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Muscle-type Nicotinic Acetylcholine Receptor Delta Subunit Determines Sensitivity to Noncompetitive Inhibitors, While Gamma Subunit Regulates Divalent Permeability

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Summary—Heterologous expression of nicotinic acetylcholine receptor (nAChR) RNAs in *Xenopus* oocytes was used to examine the structural basis for pharmacological and physiological differences between muscle-type and neuronal nAChRs. Neuronal nAChRs have a higher permeability to calcium than muscle-type nAChRs and display inward rectification, while muscle-type nAChRs have a linear current–voltage relation. In addition, neuronal nAChRs are more sensitive to inhibition by a class of compounds known as "ganglionic blockers".

It has been shown previously that neuronal-muscle hybrid receptors show increased sensitivity to the use-dependent inhibitor of neuronal nAChRs, BTMPS, based on the presence of a neuronal beta subunit. In this study, we report that omission of gamma subunit RNA has a similar effect. $\alpha\beta\delta$ receptors exhibit prolonged inhibition by BTMPS, show a significant permeability to divalent ions, display inward rectification and are more sensitive to mecamylamine. However, while pharmacological effects are associated with the presence of an additional delta subunit, the physiological changes described seem to be associated with the presence or absence of a gamma subunit. These results suggest that, for nAChRs, as is also the case for non-NMDA ionotropic glutamate receptors, the crucial functional property of limiting calcium permeability can be served by a single subunit. Copyright © 1996 Elsevier Science Ltd

Keywords—Use-dependent inhibition, calcium permeability, *Xenopus* oocytes, acetylcholine nicotinic receptor (nAChR).

Muscle-type nicotinic acetylcholine receptors (nAChRs) are the best characterized of the ligand-gated ion channels and as such are a model system for the study of structure–function relationships in this receptor family. These receptors mediate ion permeation at the endplate of the neuromuscular junction. Muscle-type nAChRs are pentameric complexes, consisting of four distinct protein subunits ($\alpha 1\beta 1\gamma\delta$) in the ratio of 2α:1β:1γ:1δ. An epsilon subunit is substituted for the gamma subunit during development, altering the functional response of the receptor (Camacho *et al*., 1993; Mishina *et al*., 1986). The binding of two molecules of acetylcholine to specific sites at the interface of the alpha subunits with the gamma and delta subunits causes a conformational change in the receptor protein allowing ion permeation (Blount and Merlie, 1989; Pedersen and Cohen, 1990; Sine and Claudio, 1991; Unwin, 1995).

Each subunit shares a characteristic configuration containing a large N-terminal hydrophilic sequence, followed by four putative transmembrane domains (Devillers-Thiery *et al*., 1982). Previous studies have demonstrated that the second transmembrane domain (TM2) of each subunit lines the ion channel pore (for review see Changeux *et al*., 1992a,b). This region plays a role in determining the divalent permeability of the receptor as well as determining pharmacological properties (Changeux *et al*., 1992a,b). Charged loop regions at both ends of TM2 regulate the single channel conductance and rectification properties of nAChRs (Imoto *et al*., 1988). Muscle-type nAChRs have a low, but potentially significant, permeability to calcium and

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display a linear current–voltage relation (Decker and Dani, 1990; Vernino *et al*., 1994).

Neuronal nAChRs are believed to exist as pentameric complexes and are thought to have roughly equivalent membrane topographies to muscle-type nAChRs, but may be composed of only alpha and beta subunits. However, since eight different genes encoding putative neuronal alpha subunits and three different genes coding for putative beta subunits have been cloned to date, a number of subtypes of heteromeric nAChRs may exist in the nervous system (for review, see Papke, 1993). Neuronal nicotinic receptor channels in general show greater inward rectification of current than muscle-type channels. The rectification may be due in part to an ability of intracellular Mg^{2+} to block the channel when there is a driving force for outward current (Ifune and Steinbach, 1990; Mathie *et al*., 1990; Neuhaus and Cachelin, 1990), although other factors such as a voltage dependence for channel gating may also be important (Ifune and Steinbach, 1992; Mathie *et al*., 1990). Neuronal channels also tend to have a higher permeability for Ca^{2+} than do muscle nicotinic receptors (Fieber and Adams, 1991; Mulle *et al*., 1992; Vernino *et al*., 1992).

Noncompetitive inhibitors (NCIs) prevent receptor function by binding to sites which are believed to block the ion conduction pathway and which are distinct from the binding sites for agonist activation. BTMPS (bis (2,2,6,6-tetramethyl-4-piperidinyl) sebacate), inhibits nicotinic acetylcholine receptors expressed in *Xenopus* oocytes in a use-dependent manner (Papke *et al*., 1994). This compound is a symmetrical conjugate of methylated piperidines which are themselves ganglionic blockers (Spinks and Young, 1958). Previous studies of nAChRs expressed in *Xenopus* oocytes in which a neuronal beta subunit (β4) was substituted for the muscle beta subunit (β1) creating an $\alpha 1\beta 4\gamma\delta$ neuronal–muscle hybrid receptor (N–M hybrid), showed that introduction of a neuronal beta subunit produced a form of the receptor similar to neuronal subtypes in regard to kinetics of inhibition by BTMPS (Papke *et al*., 1994). This result demonstrated that the part of the binding site for BTMPS that is important for slow kinetics of recovery is common to neuronal beta subunits and not shared by the muscle beta subunit. In addition, this finding suggested that BTMPS inhibits neuronal receptors with slowly reversible kinetics because of the potential for binding to multiple beta subunits within a neuronal receptor complex.

The present study further defines the role of individual subunits in defining time-course of inhibition by noncompetitive inhibitors and, in addition, examines the contribution of individual subunits in determining the divalent permeability of the pentameric receptor. Functional nAChRs can be formed by the muscle receptor subunits, even in the absence of either the gamma or delta subunits (Jackson *et al*., 1990; Kullberg *et al*., 1990; White *et al*., 1985). By expressing $\alpha\beta\delta$, $\alpha\beta\gamma$ and N–M hybrid nAChRs in *Xenopus* oocytes, we have been able to examine directly the roles of the gamma and delta subunits in determining sensitivity to block by noncompetitive inhibitors and in regulating the divalent permeability of nAChRs.

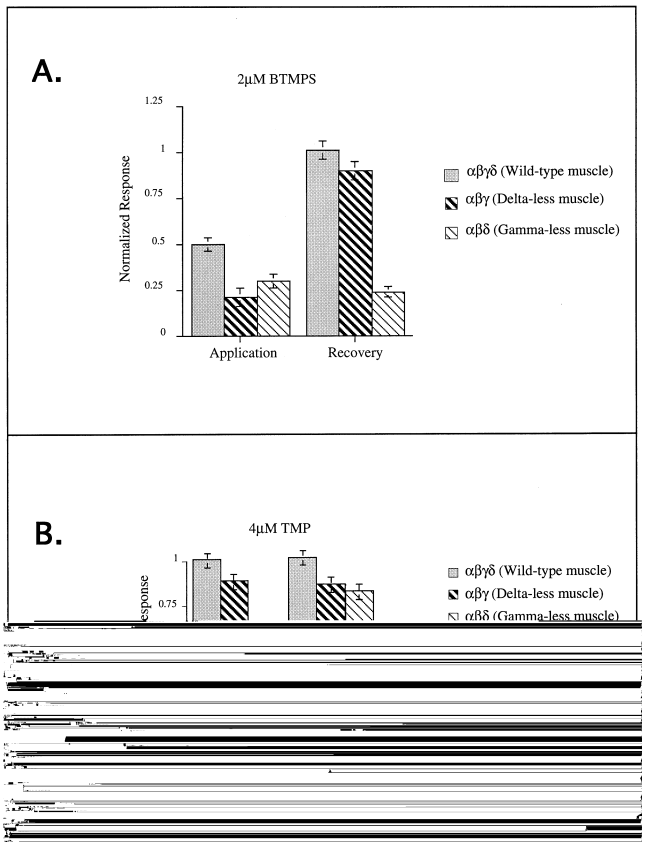

Fig. 1. The blockade of $\alpha\beta\delta$ nAChRs by (A) BTMPS and (B) TMP. The cluster of bars on the left represents the average normalized responses ($\pm$SEM) of oocytes to the co-application of 10 μM ACh and the inhibitor, and the cluster of bars on the right represents the normalized responses to 10 μM ACh alone after a 5-min wash period. The response of each oocyte was normalized to its response to 10 μM ACh applied 5 min prior to the co-applications represented in the figure.

METHODS

Preparation of RNA and expression in Xenopus *oocytes*

The preparation of *in vitro* synthesized cRNA transcripts and oocyte injection have been described previously (de Fiebre *et al*., 1995). Briefly, 2–3 ovarian lobes were surgically removed and then cut open to expose the oocytes. The ovarian tissue was then treated with collagenase from Worthington Biochemical (in calcium-free Barth's solution: 88 mM NaCl, 10 mM HEPES pH 7.6, 0.33 mM $MgSO_4$, 0.1 mg/ml gentamicin sulfate) for 2 hr at room temperature. Subsequently, stage 5 oocytes were isolated and injected with 5 ng each of the appropriate subunit cRNAs following harvest. Record-

ings were made 2–7 days after injection, depending on the cRNAs being tested.

Electrophysiology methods

Current responses to drug application were studied under two-electrode voltage clamp at a holding potential of −50 mV. Recordings were made using a Warner Instruments OC-725 oocyte amplifier interfaced with National Instruments Labview software. Oocytes were placed in a Lucite recording chamber with a total volume of 0.5 ml and were perfused at room temperature by frog Ringers (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES pH 7.3, 1.8 mM $CaCl_2$) containing 1 μM atropine to block potential muscarinic responses. A Mariotte flask filled with Ringers was used to maintain a constant hydrostatic pressure for drug deliveries and washes. Drugs were diluted in perfusion solution and were applied following the pre-loading of a 1.8-ml length of tubing at the terminus of the perfusion system. This system permitted a bolus dose drug application of approximately 10-sec duration. This protocol was sufficient to achieve the maximal concentration-dependent peak responses. Specifically, when 10-sec (1.8 ml) agonist applications were compared to responses obtained with 30-sec (6 ml) agonist applications, no significant increase in peak responses was observed (paired *t*-test; $p = 0.38$). Moreover, the longer application protocol tended to increase rundown (or protracted desensitization) compared to our method of choice.

TMP was purchased from Aldrich and BTMPS was obtained from Ciba-Geigy. All other chemicals for electrophysiology were purchased from Sigma Chemical Company unless otherwise noted.

Current electrodes were filled with a solution containing 250 mM CsCl, 250 mM CsF and 100 mM EGTA and had resistances of 0.5–2 MΩ. Voltage electrodes were filled with 3 M KCl and had resistances of 1–3 MΩ. Oocytes with resting membrane potentials of less than −30 mV were rejected. Currents were normalized to the initial response to application of 10 μM ACh and applications of drug were separated by 5-min intervals. Data represent the means (±SEM) that were calculated from the normalized responses of at least four oocytes to each experimental condition.

For experiments measuring divalent permeability, oocytes were perfused with barium Ringers (low barium: 90.7 mM NaCl, 2.5 mM KCl, 10 mM HEPES pH 7.3, 1.8 mM $BaCl_2$, 48.6 mM sucrose; high barium: 90.7 mM NaCl, 2.5 mM KCl, 10 mM HEPES pH 7.3, 18 mM $BaCl_2$). Recordings were made using pClamp6 software (Axon Instruments). Records were obtained at the plateau of the response to prolonged applications of agonist. Shifts in reversal potential were measured by ramping the holding potential from −50 mV to +50 mV. A potential flaw in any paradigm measuring divalent permeability based solely on reversal potential shift is the interaction of changing divalent strength of solution with surface charge. In order to limit any contribution of surface charge effects to our measurements and minimize increases in intracellular divalent ion concentration, recordings were made first in low and then in high barium Ringers solution. Barium was used instead of calcium to minimize the contribution of endogenous calcium-activated chloride channels (Sands *et al.*, 1993). Holding currents in the absence of agonist were point to point subtracted. Oocytes were given 5 min to equilibrate after solution changes. Chloride-free Ringers (low calcium (mM): 90.7 NaOH, 2.5 KOH, 10 HEPES pH 7.3, 1.8 $CaOH_2$, 48.6 sucrose; high calcium (mM): 90.7 NaOH, 2.5 KOH, 10 HEPES pH 7.3, 18 $CaOH_2$) was used in some experiments measuring reversal potential shift as a second control for the possible contribution of endogenous calcium-activated chloride channels. The methanesulfonate anion was added in the form of methylsulfonic acid to substitute for chloride (Seguela *et al.*, 1993).

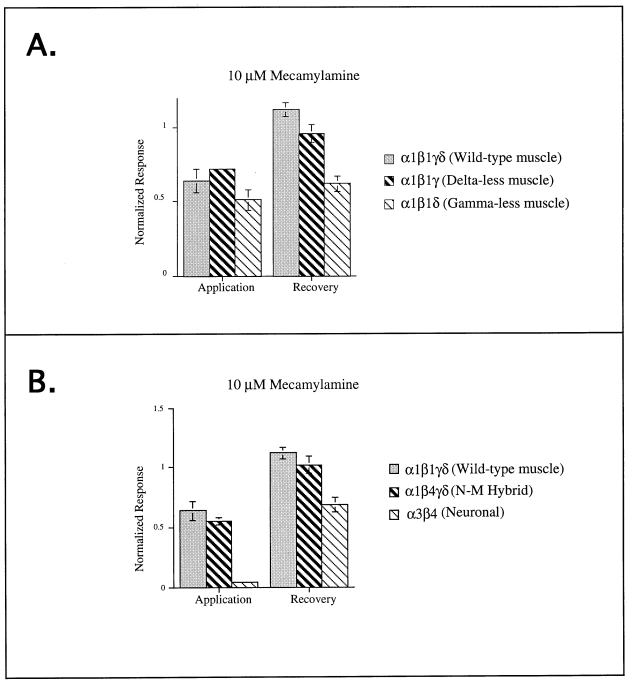

Fig. 2. The blockade of $\alpha\beta\gamma$, $\alpha\beta\delta$ and muscle-type receptors (A) and muscle-type, N–M Hybrid ($\alpha1\beta4\gamma\delta$) and neuronal ($\alpha3\beta4$) receptors (B) by mecamylamine. The cluster of bars on the left represents the average normalized responses (±SEM) of oocytes to the co-application of 10 μM ACh and the inhibitor, and the cluster of bars on the right represents the normalized responses to 10 μM ACh alone after a 5-min wash period. The response of each oocyte was normalized to its response to 10 μM ACh applied 5 min prior to the co-applications represented in the figure.

RESULTS

BTMPS inhibits $\alpha\beta\delta$ receptors with slowly reversible kinetics

Previously published experiments have shown that inhibition of neuronal nAChRs by BTMPS is prolonged

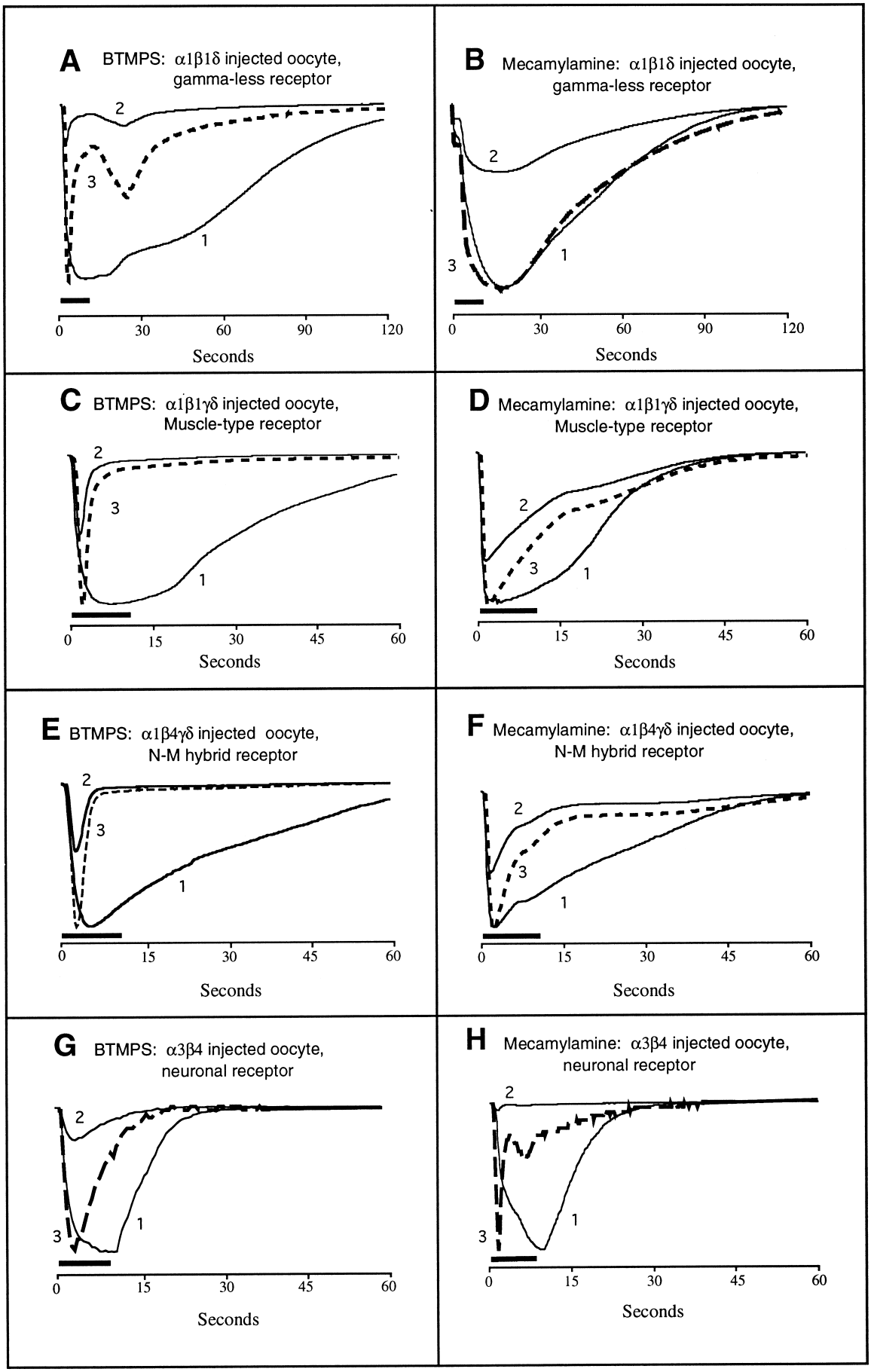

Fig. 3—*Caption opposite.*

and continues to increase throughout agonist application, such that measurements of the peak responses 5 min later show increased inhibition compared to the co-application response (Papke *et al*., 1994). In the current set of experiments, muscle-type and $\alpha\beta\gamma$ receptors showed significant recovery from inhibition over 5 min, while $\alpha\beta\delta$ receptors displayed prolonged inhibition as measured 5 min after co-application of ACh and BTMPS. The mean post-application response of $\alpha\beta\delta$ receptors was $24 \pm 3\%$ of the response to the initial control application of 10 μM ACh ($n = 8$), while the mean post-application responses of $\alpha\beta\gamma$ ($n = 4$) and wild-type muscle receptors ($n = 6$) were $90 \pm 5\%$ and $101 \pm 5\%$ of control responses (Fig. 1(A)). Additional experiments in which the agonist application times were more prolonged were in agreement with these findings (see Methods). These results demonstrate that omission of gamma subunit RNA has effects on the kinetics of inhibition by BTMPS comparable to the effects seen previously after substitution of neuronal beta subunit RNA (β4) for muscle beta subunit RNA (β1).

$\alpha\beta\delta$ receptors also showed increased sensitivity to the monofunctional inhibitor TMP (2,2,6,6-tetramethylpiperidine) at the time of co-application, as compared to wild-type muscle and $\alpha\beta\gamma$ receptors (Fig. 1(B)). After co-application of 10 μM ACh with 4 μM TMP to $\alpha\beta\delta$ receptors, the mean peak current was $56 \pm 2\%$ of control responses to 10 μM ACh alone ($n = 4$), while the responses of normal muscle-type receptors were unchanged and the responses of $\alpha\beta\gamma$ receptors were $89 \pm 5\%$ of controls.

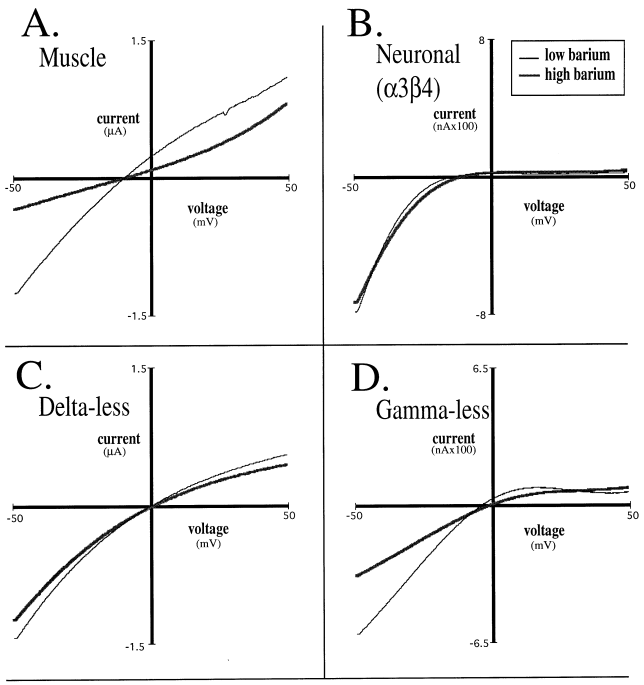

Fig. 4. Representative current–voltage relations for (A) muscle-type, (B) neuronal $\alpha 3\beta 4$, (C) $\alpha\beta\gamma$ and (D) $\alpha\beta\delta$ nAChRs. The holding potential was ramped from −50 mV to +50 mV in the plateau phase of the response to a prolonged application of ACh. Reversal potential measurements were made in first low (1.8 mM barium) and then high (18 mM barium) divalent Ringers solution to assess divalent permeability. Sucrose was substituted for barium in the low divalent solution to control for differences in osmolarity. See Table 1 for shift means.

$\alpha\beta\delta$ receptors recover rapidly from block by TMP

The mean response of $\alpha\beta\delta$ receptors to application of ACh alone, measured 5 min after co-application of agonist and inhibitor, was $83 \pm 5\%$ of control responses to ACh alone in the case of TMP co-applied with ACh ($n = 4$) while, in the case of BTMPS co-applied with ACh, the mean response to ACh measured 5 min after co-application was only $24 \pm 3\%$ of control responses to ACh alone ($n = 8$). This finding indicates a longer time course of recovery from inhibition by the conjugated inhibitor BTMPS as compared to the time course of recovery from inhibition by TMP, and is in keeping with results obtained with neuronal nAChRs (Papke *et al*., 1994). The results of the present study in conjunction with the previously published results involving N–M hybrid and neuronal receptors, lend further support to the hypothesized two-site binding model for BTMPS activity.

$\alpha\beta\delta$ receptors display increased sensitivity to mecamylamine

Omission of gamma subunit RNA also leads to increased sensitivity to inhibition by mecamylamine. Co-application of 10 μM ACh with 10 μM mecamyl-

Fig. 3. Inhibitor effects on the kinetics of macroscopic currents. Representative waveforms of responses from $\alpha\beta\delta$ (A and B), wild-type muscle (C and D), N–M hybrid (E and F) and neuronal (G and H) receptors to a pulse of 30 μM ACh alone (line 1), and a co-application with either 2 μM BTMPS (traces on left) or 10 μM mecamylamine (traces on right) and 30 μM ACh (line 2). For all the traces, the thick broken line (3) plots the inhibited current scaled to the same peak value as the control in order to visualize the kinetics of inhibition. The thick bars under the traces represent the period of drug application. Note that due to the prolonged response of $\alpha\beta\delta$ receptors, the time scale in (A) and (B) is expanded. The time to peak of the response in the presence of BTMPS is less than that of the control application of ACh alone in all cases (A, C, E and G) whereas, in the presence of mecamylamine, the time to peak of the co-application response is less than that of the control response to ACh alone in only neuronal receptors (H).

Table 1. Summary of reversal potential shifts in barium Ringers solution

Receptor type	$\alpha1\beta1\gamma\delta$	$\alpha1\beta4\gamma\delta$	$\alpha1\beta1\delta\varepsilon$	$\alpha1\beta1\gamma\varepsilon$	$\alpha1\beta1\gamma$	$\alpha3\beta4$	$\alpha1\beta1\delta$
Reversal potential shift (mV ± SEM)	−1.0 ± 1.4 $n = 4$	0.5 ± 0.6 $n = 11$	−0.6 ± 1.3 $n = 5$	1.7 ± 0.5 $n = 4$	3.1 ± 0.7 $n = 11$	5.7 ± 1.1 $n = 7$	7.8 ± 0.7 $n = 9$

amine caused prolonged inhibition of $\alpha\beta\delta$ receptors compared to normal muscle-type and $\alpha\beta\gamma$ receptors (Fig. 2(A)), while $\alpha3\beta4$ neuronal receptors show more pronounced inhibition by mecamylamine than any of the non-neuronal or N–M hybrid receptors (Fig. 2(B)). However, the waveform of the inhibition response of $\alpha\beta\delta$ receptors was qualitatively different from the response waveform produced by co-application of the purely use-dependent inhibitor BTMPS with ACh (Fig. 3(A, B)). Specifically, the time to peak of the inhibition response of $\alpha\beta\delta$ receptors to co-application of the use-dependent inhibitor BTMPS with ACh was significantly less than the time to peak of the response to ACh alone (paired *t*-test, $p < 0.01$). However, the time to peak of the response to co-application of ACh with mecamylamine was not significantly different from the time to peak of the response to ACh alone (Fig. 3(B)). The decreased time to peak of the response seen in the presence of BTMPS is characteristic of use-dependent inhibition (Papke *et al*., 1994) and is seen with all receptor subtypes (Fig. 3(A, C, E, G)). In the case of mecamylamine, however, the time to peak of the response is decreased only with neuronal receptors (Fig. 3(H)). These observations indicate that mecamylamine may not act by a purely use-dependent mechanism on non-neuronal receptors.

In some cases where inhibition is clearly use-dependent, a secondary peak is observed in the waveform of the co-application response (Fig. 3 (A, H)). Since this secondary peak corresponds exactly with the removal of agonist and occurs only in conditons where inhibitor is present and acting in a clearly use-dependent manner, it is believed that this peak represents relief from a short-time course, low-affinity inhibition. Since this inhibition relaxes within the time course of the agonist response, it most likely represents an entirely different form of inhibition from the relatively long-term inhibition discussed above.

$\alpha\beta\delta$ receptors show increased divalent permeability and inward rectification

$\alpha\beta\delta$ receptors display significantly greater inward rectification than either normal muscle-type or $\alpha\beta\gamma$ nAChRs as measured by chord conductance ratios at −50 and +50 mV (unpaired *t*-test, $p < 0.001$) and show a significantly greater reversal potential shift (ΔE_{rev}) with 10-fold increases in the external barium concentration than either wild-type muscle or $\alpha\beta\gamma$ receptors (one-tailed *t*-test: $p < 0.05$; Fig. 4 and Table 1). A much slighter increase in inward rectification is also seen in the case of $\alpha\beta\gamma$ receptors and, for both receptor types, may reflect an increased likelihood of voltage-dependent block by divalent ions in proportion to their ability to enter the channel. Thus, receptors with a larger divalent permeability also show more pronounced inward rectification, as is seen in the case of $\alpha\beta\delta$ receptors.

Some experiments were also conducted in chloride-free methanesulfonate-based Ringers. Although the reversal potential shift for $\alpha\beta\delta$ receptors observed in the chloride-free solution (+3.4 ± 1.0 mV) is reduced from that seen in barium Ringers solution (+7.8 ± 0.7 mV; see Table 1), $\alpha\beta\gamma$ receptors show a comparable decrease in reversal potential shift and are no longer significantly different from $\alpha1\beta1\gamma\delta$ receptors in the chloride-free solution. It is unclear whether this reduction in reversal potential shift arises as a product of the possible activation of chloride currents in the barium-containing solution, or is the result of poorly compensated junction potentials in the chloride-free conditions.

Substitution of a neuronal beta subunit ($\beta4$), which is permissive to divalent ions in the neuronal receptor, for the muscle beta subunit ($\beta1$) does not significantly increase the reversal potential shift or alter the current–

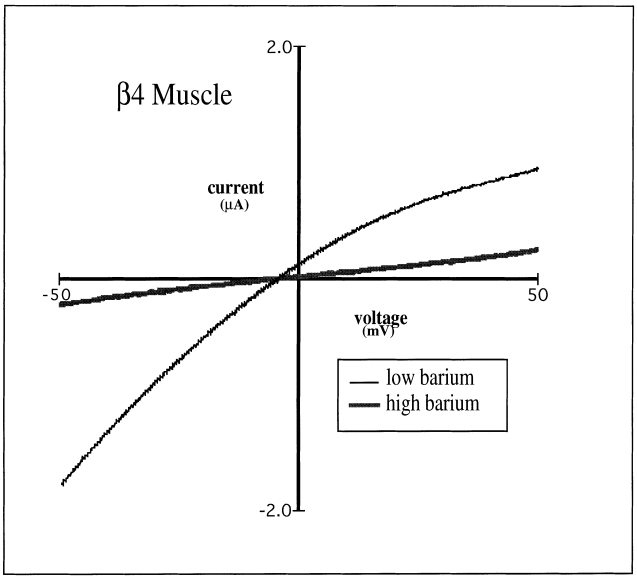

Fig. 5. N–M hybrids do not display a significant shift in reversal potential. Substitution of $\beta4$ subunit RNA for $\beta1$ subunit RNA does not significantly increase the divalent permeability or alter the current–voltage relation of nAChRs when the gamma subunit is present (compare with representative trace in Fig. 4(A)). Thin black lines are the responses obtained in the presence of the normal divalent ion concentration (1.8 mM). The thick gray lines are the responses obtained in the presence of 10-fold higher divalent ion concentration (18 mM).

voltage relation of N–M hybrid receptors when the gamma subunit is present (Fig. 5; see Table 1). In addition, injection of epsilon subunit mRNA in place of either gamma or delta subunit mRNA (creating $\alpha 1\beta 1\gamma\varepsilon$ or $\alpha 1\beta 1\delta\varepsilon$ receptors) provides for the expression of receptors that are not significantly different from fetal muscle-type ($\alpha 1\beta 1\gamma\delta$) in terms of current–voltage relation or apparent divalent permeability. Since the mouse epsilon subunit shares a high degree of sequence identity with the gamma subunit in channel-forming domains (see sequence below), these results are consistent with our observation that receptors lacking a gamma subunit show an increased permeability to divalent ions and indicate that the gamma and/or epsilon subunit may serve to limit the divalent permeability of muscle-type nAChRs.

	Membrane spanning II
DELTA	FYLPGDCG EKTSVAISVLLAQSVFLLLISKRLPAT
BETA2	FYLPSDCG EKMTLCISVLLALTVFLLLISKIVPPT
BETA4	FYLPSDCG EKMTLCISVLLALTFFLLLISKIVPPT
	ECL
BETA1	FYLPQDAG EKMGLSIFALLTLTVFLLLLADKVPET
GAMMA	FYLPAKAGGQKCTVATNVLLAQTVFLFLVAKKVPET
EPSILON	YFLPAQAGGQKCTVSINVLLAQTVFLFLIAQKIPET

DISCUSSION

Omission of gamma subunit RNA has an effect on the receptor's pharmacology that is equivalent to substitution of a neuronal beta subunit ($\beta 4$) for the muscle beta subunit ($\beta 1$). This result may indicate that neuronal beta subunits and muscle delta subunits share a common structural motif which provides a site for the binding of BTMPS and is not present in the gamma subunit. As has been demonstrated previously by Jackson *et al.* (1990) for bovine receptors, mouse $\alpha\beta\delta$ receptors also show a larger divalent permeability. One possible interpretation of these data is that domains which determine ionic permeability also regulate pharmacological sensitivity. However, our observation that $\beta 4$-containing N–M hybrid receptors do not exhibit increased divalent permeability compared to wild-type muscle receptors argues for a model in which sensitivity to noncompetitive inhibition by BTMPS is determined by the delta subunit, while permeability to divalent ions is regulated by the presence or absence of the gamma subunit. In the absence of the gamma (or epsilon) subunits, the receptor formed from the other muscle receptor subunits has a greater permeability to divalent ions and contributes to a whole-cell current which shows inward rectification. A number of reports from other investigators (Bertrand *et al*., 1993; Ferrer-Montiel and Montal, 1993; Imoto *et al*., 1988, 1991; Konno *et al*., 1991) indicate that both of these effects may be determined by sequence in the channel-forming regions.

Receptors incorporating an additional delta subunit have additional binding site(s) for noncompetitive inhibitors

Based on published results of the binding sites of other noncompetitive inhibitors (Changeux *et al*., 1992a,b; Charnet *et al*., 1990; Pedersen *et al*., 1992), we believe that BTMPS may bind to site(s) in the channel-forming domains which include TM2 and the extracellular loop region (ECL) between TM2 and TM3. Sequence similarity was observed between neuronal beta subunits and muscle delta subunits in the channel-forming regions while gamma subunits were observed to be more similar to muscle beta subunits in these regions (see underline below).

Our finding that BTMPS produces prolonged inhibition of $\alpha\beta\delta$ receptors may indicate that incorporation of an additional delta subunit provides a second binding site for the bi-functional inhibitor. This site would presumably be located in the channel-forming region as was previously hypothesized for N–M hybrid receptors (Papke *et al*., 1994). In this model, BTMPS would bind to sites on separate delta subunits, allowing the molecule to span the ion channel and block ion conduction. Thus, all receptor subtypes incorporating multiple subunits with high-affinity BTMPS binding sites (e.g. $\alpha 3\beta 4$, $\alpha 1\beta 4\gamma\delta$, $\alpha\beta\delta$) would show prolonged recovery from inhibition by BTMPS as a result of the molecule binding to separate subunits. All of our data to date support this model.

The increased sensitivity of $\alpha\beta\delta$ receptors to the monofunctional inhibitor TMP at the time of co-application with ACh may indicate that addition of a second delta subunit into the receptor pentamer introduces additional potential binding sites for TMP and thereby increases the probability of any single receptor to be inhibited. The increased sensitivity of $\alpha\beta\delta$ receptors to block by mecamylamine may result from a similar mechanism. However, we show the block of $\alpha\beta\delta$ and $\alpha\beta\gamma$ receptors by mecamylamine is qualitatively unlike the inhibition by BTMPS. Specifically, inhibition of muscle-related receptors by mecamylamine may not have the same use-dependence as is the case for inhibition of nAChRs by the TMP compounds (Fig. 3). Therefore, it is unlikely that BTMPS and mecamylamine are binding to the same sites in these subunits. The inhibition of neuronal receptors ($\alpha 3\beta 4$) by mecamylamine is qualitatively similar to the inhibition by BTMPS and in this case does seem to be use-dependent (Fig. 3(H)). Our results indicate that the inhibition of N–M hybrid receptors by

mecamylamine is not purely use-dependent (Fig. 3(F)); however, it should be noted that a neuronal receptor may contain as many as three beta subunits while an N–M hybrid receptor contains only a single neuronal beta subunit. Thus, the inclusion of multiple neuronal beta subunits may provide additional use-dependent binding sites.

Role of gamma subunit in determining calcium permeability

Previous analyses of the effects of site-directed mutations in the putative pore-forming domain on channel conductance have indicated that the gamma subunit is arranged asymmetrically in the ion channel pore (Imoto *et al*., 1991). The presence of an additional glycine residue near the cytoplasmic face of the pore-forming domain is thought to be in part responsible for this hypothesized asymmetrical arrangement (see above sequence). The specialized placement of the gamma subunit may provide a mechanism whereby this subunit could selectively regulate permeability to divalent ions. This hypothesis is supported by our observation that the mouse epsilon subunit also acts to limit calcium permeability and also contains the extra glycine residue believed to be critical for the asymmetrical placement of the subunit in the ion channel pore. In addition, this property of the gamma subunit might underlie the reversal potential shift seen with $\alpha\beta\gamma$ receptors in barium Ringers solution; that is, inclusion of two asymmetrically oriented gamma subunits in the muscle receptor pentamer may result in the formation of nAChR with an uncharacteristic subunit arrangement and thereby form an ion channel slightly more permissive to divalent ions.

Although we show that mouse $\alpha 1\beta 1\delta\varepsilon$ receptors are not significantly different from $\alpha 1\beta 1\gamma\delta$ receptors in terms of divalent permeability, it should be noted that Villaroel and Sakmann (1995) report that adult rat muscle receptors containing the epsilon subunit have a higher divalent permeability than fetal forms of the receptor. Since the sequence of the putative pore-lining transmembrane region of the epsilon subunit is identical across species from rat to mouse, this apparent difference in divalent permeability is somewhat unexpected. However, differences between the findings of Villaroel and Sakmann's study and the present study may arise from alterations in the pentameric protein structure due to interactions between subunits, from differential regulation by intracellular factors or from the use of cesium as the sole monovalent cation in Villaroel and Sakmann's study.

Significance of calcium influx via muscle-type nAChRs

The regulation of calcium permeability is arguably one of the most important aspects driving the evolution of diversity in the families of neurotransmitter receptors. A striking example of this is the evolution of RNA editing mechanisms that ultimately determine the calcium permeability properties of non-NMDA type glutamate receptors. Recent work has shown that the divalent permeability of certain types of glutamate receptors (AMPA and kainate selective varieties) is regulated by RNA editing (Kohler *et al*., 1993; Sommer *et al*., 1991), resulting in the inclusion of either a glutamine or an arginine at a site in the putative pore-lining domain of the GluR2, GluR5 and GluR6 subunits known as the Q/R site (Hume *et al*., 1991). The major consequence of inclusion of an arginine at this site (instead of the glutamine encoded for by the genomic DNA) seems to be reduced calcium permeability (Hume *et al*., 1991). Receptors incorporating a subunit with a positively charged arginine residue in the pore-lining domain had linear current–voltage relationships and low calcium permeability, while receptors without this subunit showed inward-rectification and increased calcium permeability (Hume *et al*., 1991).

Our results suggest that, in the case of nAChR also, some subunits may have evolved to serve the crucial role of limiting calcium permeability. It has been demonstrated that calcium influx via muscle-type nAChRs could be physiologically significant and may mediate local increases in intracellular calcium in a situation like that at the endplate where nAChRs are concentrated (Decker and Dani, 1990). In addition, it has been shown that calcium entry via nAChRs in rat myotubes mediates autophosphorylation of the receptor (Miles *et al*., 1994). This phosphorylation could, in turn, affect the functional properties of the receptor or could be critical in development (Huganir and Greengard, 1990; Qu *et al*., 1990). Furthermore, it has been demonstrated that electrical activity can alter the expression of muscle-type nAChR genes via a calcium- and protein kinase C-dependent mechanism (Huang *et al*., 1992; Klarsfeld *et al*., 1989). With these results in mind, it seems that calcium influx via muscle-type nAChRs may be physiologically relevant and that limiting this permeability might serve as a safety mechanism whereby repeated subthreshold stimulation of the post-synaptic cell does not result in local increases in intracellular calcium and inappropriate effects on calcium-dependent cellular processes. Following this line of reasoning, it may be the case that a functional segregation of ligand-gated ion channels has evolved, such that some classes mediate both calcium and voltage effects whereas other classes primarily serve to mediate the propagation of electrical signals. This has been shown previously to be the case for ionotropic glutamate receptors and the results of the present study would seem to indicate that there could be a similar stratification of function amongst nAChRs.

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