

# Differential Modulation of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ Neuronal Nicotinic Receptors Expressed in *Xenopus* Oocytes by Flufenamic Acid and Niflumic Acid

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**Effects of flufenamic acid (FFA) and niflumic acid (NFA), which are often used to block  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current, have been investigated in voltage-clamped *Xenopus* oocytes expressing  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nicotinic ACh receptors (nAChRs). NFA and FFA inhibit  $\alpha 3\beta 2$  nAChR-mediated inward currents and potentiate  $\alpha 3\beta 4$  nAChR-mediated inward currents in normal,  $\text{Cl}^-$ -free and  $\text{Ca}^{2+}$ -free solutions to a similar extent. The concentration-dependence of the inhibition of  $\alpha 3\beta 2$  nAChR-mediated ion current yields  $\text{IC}_{50}$  values of 90  $\mu\text{M}$  for FFA and of 260  $\mu\text{M}$  for NFA. The potentiation of  $\alpha 3\beta 4$  nAChR-mediated ion current by NFA yields an  $\text{EC}_{50}$  value of 30  $\mu\text{M}$ , whereas the effect of FFA does not saturate for concentrations of up to 1 mM. At 100  $\mu\text{M}$ , FFA reduces the maximum of the concentration–effect curve of ACh for  $\alpha 3\beta 2$  nAChRs, but leaves the  $\text{EC}_{50}$  of ACh unaffected. The same concentration of FFA potentiates  $\alpha 3\beta 4$  nAChR-mediated ion currents for all ACh concentrations and causes a small shift of the concentration–effect curve of ACh to lower agonist concentrations. The potentiation, like the inhibition, is most likely due to a noncompetitive effect of FFA. Increasing ACh-induced inward current either by raising the agonist concentration from 10  $\mu\text{M}$  to 200  $\mu\text{M}$  or by coapplication of 10  $\mu\text{M}$  ACh and 200  $\mu\text{M}$  FFA causes a similar enhancement of block of the  $\alpha 3\beta 4$  nAChR-mediated ion current by  $\text{Mg}^{2+}$ . This suggests that the effects of FFA and of an increased agonist concentration result in a similar functional modification of the  $\alpha 3\beta 4$  nAChR-operated ion channel. It is concluded that  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$  nAChRs are oppositely modulated by FFA and NFA through a direct  $\beta$ -subunit-dependent effect.**

**[Key words: neuronal nicotinic ACh receptors, *Xenopus* oocytes, inhibition, potentiation, chloride channel blockers, flufenamic acid, niflumic acid, voltage clamp]**

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Neuronal nicotinic acetylcholine receptors (nAChRs) belong to the family of ligand-gated ion channels and are found throughout the CNS and PNS (Luetjck et al., 1990). Molecular cloning studies have provided evidence for the existence of at least six different types of  $\alpha$ -subunits of mammalian neuronal nAChRs, named  $\alpha 2$ – $\alpha 7$  (Wada et al., 1988; Boulter et al., 1986, 1990; Goldman et al., 1987; Lamar et al., 1990; Séguéla et al., 1993) and three different types of  $\beta$ -subunits,  $\beta 2$ – $\beta 4$  (Deneris et al., 1988a,b; Duvoisin et al., 1989). Oocyte expression studies have demonstrated that each of the  $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 4$  subunits may be combined with  $\beta 2$  or  $\beta 4$  to obtain functional hetero-oligomeric nAChRs (Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989; Luetjck and Patrick, 1991). The  $\alpha 7$  subunit forms homo-oligomeric channels in oocytes (Couturier et al., 1990; Schoepfer et al., 1990; Séguéla et al., 1993) and the functional significance of  $\alpha 5$ ,  $\alpha 6$ , and  $\beta 3$  is presently unknown (Deneris et al., 1991). By analogy to the pentameric end-plate nicotinic receptor, the hetero-oligomeric neuronal nAChRs are supposed to contain two  $\alpha$ - and three  $\beta$ -subunits (Anand et al., 1991; Cooper et al., 1991).

The expression of different combinations of subunits in *Xenopus* oocytes results in neuronal nAChRs with distinct pharmacological and physiological properties. The features of  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  subunit combinations have been investigated to some detail. As compared to the  $\alpha 3\beta 2$  nAChR, the  $\alpha 3\beta 4$  nAChR is more sensitive to the agonists ACh and cytisine (Cachelin and Jaggi, 1991; Luetjck and Patrick, 1991), is much less sensitive to block by the antagonist neuronal bungarotoxin (Duvoisin et al., 1989; but see Papke et al., 1993) and by substance P (Stafford et al., 1994), desensitizes much more slowly (Cachelin and Jaggi, 1991), and exhibits prolonged single channel bursting and a higher single channel conductance (Papke and Heinemann, 1991).

In various types of mammalian cells the ion channels of native neuronal nAChRs appear permeable to  $\text{Ca}^{2+}$  (Fieber and Adams, 1991; Sands and Barish, 1991; Mulle et al., 1992a; Vernino et al., 1992; Trouslard et al., 1993; Zhou and Neher, 1993). However, multiple types of nAChR may be expressed in the same cell (Vernallis et al., 1993) and the subunit composition of native nAChRs is unknown. In oocytes it has been demonstrated that the homo-oligomeric  $\alpha 7$  nAChR is much more permeable to  $\text{Ca}^{2+}$  than to  $\text{Na}^+$  (Galzi et al., 1992; Bertrand et al., 1993; Séguéla et al., 1993). Although the relative  $\text{Ca}^{2+}$  permeabilities of defined hetero-oligomeric nAChRs remains to be established, they may carry sufficient  $\text{Ca}^{2+}$  current (Vernino et al., 1992) to activate an endogenous  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  [ $\text{Cl}(\text{Ca})$ ] current in oocytes (Barish, 1983). Apart from permeating through the ion

channel, external divalent cations allosterically modulate neuronal nAChR function. Increasing the external  $\text{Ca}^{2+}$  concentration causes a simultaneous reduction of the single channel conductance and increase of the channel opening frequency, resulting in a net enhancement of the amplitude of the whole-cell ACh response (Fieber and Adams, 1991; Mulle et al., 1992b; Vernino et al., 1992). A similar effect of external  $\text{Mg}^{2+}$  on single channel conductance has been reported, but  $\text{Mg}^{2+}$  hardly affects the whole-cell ACh response (Mulle et al., 1992b).

In order to investigate  $\text{Ca}^{2+}$  permeable channels in *Xenopus* oocytes, the endogenous Cl(Ca) current is routinely suppressed by high concentrations of flufenamic acid (FFA;  $\text{IC}_{50} = 28 \mu\text{M}$ ) and niflumic acid (NFA;  $\text{IC}_{50} = 17 \mu\text{M}$ ) (Leonard and Kelso, 1990; White and Aylwin, 1990; Vernino et al., 1992; Séguéla et al., 1993). Alternatively, the endogenous Cl(Ca) current can be eliminated by replacement of external  $\text{Ca}^{2+}$  by  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ , or  $\text{Ba}^{2+}$  and by chelation of intracellular  $\text{Ca}^{2+}$  (Barish, 1983; Mileli and Parker, 1984; Galzi et al., 1992; Sands et al., 1993). In addition, methanesulfonate does not permeate through the Cl(Ca) channel in *Xenopus* oocytes (Barish, 1983).

As fenamates have also been shown to act as noncompetitive antagonists of NMDA receptors in primary cultured rat spinal neurons (Lerma and Martín del Río, 1992) and of rat cerebral cortex GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes (Woodward et al., 1994), we have investigated potential effects of FFA and NFA on nAChRs. The results indicate that the chloride channel blockers cause direct and opposite effects on  $\alpha\beta\beta 2$  and  $\alpha\beta\beta 4$  nAChRs.

## Materials and Methods

**Materials.** Mature female specimens of *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI). Acetylcholine chloride, atropine sulfate, 3-aminobenzoic acid ethyl ester, collagenase type I, flufenamic acid, and niflumic acid (Fig. 1) were purchased from Sigma (St. Louis, MO) and methanesulfonate from Aldrich (Milwaukee, WI).

**Expression in *Xenopus* oocytes.** Frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester. Oocytes were surgically removed and defolliculated after treatment with 1.5 mg/ml collagenase type I in  $\text{Ca}^{2+}$ -free modified Barth's saline for 2 hr at room temperature. cDNAs for  $\alpha 3$ ,  $\beta 2$ , and  $\beta 4$  were ligated into the pSM plasmid vector containing the SV40 viral promoter. The  $\alpha 3$  construct and either one of the two  $\beta$ -constructs were coinjected at a molar ratio of 1:1 into the nuclei of stage V and VI oocytes within 24 hr after harvesting using a Drummond microinjector (Bertrand et al., 1991). Subunits were dissolved in distilled water and the total injection volume was about 20 nl per oocyte, corresponding to 7–10 ng DNA of each subunit. Injected oocytes were incubated at 19°C for 2–5 d in  $\text{Cl}^-$ -containing or  $\text{Cl}^-$ -free modified Barth's saline, to be investigated under  $\text{Cl}^-$  or  $\text{Cl}^-$ -free conditions, respectively.

**Solutions.** Modified Barth's saline contained (in mM) 88 NaCl, 1 KCl, 2.4  $\text{NaHCO}_3$ , 0.3  $\text{Ca}(\text{NO}_3)_2$ , 0.41  $\text{CaCl}_2$ , 0.82  $\text{MgSO}_4$ , 15 HEPES, and 100  $\mu\text{g}/\text{ml}$  gentamicin (pH = 7.6 with NaOH).  $\text{Cl}^-$ -free modified Barth's saline contained (in mM) 90 NaOH, 2.5 KOH, 10 HEPES, 2  $\text{Ca}(\text{OH})_2$ , 0.8  $\text{Mg}(\text{OH})_2$ , and 100  $\mu\text{g}/\text{ml}$  gentamicin (pH = 7.6 with methanesulfonate). During voltage clamp oocytes were superfused with  $\text{Cl}^-$  saline containing (in mM) 115 NaCl, 2.5 KCl, 1  $\text{CaCl}_2$ , 10 HEPES (pH = 7.2 with NaOH) or with  $\text{Cl}^-$ -free saline containing (in mM) 115 NaOH, 2.5 KOH, 1  $\text{Ca}(\text{OH})_2$ , 10 HEPES (pH = 7.2 with methanesulfonate). All superfusates contained 1  $\mu\text{M}$  atropine to block endogenous muscarinic responses.

**Electrophysiology.** Oocytes were voltage clamped by the standard two microelectrode voltage-clamp technique using an Axoclamp 2A voltage-clamp amplifier (Axon Instruments, Burlingame, CA). Microelectrodes (0.5–2.5 M $\Omega$ ) were filled with 3 M KCl under standard conditions or with 3 M K-methanesulfonate and 50 mM KCl under  $\text{Cl}^-$ -free conditions. The holding potential was  $-60$  mV, unless otherwise indicated. All experiments were performed at room temperature (20–23°C).

Voltage clamped oocytes were continuously superfused with saline solution at a rate of approximately 20 ml/min. Aliquots of stock solu-

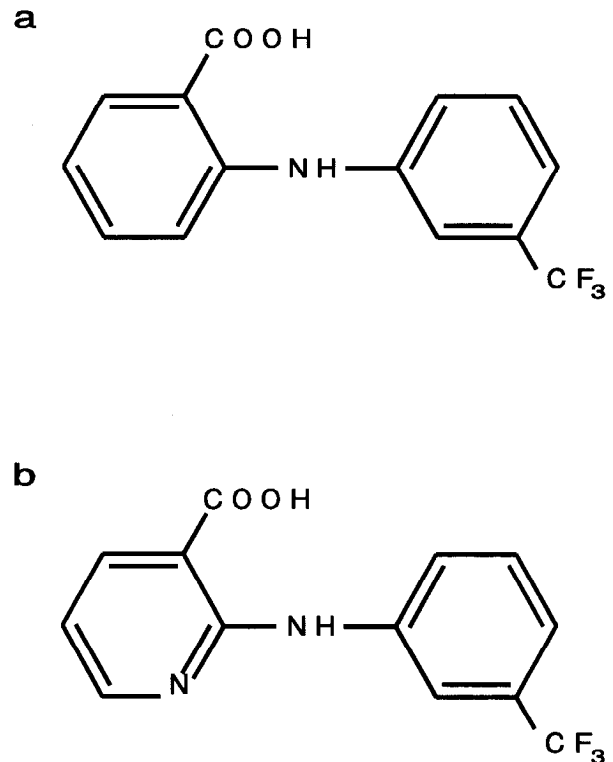


Figure 1. Structural formulas of flufenamic acid (a) and of niflumic acid (b).

tions of 0.1–1 M of niflumic acid and flufenamic acid in dimethylsulfoxide (DMSO) and of ACh in distilled water were added to the saline immediately before the experiments. Drugs were applied by switching between control and drug-containing saline using a solenoid valve. Agonist applications were alternated with 5 min of superfusion with agonist-free saline to allow the receptors to recover completely from desensitization. Final DMSO concentrations never exceeded 0.2% (v/v), which had no effect on  $\alpha\beta\beta 2$  and  $\alpha\beta\beta 4$  nAChR-mediated inward currents in control experiments.

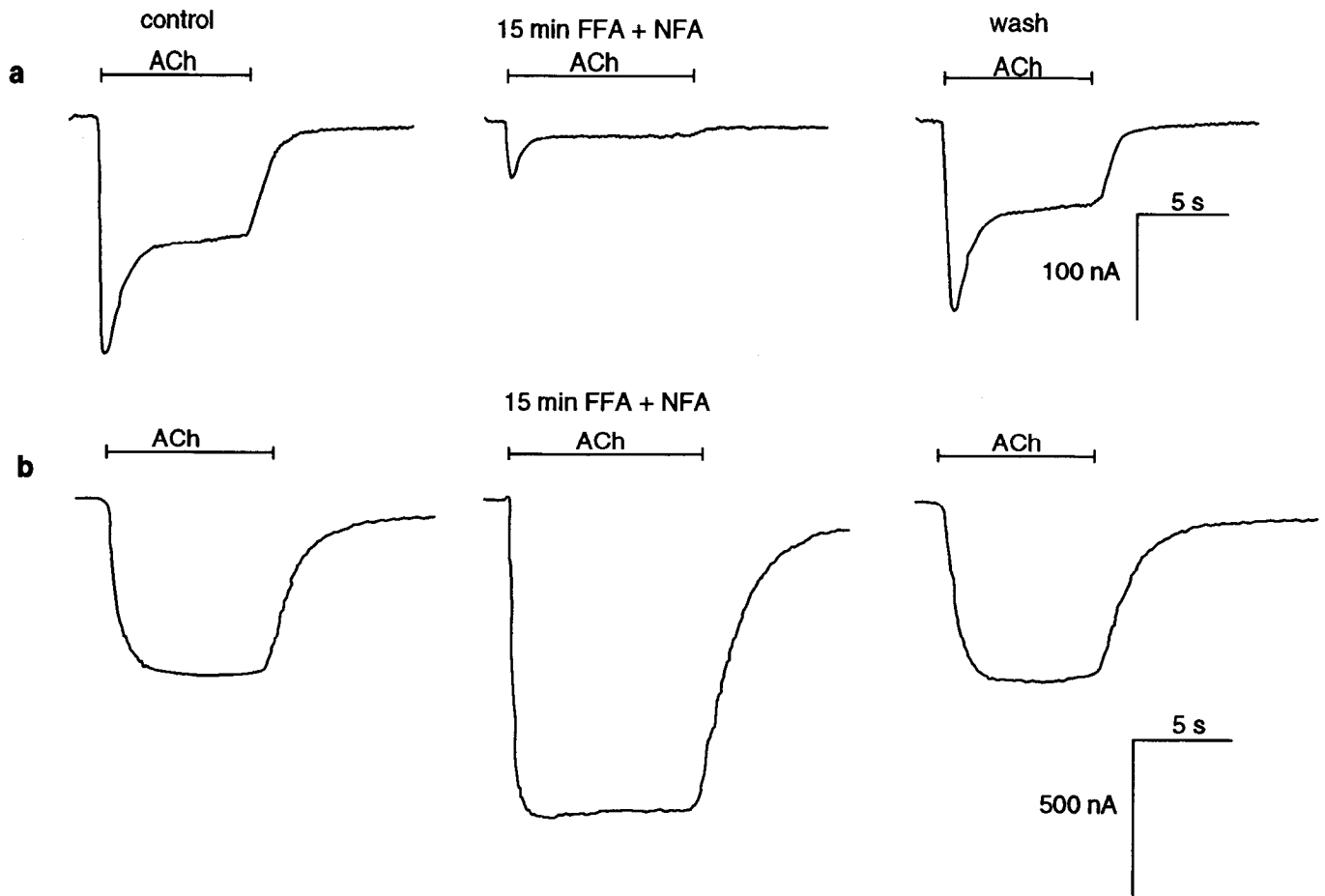
Currents were low-pass filtered (eight-pole Bessel, Frequency Devices, Haverhill, MA) at 150 Hz unless otherwise indicated, digitized and stored by a Macintosh Ixi computer using data acquisition software (LABVIEW, National Instruments, Austin, TX and LIB I, University of Arizona, Tucson, AZ).

Concentration–effect curves were fitted by a nonlinear least-squares algorithm (Marquardt, 1963) according to the equation  $i = i_{\text{max}} / (1 + \{\text{EC}_{50} / [\text{conc}]\}^n)$ , where  $n$  and  $\text{EC}_{50}$  represent the slope factor and the agonist concentration producing half-maximal response, respectively. Results are expressed as mean  $\pm$  SD of  $n$  independent experiments.

## Results

### Direct effects of flufenamic acid and niflumic acid on neuronal nAChR-mediated ion current

Under standard  $\text{Cl}^-$  conditions the superfusion of oocytes with 100  $\mu\text{M}$  and 10  $\mu\text{M}$  ACh, that is, nearly equipotent concentrations for  $\alpha\beta\beta 2$  and  $\alpha\beta\beta 4$  nAChRs, respectively (Cachelin and Jaggi, 1991), evoked the typical desensitizing and nondesensitizing inward currents (Fig. 2). After 15 min of superfusion with a mixture of 100  $\mu\text{M}$  FFA and 100  $\mu\text{M}$  NFA, which is commonly used to suppress endogenous Cl(Ca) current in *Xenopus* oocytes (Vernino et al., 1992; Séguéla et al., 1993), the peak amplitude of the  $\alpha\beta\beta 2$  nAChR-mediated inward currents was steadily reduced to  $23 \pm 3\%$  ( $n = 3$ ) of control values (Fig. 2a). Conversely, after 15 min of superfusion with the same mixture, the peak amplitude of  $\alpha\beta\beta 4$  nAChR-mediated inward currents was potentiated to  $197 \pm 25\%$  ( $n = 5$ ) of control values (Fig. 2b).



**Figure 2.** Differential effects of FFA and NFA on  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs. A mixture of 100  $\mu\text{M}$  NFA and 100  $\mu\text{M}$  FFA inhibits  $\alpha 3\beta 2$  nAChR- (a) and potentiates  $\alpha 3\beta 4$  nAChR-mediated inward currents (b) recorded in  $\text{Cl}^-$  saline. Inward currents were evoked by superfusion of 100  $\mu\text{M}$  ACh (a) and 10  $\mu\text{M}$  ACh (b) as indicated by horizontal bars. After 15 min of superfusion with NFA and FFA, the peak amplitude of the  $\alpha 3\beta 2$  nAChR-mediated inward current was reduced to 25% of the control value, whereas the peak amplitude of the  $\alpha 3\beta 4$  nAChR-mediated inward current was increased to 176% of the control value. Both effects were reversed after the removal of NFA and FFA by washing with  $\text{Cl}^-$  saline.

The inhibitory and potentiating effects were both reversed on washing with  $\text{Cl}^-$  saline (Fig. 2a,b).

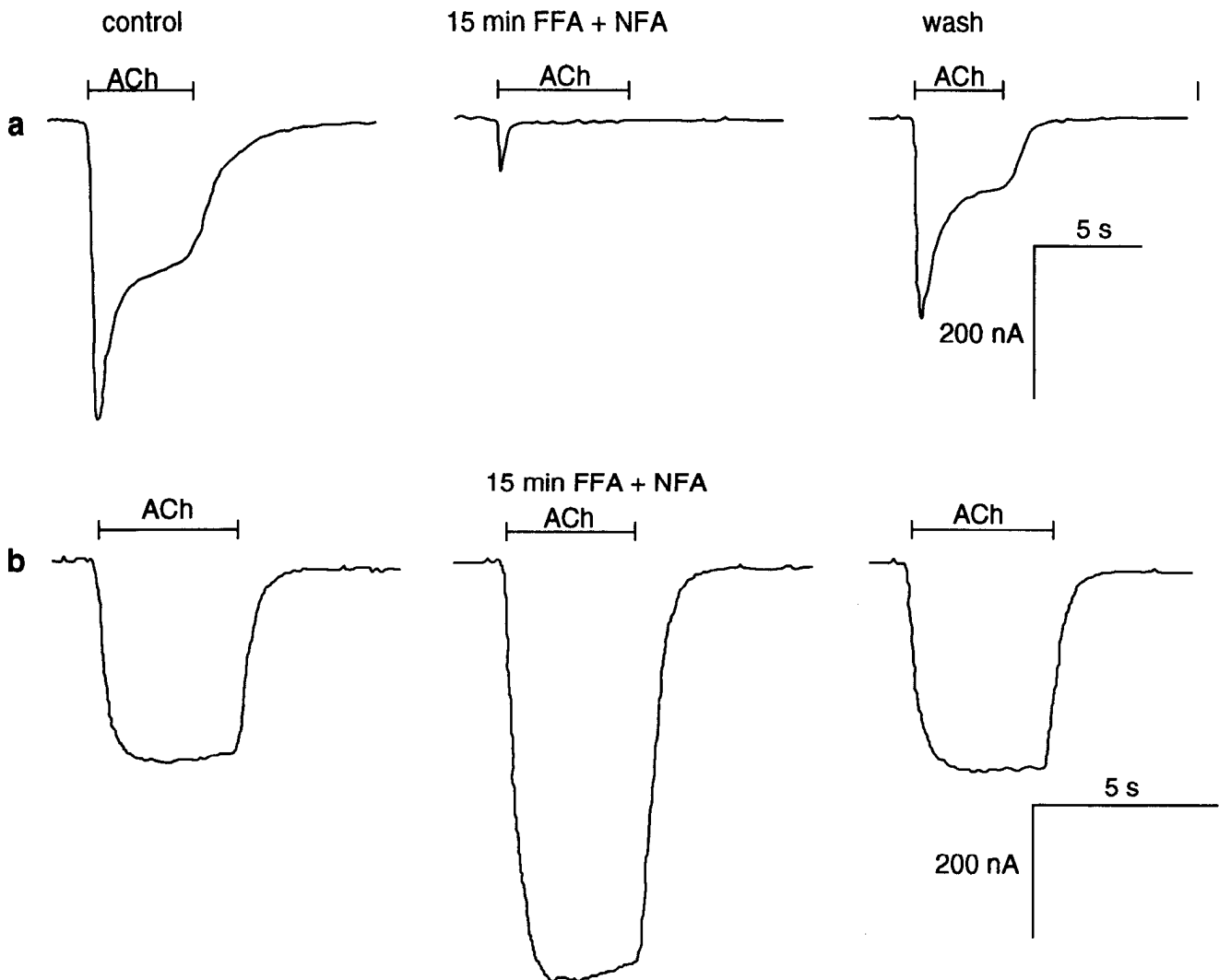
The effects of the  $\text{Cl}(\text{Ca})$  current blockers were further investigated in oocytes incubated in  $\text{Cl}^-$ -free modified Barth's solution and voltage clamped in  $\text{Cl}^-$ -free saline. In the experiments of Figure 3,  $\text{Ca}^{2+}$  was replaced by 1 mM  $\text{Mg}^{2+}$  or 1 mM  $\text{Ba}^{2+}$ , which are ineffective in activating  $\text{Cl}(\text{Ca})$  current in oocytes (Barish, 1983; Miledi and Parker, 1984). These experimental conditions are most unfavorable for the activation of  $\text{Cl}(\text{Ca})$  current. Even under these conditions the mixture of 100  $\mu\text{M}$  FFA and 100  $\mu\text{M}$  NFA reduced the peak amplitude of  $\alpha 3\beta 2$  nAChR-mediated ion currents to  $22 \pm 5\%$  ( $n = 3$ ) of the control values (Fig. 3a) and increased that of the  $\alpha 3\beta 4$  nAChR-mediated ion current to  $169 \pm 24\%$  ( $n = 3$ ) of control values (Fig. 3b). These effects are qualitatively the same as and cannot be distinguished from those obtained under standard conditions within experimental error (Student's *t* test,  $p = 0.78$  and 0.17, respectively).

Figure 4 shows the effects of coapplication of ACh with the mixture of 100  $\mu\text{M}$  FFA and 100  $\mu\text{M}$  NFA on  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChR-mediated inward currents. The effect of the mixture on the  $\alpha 3\beta 2$  nAChR was incomplete on coapplication with ACh (Fig. 4a). Steady-state inhibition was not reached until 5–10 min of superfusion of the oocytes with NFA and FFA (not shown). Conversely, the potentiation of  $\alpha 3\beta 4$  nAChR-mediated ion cur-

rents by the mixture of FFA and NFA appeared already complete on the first coapplication and amounted to  $187 \pm 15\%$  ( $n = 4$ ). This effect is the same as that observed after 15 min of superfusion with FFA and NFA under standard  $\text{Cl}^-$  conditions and under  $\text{Ca}^{2+}$ -free and  $\text{Cl}^-$ -free conditions. When applied alone, neither the mixture of FFA and NFA nor the individual compounds caused detectable changes in membrane current (not shown).

#### Concentration-dependent effects of FFA and of NFA

In order to investigate the concentration-dependent effects of FFA and of NFA on  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs, oocytes were superfused with various concentrations of the individual compounds under  $\text{Cl}^-$ -free conditions. The peak amplitude of  $\alpha 3\beta 2$  nAChR-mediated inward currents was reduced after 10 min of superfusion of FFA (Fig. 5a) and NFA (Fig. 5b) in a concentration-dependent manner. In addition, the decay of 100  $\mu\text{M}$  ACh-induced inward currents became faster with increasing concentrations of FFA and NFA, and the amplitude of the steady inward current component was reduced by FFA and NFA. The concentration–effect curves for the reduction of peak amplitudes of the  $\alpha 3\beta 2$  nAChR-mediated inward currents by FFA and NFA are shown in Figure 5c. The fitted concentration–effect curves yielded estimates for the  $\text{IC}_{50}$  and slope factor of  $90 \pm 4 \mu\text{M}$  and



**Figure 3.** Effects of FFA and NFA on  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs under  $\text{Ca}^{2+}$ -free and  $\text{Cl}^{-}$ -free conditions. The mixture of  $100 \mu\text{M}$  NFA and  $100 \mu\text{M}$  FFA inhibits  $\alpha 3\beta 2$  nAChR- (*a*) and potentiates  $\alpha 3\beta 4$  nAChR-mediated inward currents (*b*) recorded under  $\text{Ca}^{2+}$ -free and  $\text{Cl}^{-}$ -free conditions. Inward currents were evoked by superfusion of  $100 \mu\text{M}$  (*a*) and  $10 \mu\text{M}$  ACh (*b*) as indicated by horizontal bars. After 15 min of superfusion of NFA and FFA, the peak amplitude of the  $\alpha 3\beta 2$  nAChR-mediated inward current was reduced to 15% of the control value, whereas the peak amplitude of the  $\alpha 3\beta 4$  nAChR-mediated inward current was increased to 206% of the control value. The effects were reversed after the removal of NFA and FFA by washing with  $\text{Ca}^{2+}$ -free and  $\text{Cl}^{-}$ -free saline.

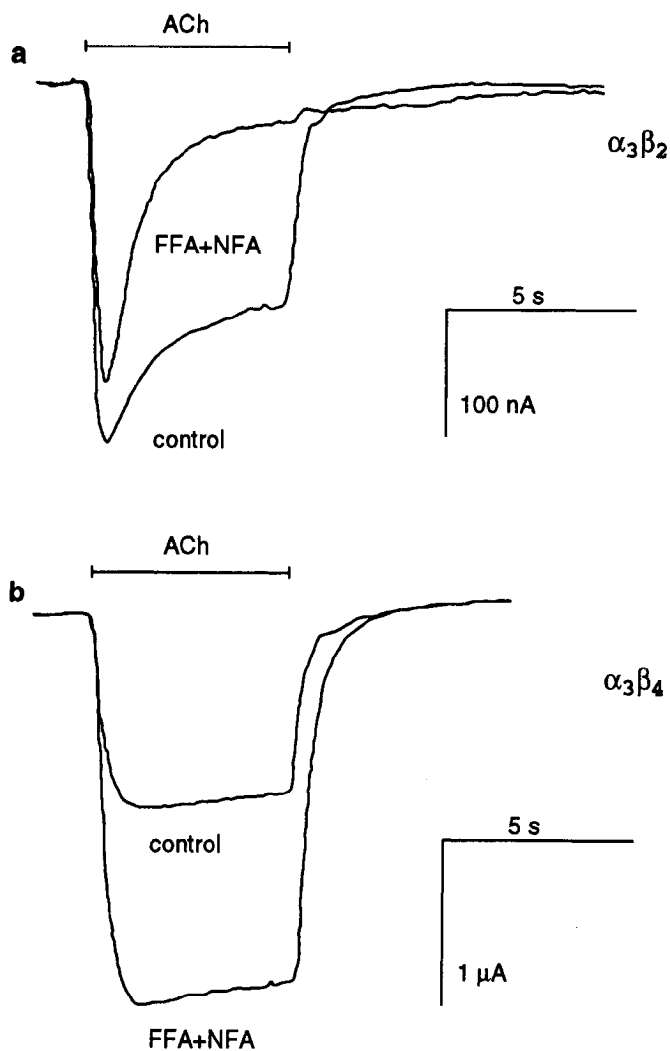
$-1.3 \pm 0.1$  for FFA, and  $260 \pm 30 \mu\text{M}$  and  $-1.5 \pm 0.2$  for NFA.

The potentiating effects of FFA and NFA on  $\alpha 3\beta 4$  nAChR-mediated inward currents are shown in Figure 6. Peak amplitudes of inward currents were increased on coapplication of  $10 \mu\text{M}$  ACh with FFA (Fig. 6*a*) and NFA (Fig. 6*b*) in a concentration-dependent manner. Control experiments showed that the potentiating effect of  $1 \mu\text{M}$  FFA also reached steady-state on coapplication with ACh. The concentration-effect curve fitted to the data obtained with NFA (Fig. 6*c*) yielded an  $\text{EC}_{50}$  value of  $30 \pm 13 \mu\text{M}$ , a slope factor of  $0.88 \pm 0.22$  and a maximum response amplitude of  $159 \pm 4\%$  relative to control. The data in Figure 6*c* show that the potentiating effect of FFA did not saturate up to the highest concentration used, which was at the solubility limit. Therefore, a concentration-effect curve could not be fitted for FFA. At concentrations of  $1 \text{ mM}$ , FFA and NFA potentiated the  $\alpha 3\beta 4$  nAChR-mediated inward currents, evoked

by superfusion of  $10 \mu\text{M}$  ACh, to  $328 \pm 10\%$  ( $n = 3$ ) and  $156 \pm 12\%$  ( $n = 4$ ) of control values, respectively.

#### Effects of FFA on the concentration-effect curves of ACh

The inhibitory and potentiating effects of FFA on  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs have been studied in more detail. Inward currents mediated by  $\alpha 3\beta 2$  nAChRs evoked by various concentrations of ACh in the absence and presence of  $100 \mu\text{M}$  FFA in  $\text{Ca}^{2+}$ -free  $\text{Cl}^{-}$  saline containing  $1 \text{ mM}$   $\text{Ba}^{2+}$  are shown in Figure 7*a*. The dependence of the peak inward current amplitude on ACh concentration under control conditions and in the presence of  $100 \mu\text{M}$  FFA is shown in Figure 7*b*. The results show that FFA causes a reduction of the peak amplitudes of  $\alpha 3\beta 2$  nAChR-mediated inward currents at all ACh concentrations. The mean of the  $\text{EC}_{50}$  values and slope factors of concentration-effect curves fitted to the data obtained from three oocytes were  $30 \pm 14 \mu\text{M}$  ACh and  $0.85 \pm 0.18$  in control experiments, and  $41 \pm$



**Figure 4.** Onset of inhibitory and potentiating effects. Effects of coapplication of 100  $\mu\text{M}$  NFA and 100  $\mu\text{M}$  FFA with 100  $\mu\text{M}$  ACh on  $\alpha 3\beta 2$  (*a*) and with 10  $\mu\text{M}$  ACh on  $\alpha 3\beta 4$  nAChR-mediated inward current (*b*). The coapplications show only the onset of the inhibitory effect on  $\alpha 3\beta 2$  nAChR-mediated inward current, whereas the potentiating effect on  $\alpha 3\beta 4$  nAChR-mediated inward current cannot be distinguished from the steady-state effect.

30  $\mu\text{M}$  ACh and  $0.73 \pm 0.23$  in the presence of 100  $\mu\text{M}$  FFA. On average the maximum of the concentration–effect curve of ACh was reduced by 100  $\mu\text{M}$  FFA to  $38 \pm 8\%$  of the control value (Fig. 7*b*). The changes of the  $\text{EC}_{50}$  value and slope factor observed induced by FFA are not significant within experimental error, whereas the maximum inward current amplitude is decreased significantly (Student's *t* test,  $p < 0.001$ ).

Inward currents mediated by  $\alpha 3\beta 4$  nAChRs evoked by various concentrations of ACh in the absence and presence of 100  $\mu\text{M}$  FFA in  $\text{Cl}^-$ -free saline are shown in Figure 8*a*. The dependence of the peak inward current amplitude on ACh concentration under control conditions and in the presence of 100  $\mu\text{M}$  FFA is shown in Figure 8*b*. The results show that in the presence

of 100  $\mu\text{M}$  FFA the amplitudes of  $\alpha 3\beta 4$  nAChR-mediated inward currents are enhanced at all agonist concentrations and that tail currents on the removal of high concentrations of the agonist are also enhanced by FFA. Fitting concentration–effect curves to the data obtained from four oocytes yielded mean  $\text{EC}_{50}$  values and slope factors of  $63 \pm 23 \mu\text{M}$  ACh and  $1.47 \pm 0.16$  in the control situation, and of  $37 \pm 14 \mu\text{M}$  ACh and  $1.48 \pm 0.35$  in the presence of 100  $\mu\text{M}$  FFA. On average 100  $\mu\text{M}$  FFA enhances the maximum of the concentration–effect curve of ACh to  $118 \pm 9\%$  of the control value (Fig. 8*b*). The changes of the  $\text{EC}_{50}$  value and slope factor observed induced by FFA are not significant within experimental error, whereas the maximum inward current amplitude is enhanced significantly (Student's *t* test,  $p < 0.001$ ).

#### Relation between FFA effect on $\alpha 3\beta 4$ nAChR and divalent cation concentration

Since FFA and divalent cations both modulate the responses mediated by neuronal type nAChRs the interaction between the two was also investigated. Experiments were performed under  $\text{Cl}^-$ -free conditions and the concentration of  $\text{Mg}^{2+}$  was varied in order to maintain a low probability of  $\text{Cl}(\text{Ca})$  channel activation. In control experiments  $\alpha 3\beta 4$  nAChR-mediated ion currents were evoked by coapplications of 10  $\mu\text{M}$  ACh and 1–30 mM  $\text{Mg}^{2+}$ . A slight, but significant potentiation was observed with increasing  $\text{Mg}^{2+}$  concentration (Fig. 9*a*), whereas concentration-dependent block was observed in the continuous presence of 200  $\mu\text{M}$  FFA (Fig. 9*b*). A similar concentration-dependent block by  $\text{Mg}^{2+}$  was observed when responses were evoked by 200  $\mu\text{M}$  ACh instead of 10  $\mu\text{M}$  ACh (Fig. 9*c*).

#### Discussion

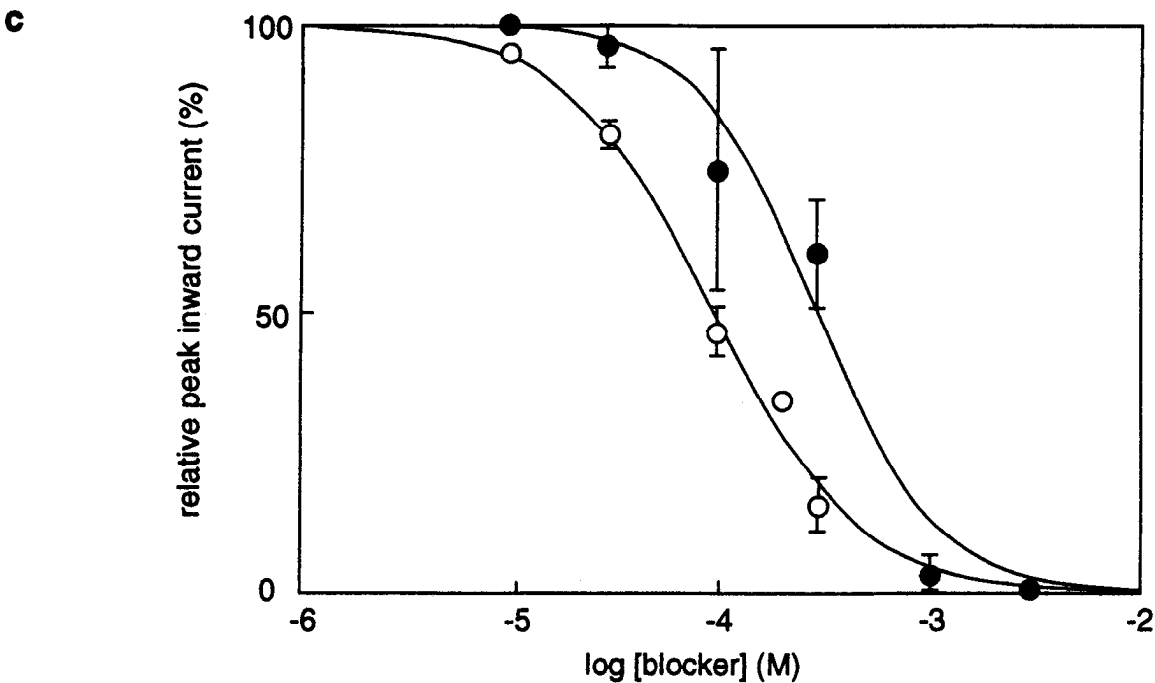
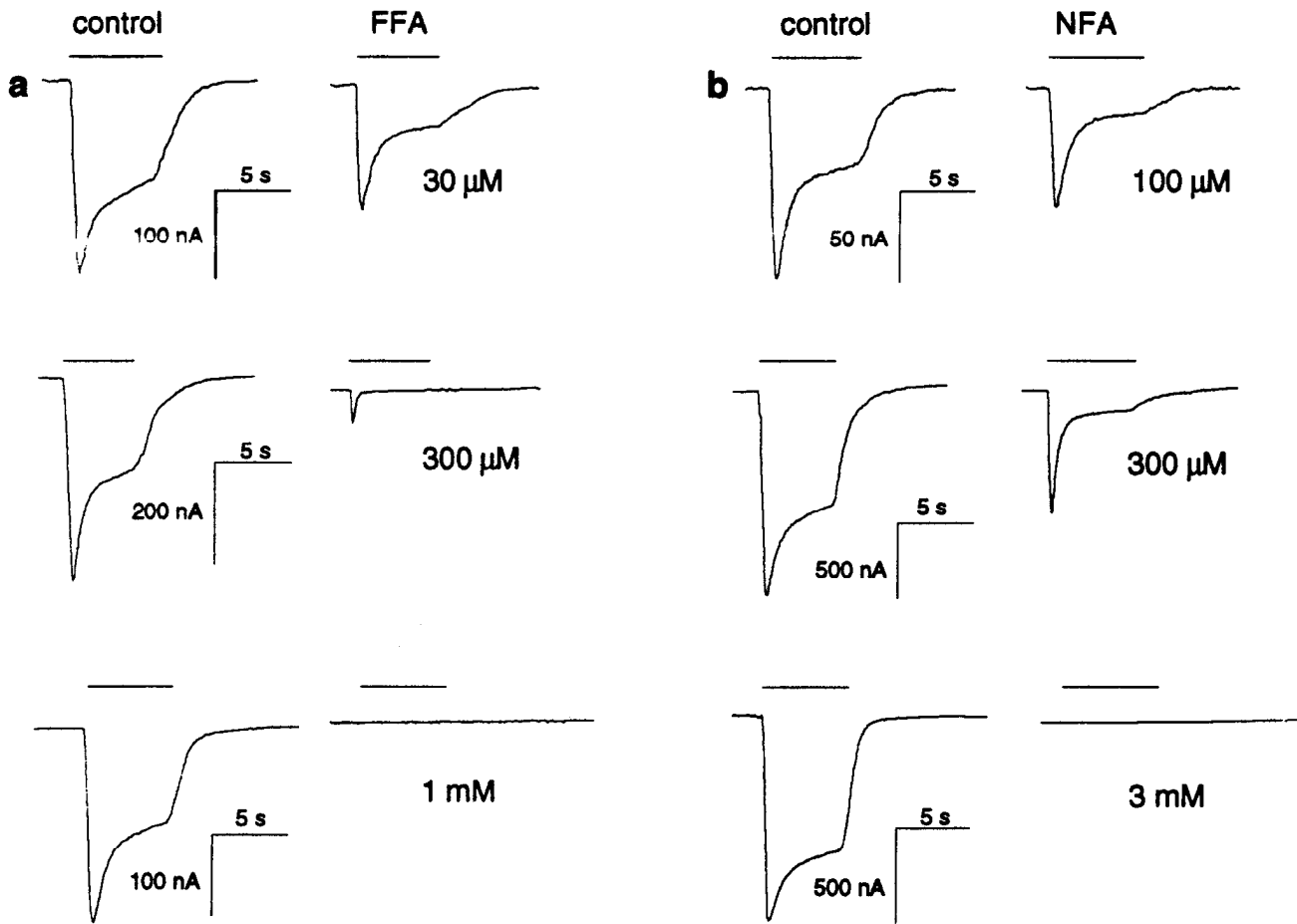
##### Direct effects of niflumic acid and flufenamic acid on neuronal nAChRs

In this article we demonstrate that the compounds NFA and FFA, which are commonly used to block endogenous  $\text{Cl}(\text{Ca})$  current in *Xenopus* oocytes, differentially affect  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$  neuronal nAChRs. Surprisingly,  $\alpha 3\beta 4$  nAChR-mediated inward currents are potentiated by these compounds, whereas the  $\alpha 3\beta 2$  nAChR-mediated inward currents are reduced.

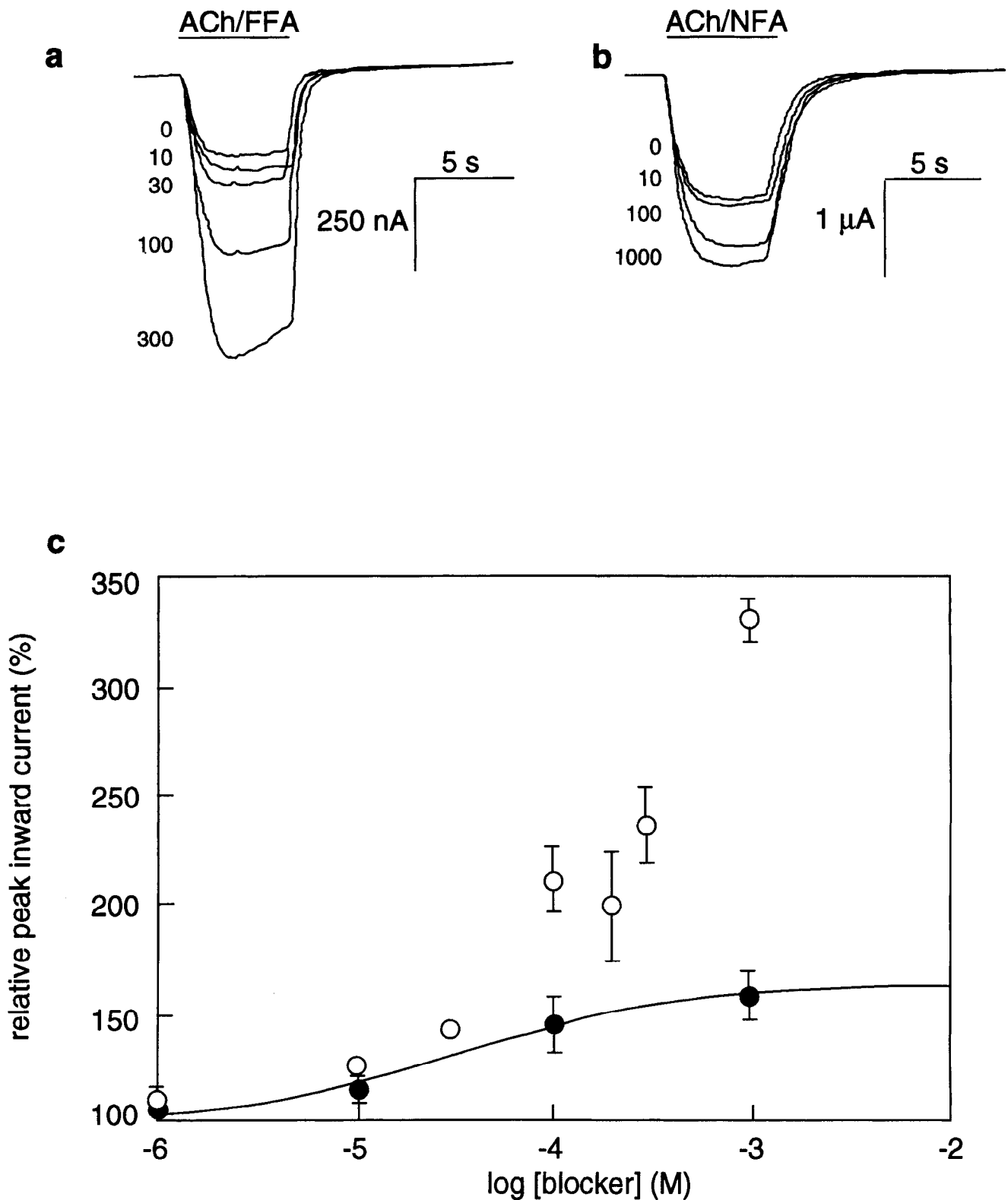
The effects of NFA and FFA appear to originate from a direct interaction with the nAChRs for a number of reasons. The potentiation of  $\alpha 3\beta 4$  nAChR-mediated inward currents under standard  $\text{Cl}^-$  conditions (Fig. 2) cannot be explained by  $\text{Cl}(\text{Ca})$  current block.  $\text{Cl}(\text{Ca})$  current block at the holding potential of  $-60$  mV should have resulted in a reduction of the amplitude of the ACh-induced inward current, since the  $\text{Cl}^-$  current reversal potential in oocytes is about  $-25$  mV (Barish, 1983). In addition, the differential effects on  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$  nAChRs cannot be accounted for at the same time by block of  $\text{Cl}(\text{Ca})$  current and were also observed at potentials near the reversal potential for  $\text{Cl}^-$  (not shown). Potentiation and inhibition were similar under standard  $\text{Cl}^-$  conditions (Fig. 2),  $\text{Cl}^-$ -free conditions (Figs. 5, 6) and even when external  $\text{Ca}^{2+}$  was replaced by  $\text{Mg}^{2+}$  or  $\text{Ba}^{2+}$  under  $\text{Cl}^-$ -free conditions (Fig. 3). The complete block of  $\alpha 3\beta 2$  nAChR-mediated currents by high concentrations of NFA and

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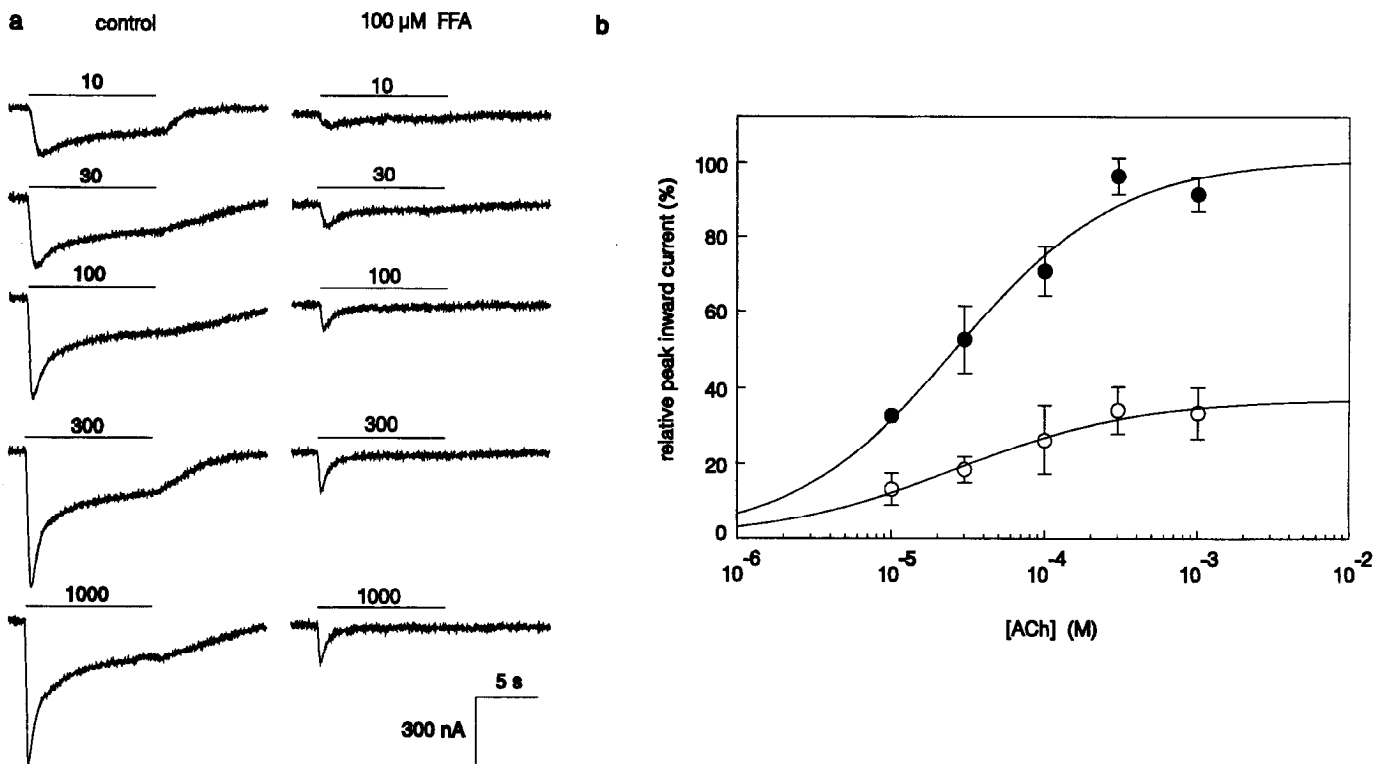
**Figure 5.** Concentration-dependent effects of FFA and NFA on  $\alpha 3\beta 2$  nAChRs. *a*, Left column shows control, 100  $\mu\text{M}$  ACh-induced inward currents and the right column shows the 100  $\mu\text{M}$  ACh-induced inward currents in the same oocytes after 10 min of superfusion with FFA at concentrations of 30, 300, and 1000  $\mu\text{M}$  as indicated. *b*, Left column shows control, 100  $\mu\text{M}$  ACh-induced inward currents and the right column



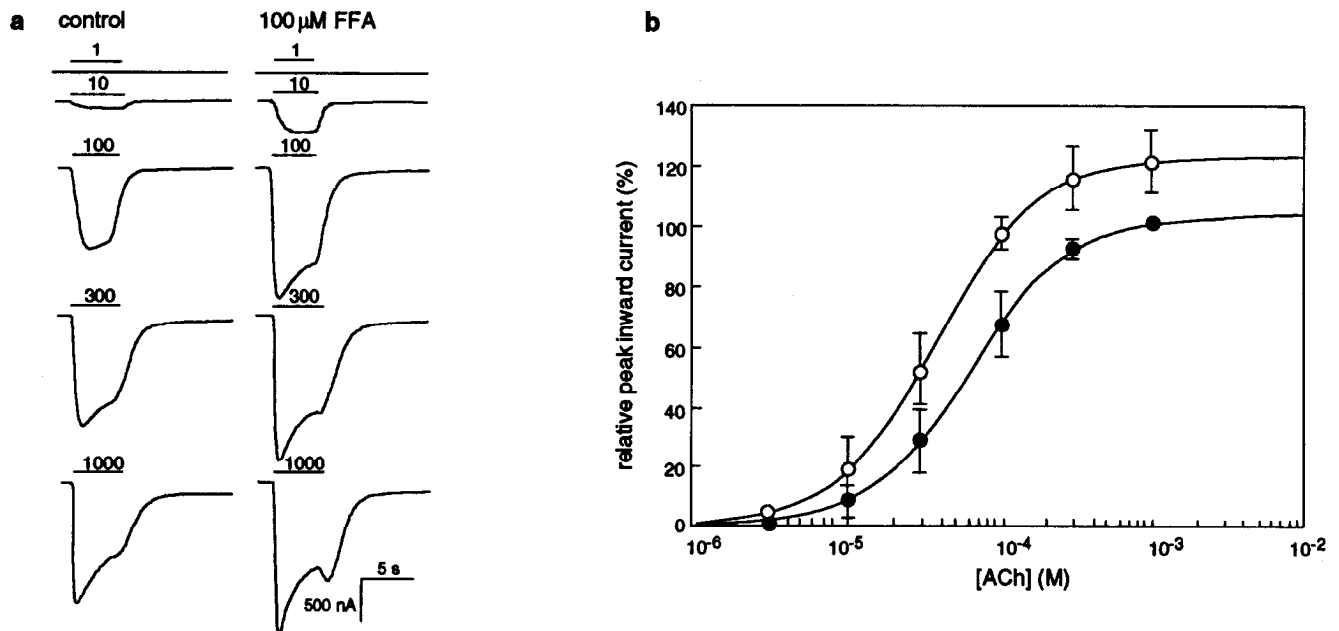
shows the 100  $\mu\text{M}$  ACh-induced inward currents in the same oocytes after 10 min of superfusion with NFA at concentrations of 100, 300, and 3000  $\mu\text{M}$  as indicated. *c*, Concentration-effect curves of FFA (*open circles*) and NFA (*solid circles*) for the reduction of the peak amplitude of 100  $\mu\text{M}$  ACh-induced inward current mediated by  $\alpha\beta 2$  nAChRs. Data points represent mean  $\pm$  SD (error bars) of three to five oocytes.



**Figure 6.** Concentration-dependent effects of FFA and NFA on  $\alpha 3\beta 4$  nAChRs. *a*, Control,  $10 \mu\text{M}$  ACh-induced inward current and superimposed inward currents recorded from the same oocyte during coapplication of  $10 \mu\text{M}$  ACh and FFA at concentrations of 10, 30, 100, and  $300 \mu\text{M}$  as indicated. *b*, Control,  $10 \mu\text{M}$  ACh-induced inward current and superimposed inward currents recorded from the same oocyte during coapplication of  $10 \mu\text{M}$  ACh and NFA at concentrations of 10, 100, and  $1000 \mu\text{M}$  as indicated. *c*, Concentration-effect curves of FFA (open circles) and NFA (solid circles) for the potentiation of the peak amplitude of  $10 \mu\text{M}$  ACh-induced inward current mediated by  $\alpha 3\beta 4$  nAChRs. Data points represent mean  $\pm$  SD (error bars) of three to seven oocytes.

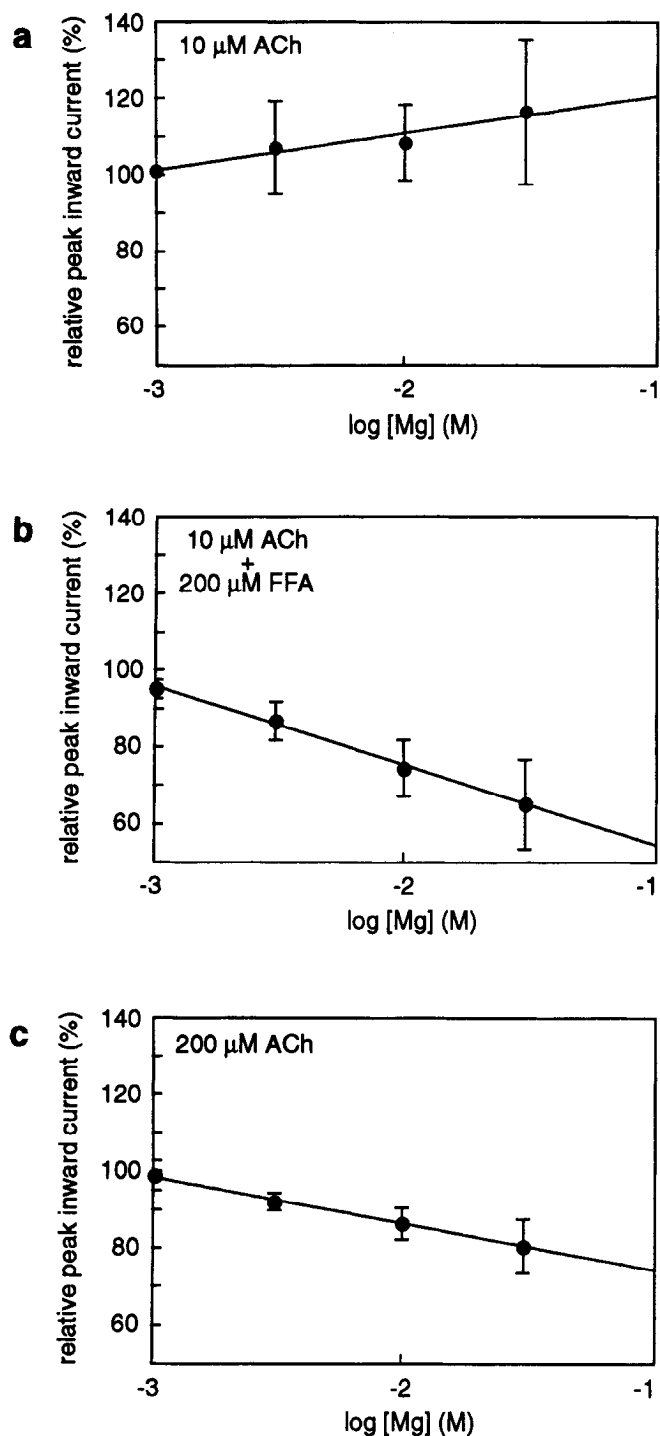


**Figure 7.** Effect of 100  $\mu$ M FFA on the concentration-effect curve of ACh on  $\alpha$ 3 $\beta$ 2 nAChR. *a*, Inward currents evoked by superfusion of 10, 30, 100, 300, and 1000  $\mu$ M ACh (as indicated by horizontal bars) under control conditions and after 15 min of superfusion with 100  $\mu$ M FFA. Current traces were filtered at 1 kHz. *b*, Concentration-effect curves of ACh under control conditions (solid circles) and in the presence of 100  $\mu$ M FFA (open circles). Data points represent mean  $\pm$  SD (error bars) of three oocytes. For each oocyte a control concentration-effect curve was fitted with a maximum of 100 and the maximum of the concentration-effect curve in the presence of FFA was normalized to control. The lines drawn represent the mean concentration-effect curves with the mean of the parameters fitted to the data from individual oocytes. Complete reversal of the effect was obtained only after 10–15 min of washing (not shown).



**Figure 8.** Effect of 100  $\mu$ M FFA on the concentration-effect curve of ACh on  $\alpha$ 3 $\beta$ 4 nAChR. *a*, Inward currents evoked by superfusion of 1, 10, 100, 300, and 1000  $\mu$ M ACh (as indicated by horizontal bars) under control conditions and in the presence of 100  $\mu$ M FFA. *b*, Concentration-effect curves of ACh under control conditions (solid circles) and in the continuous presence of 100  $\mu$ M FFA (open circles). Data points represent mean  $\pm$  SD (error bars) of four oocytes. For each oocyte, all peak inward current amplitudes were normalized to that of the inward current evoked by 1 mM ACh in the absence of FFA and plotted against ACh concentration. The concentration-effect curves after washout of 100  $\mu$ M FFA were the same as those obtained before application of FFA (result not shown). In order to maintain the quality of voltage clamp during the large inward currents mediated by the  $\alpha$ 3 $\beta$ 4 nAChR at high concentrations of the agonist, the membrane potential was held at  $-30$  mV in these experiments.





**Figure 9.** Relation between FFA effect and external  $\text{Mg}^{2+}$  concentration on  $\alpha 3\beta 4$  nAChRs. *a*, Under control conditions  $\text{Mg}^{2+}$  causes only a slight, concentration-dependent potentiation of 10  $\mu\text{M}$  ACh-induced inward currents. Data points represent mean  $\pm$  SD (error bars) of three oocytes. For each oocyte amplitudes were normalized to that of the 10  $\mu\text{M}$  ACh-induced inward current in  $\text{Cl}^-$ -free saline. Log-linear regression showed no deviation from linearity ( $p = 0.81$ ) and the hypothesis of no regression was rejected ( $p < 0.01$ ). *b*, In the continuous presence of 200  $\mu\text{M}$  FFA  $\text{Mg}^{2+}$  blocks 10  $\mu\text{M}$  ACh-induced inward currents in a concentration-dependent manner. Data points represent mean  $\pm$  SD (error bars) of three oocytes. For each oocyte amplitudes were normalized to that of the 10  $\mu\text{M}$  ACh-induced inward current in  $\text{Cl}^-$ -free saline containing 200  $\mu\text{M}$  FFA. Log-linear regression showed no deviation from linearity ( $p = 0.67$ ) and the hypothesis of no regression was rejected ( $p < 0.001$ ). *c*, Under control conditions 200  $\mu\text{M}$  ACh-induced inward currents are also blocked by  $\text{Mg}^{2+}$  in a concentration-dependent

of FFA (Fig. 5) also indicates that the effects are directly on the nAChR. The  $\beta 2$  and  $\beta 4$  subunits are involved in the determination of agonist and antagonist sensitivity as well as of single channel parameters of nAChRs (see introductory section). The agonist cytosine, which is a potent agonist of  $\beta 4$ -containing nAChR, acts as a partial agonist on  $\beta 2$ -containing nAChR and thereby inhibits the effects of full agonists on these receptors (Luetje and Patrick, 1991; Papke and Heinemann, 1994). However,  $\beta$ -subunit-dependent, opposite effects as presently observed for FFA and NFA have not been reported thus far. Together with the observation, that  $\alpha 2\beta 4$  nAChR-mediated ion current is also potentiated by FFA (R. Zwart, unpublished result), the results suggest that the differential modulation of nAChR-mediated ion current is dependent on the  $\beta$  subunit.

The present results show that the compounds FFA and NFA cannot be used to separate endogenous  $\text{Cl}(\text{Ca})$  current from neuronal nAChR-mediated current without adverse effects on the nAChRs. At the same time the results demonstrate that with the present methods the use of  $\text{Cl}(\text{Ca})$  current blockers is not required to investigate  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs. The lack of differences in the effects of NFA and FFA on  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  under standard  $\text{Cl}^-$  conditions and under conditions that the  $\text{Cl}(\text{Ca})$  current cannot be activated (Figs. 2, 3) indicates that  $\text{Ca}^{2+}$  influx through the  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs is not sufficient to activate a significant  $\text{Cl}(\text{Ca})$  current. Our findings contrast with the previous finding that FFA and NFA block  $\alpha 3\beta 4$  nAChR in oocytes, suggesting that  $\text{Cl}(\text{Ca})$  current is activated as a consequence of  $\alpha 3\beta 4$  nAChR stimulation in *Xenopus* oocytes (Verino et al., 1992). Although the magnitude of  $\text{Cl}(\text{Ca})$  currents may have been different, we have not been able to reproduce the result of the former study under identical experimental conditions.

#### Mechanism of potentiation and inhibition of the nAChR-mediated ion current

FFA inhibits  $\alpha 3\beta 2$  nAChR-mediated ion current at all ACh concentrations and causes a significant reduction of the maximum peak inward current amplitude to  $38 \pm 8\%$  of the control value. The  $\text{EC}_{50}$  value and the slope factor of the concentration-effect curve of ACh remain unaffected in the presence of FFA. Conversely, FFA enhances the  $\alpha 3\beta 4$  nAChR-mediated ion current at all ACh concentrations and a shift of the concentration-effect curve of ACh to lower agonist concentrations is observed (Fig. 8). The change in the  $\text{EC}_{50}$  of ACh by FFA from 63  $\mu\text{M}$  to 37  $\mu\text{M}$  appears to be within experimental error and is likely to be overestimated. Figure 8a shows that FFA causes an increase of the tail current following responses evoked by high concentrations of ACh. Tail currents indicate that ion channel block has occurred during the response and is reversed on washing (Sine and Steinbach, 1984; Oortgiesen and Vijverberg, 1989). Such a blocking effect will cause underestimation of the  $E_{\text{max}}$  and  $\text{EC}_{50}$  values of the concentration-effect curve. Enhanced open channel block in the presence of FFA (Fig. 8) may account for an apparent reduction in the  $\text{EC}_{50}$  of ACh. For this reason the potentiation of  $\alpha 3\beta 4$  nAChR-mediated inward current as well as

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manner. Data points represent mean  $\pm$  SD (error bars) of three oocytes. For each oocyte amplitudes were normalized to 200  $\mu\text{M}$  ACh-induced inward current in  $\text{Cl}^-$ -free saline. Log-linear regression showed no deviation from linearity ( $p = 0.68$ ) and the hypothesis of no regression was rejected ( $p < 0.001$ ).

the inhibition of  $\alpha 3\beta 2$  nAChR-mediated inward current by FFA are most likely due to a noncompetitive mechanism of action. Final confirmation of the noncompetitive nature of the effects of FFA would require radioligand binding experiments.

The enhancement by FFA of the  $\alpha 3\beta 4$  nAChR-mediated ion current at all agonist concentrations may be caused by a number of effects. The number of functional nAChR, the single nAChR channel open probability or the single nAChR channel conductance may be increased. The results of the experiments with  $Mg^{2+}$  provide a possible explanation for the mechanism involved.  $Mg^{2+}$  slightly potentiates ion current evoked by  $10 \mu M$  ACh, that is, at relatively low open channel probability, and blocks responses evoked by  $200 \mu M$  ACh, that is, at high open channel probability (Fig. 9). In the presence of  $200 \mu M$  FFA  $Mg^{2+}$  no longer potentiates, but blocks the  $10 \mu M$  ACh-induced inward current. This suggests that, like an increase of the agonist concentration, FFA increases the single channel open probability and thereby facilitates divalent cation block. An increase in the number of functional nAChR in the presence of FFA is a less likely explanation for the potentiating effects. In that case,  $Mg^{2+}$  should block the ion current to the same extent in the absence and in the presence of FFA. The various potentiations of neurotransmitter receptor-operated ion current reported thus far are all caused by an increase in single channel open probability. The potentiating effects of  $Ca^{2+}$  on neuronal nAChRs (Vernino, 1992; Mulle et al., 1992b), glycine on NMDA receptors (Johnson and Ascher, 1987), and benzodiazepines on GABA<sub>A</sub> receptors (Sieghart, 1992) are due to an increase in opening frequency of the channels and the potentiating effect of barbiturates on GABA<sub>A</sub>-receptors (Sieghart, 1992) is due to an increase in mean channel open time. In order to discriminate between the different possible mechanisms of potentiation by FFA and of potentiation and block by  $Mg^{2+}$  single channel studies are required.

It is concluded that  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$  nAChRs are oppositely modulated by FFA and NFA through a direct  $\beta$  subunit-dependent effect, resulting in potentiation and inhibition of agonist-induced ion currents. The results raise the important question whether endogenous ligands exist, which by non-competitive modulation of neuronal nAChRs differentially affect the functioning of cholinergic neurons *in vivo*.

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