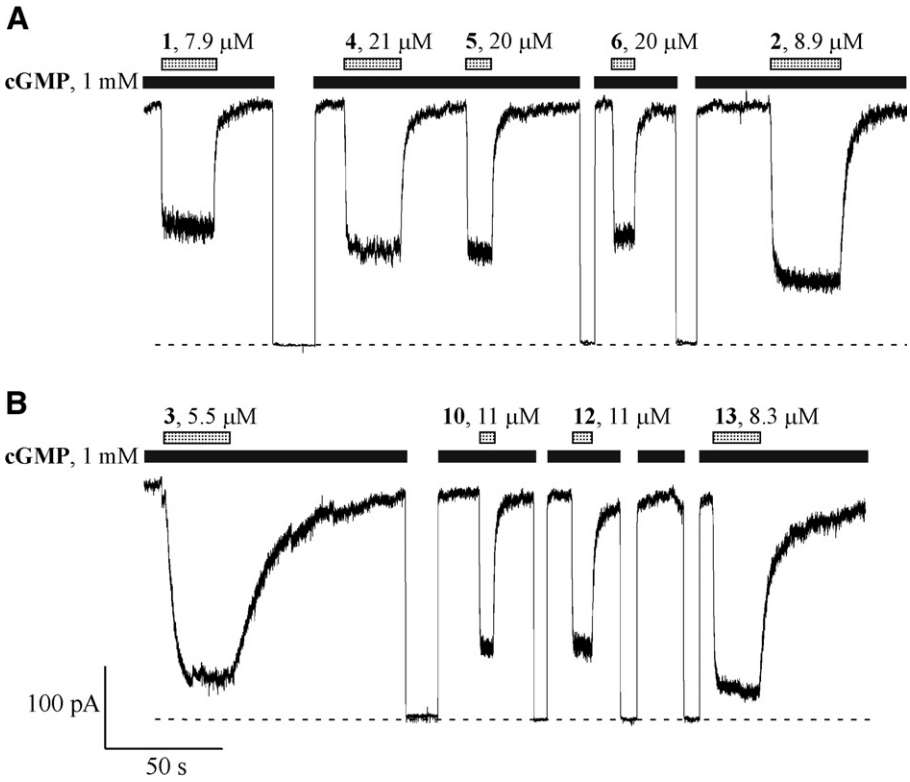
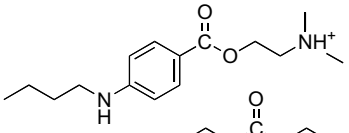
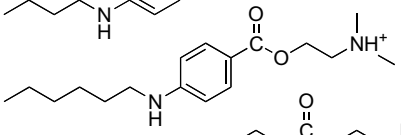
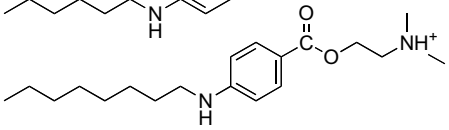
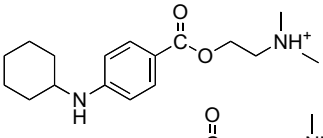
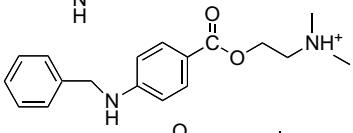
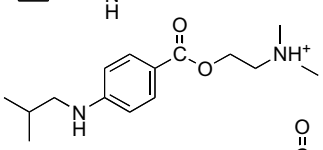
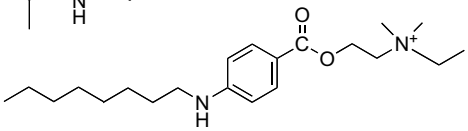
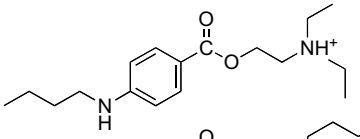
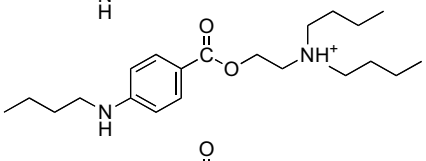
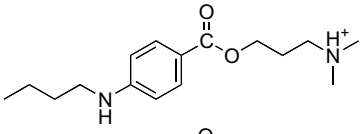
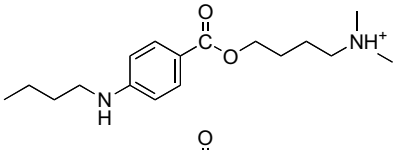
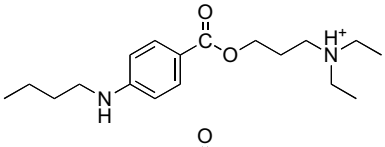
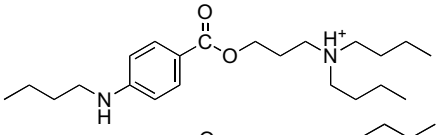
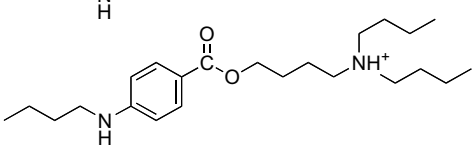


Figure 1. Blockade of retinal rod CNG channels by tetracaine derivatives: representative current traces from two different patches (A and B) showing outward currents at +40 mV. Channels were activated by 1 mM cGMP as indicated by the black bars, and tetracaine analogues were applied in addition as indicated by the stippled bars. The dashed lines indicate the zero current levels.

A panel of tetracaine derivatives was prepared in order to systematically increase the hydrophobicity of the apolar tail and tertiary amine regions according to Scheme 1. To study the effects of both chain length and branching, the tail region was modified in a two-step process. The reaction of 4-chlorobenzoic acid and an appropriate alkylamine was catalyzed with a Josiphos ligand and Pd(II) acetate to the corresponding 4-(alkylamino)benzoic acid using a synthesis adapted from Hartwig et al.[14] The resulting substituted benzoic acid derivatives

Table 1. Tetracaine analogue structures and block of heteromeric CNGA1/CNGB1 channels

Compound[a]		$K_{d(40)}$[b] (μM)	No. of patches
1		6.7 ± 1.7	16
2		3.8 ± 1.2	5
3		1.3 ± 1.2	6
4		17 ± 3	10
5		16 ± 4	10
6		20 ± 3	8
7		1.6 ± 0.8	7
8		8.5 ± 2.1	10
9		1.1 ± 0.3	7
10		5.0 ± 1.3	9
11		7.1 ± 3.1	6
12		5.6 ± 1.6	8
13		1.3 ± 0.4	8
14		1.3 ± 0.3	5

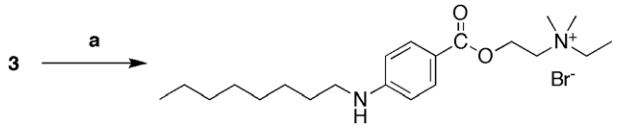
[a] The compounds are depicted here in what is expected to be the predominant protonation state at pH 7.6.

[b] $K_{d(40)}$ is the apparent dissociation constant at +40 mV calculated from the equation $I_{+B}/I_{-B} = K_{d(40)}/\{K_{d(40)} + [B]\}$, where the left side is current in the presence of blocker divided by current in the absence of blocker, and [B] is blocker concentration.

were activated with N',N'-carbonyldiimidazole and subsequently esterified using 2-*N*,*N*-dimethylaminoethanol to yield target compounds **2**–**6**.[15] Modifications of the head region extended the length between the tertiary amine and the ester and/or increased the hydrocarbon chain lengths on the tertiary amine. The head region was modified in a single synthetic step in most cases. A series of tertiary alkylamines were introduced via esterification of 4-(butylamino)benzoic acid according to the conditions outlined in step 2 of Scheme 1 to give desired products **8**–**14**.[16]

Coexpression of CNGA1 and CNGB1 in *Xenopus* oocytes yielded heteromeric retinal rod CNG channels. Channel blockade was assessed by applying tetracaine analogues to inside-out excised patches in the presence of 1 mM cGMP to fully activate the channels, as depicted in Figure 1 (electrophysiological methods are described in[17]). Lengthening the hydrocarbon tail from butyl (**1**) to hexyl (**2**) and octyl (**3**) resulted in stepwise increases in apparent affinity (Table 1). Compounds **2** and **3** were ~2- and 5-fold more potent than tetracaine, respectively. Further, there is an apparent preference for straight alkyl chains at the apolar end of tetracaine. For example, while the hexyl derivative (**2**) was ~2-fold more potent than tetracaine, the cyclohexyl derivative (**4**) was 2.5-fold less potent. The benzyl and isobutyl derivatives (**5** and **6**) displayed a similar decrease in apparent affinity relative to tetracaine (Table 1). Compound **3** was far less soluble in aqueous solution than tetracaine, requiring up to 50% methanol for a 10 mM stock concentration. To address the concern that the enhanced block might be due to increased partitioning into the membrane, we synthesized a permanently charged quaternary amine version (**7**) by reaction of **3** with bromoethane (Scheme 2). Reduced membrane partitioning by **7** was expected based on studies of tetracaine binding to model membranes that demonstrate less interaction of the charged species with the membrane relative to the neutral form.[18] Compound **7** is readily soluble in aqueous solution and displays equal potency for retinal rod CNG channel blockade as **3**, suggesting a direct block of the channel by both compounds (Table 1).

Previously, we showed that appending a butyl chain to the tertiary amine of tetracaine, thus increasing the hydrophobic content and generating a quaternary amine, resulted in ~2-fold increase in apparent affinity.[13] Here, we have increased the length of both alkyl chains at the tertiary amine from methyl (**1**) to ethyl (**8**), and butyl (**9**). While **9** was ~6-fold more potent than tetracaine, **8** was essentially equipotent with tetracaine. These results suggest that the tertiary amine of tetracaine may bind at a different position in the pore than simple tetraalkyl-ammonium derivatives. When a series of symmetrical tetraalkyl-ammonium derivatives were tested for block of CNG channels, significant increases in apparent affinity were observed with the addition of each methylene group from tetramethyl to tetrapentyl.[19]



Scheme 2. Reagents and condition: (a) $BrCH_2CH_3$, toluene, reflux (70%).

In a final set of experiments, the linker length between the tertiary amine and the ester was increased for each version of the tertiary amine. For the dimethyl version (**1**), the linker length was increased to propyl and butyl (**10** and **11**). An additional diethyl amino derivative was generated with a propyl linker in place of the ethyl (**12**). Versions of **9** with propyl and butyl linkers were also synthesized (**13** and **14**). Surprisingly, increasing the distance between the tertiary amine and the ester had no statistically significant effect on block for any of the derivatives (Table 1). Compounds **10** and **11** were equipotent with **1**; **12** was essentially equipotent with **8**; and **13** and **14** were equipotent with **9**. A lack of any effect on block, even when the linker length was doubled, suggests plasticity in the binding site for tetracaine.

This study highlights two distinct locations on the tetracaine scaffold where hydrophobic interactions are important for CNG channel binding. Increasing the hydrophobic character at the tertiary amine and the butyl tail enhanced the ability to block retinal rod CNG channels. We explored the possibility of combining increased hydrophobicity at both the tertiary amine and the butyl tail by synthesizing a derivative with an octyl chain substitution for the butyl tail and two butyl chains at the tertiary amine. Poor solubility of this analogue in aqueous solution, however, precluded its analysis by patch clamp. The effect of increasing the length of the hydrocarbon tail from butyl to octyl is relatively small compared to the magnitude of the loss of apparent affinity observed when the butyl tail is removed. Taken together with the observation that branched derivatives such as **6** are disfavored, these results suggest that the butyl tail of tetracaine may be targeted to a specific binding pocket in the pore. When used in conjunction with site-directed mutagenesis, the new tetracaine derivatives described here can be utilized to identify points of specific contact with the channel pore. Overall, the results indicate that apolar groups at two locations serve to anchor blockers that otherwise reside in the aqueous and ion-binding regions of the pore.

Acknowledgments

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