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Differential blockade of rat $\alpha_3\beta_4$ and α_7 neuronal nicotinic receptors by ω-conotoxin MVIIC, ω-conotoxin GVIA and diltiazem

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1 Rat $\alpha_3\beta_4$ or α_7 neuronal nicotinic acetylcholine receptors (AChRs) were expressed in *Xenopus laevis* oocytes, and the effects of various toxins and non-toxin Ca^{2+} channel blockers studied. Nicotinic AChR currents were elicited by 1 s pulses of dimethylphenylpiperazinium (DMPP, 100 μM) applied at regular intervals.

2 The N/P/Q-type Ca^{2+} channel blocker ω-conotoxin MVIIC inhibited $\alpha_3\beta_4$ currents with an IC_{50} of 1.3 μM; the blockade was non-competitive and reversible. The α_7 currents were unaffected.

3 At 1 μM, ω-conotoxin GVIA (N-type Ca^{2+} channel blocker) inhibited by 24 and 20% $\alpha_3\beta_4$ and α_7 currents, respectively. At 1 μM, ω-agatoxin IVA (a P/Q-type Ca^{2+} channel blocker) did not affect α_7 currents and inhibited $\alpha_3\beta_4$ currents by only 15%.

4 L-type Ca^{2+} channel blockers furnidipine, verapamil and, particularly, diltiazem exhibited a preferential blocking activity on $\alpha_3\beta_4$ nicotinic AChRs.

5 The mechanism of $\alpha_3\beta_4$ currents blockade by ω-conotoxins and diltiazem differed in the following aspects: (i) the onset and reversal of the blockade was faster for toxins; (ii) the blockade by the peptides was voltage-dependent, while that exerted by diltiazem was not; (iii) diltiazem promoted the inactivation of the current while ω-toxins did not.

6 These data show that, at concentrations currently employed as Ca^{2+} channel blockers, some of these compounds also inhibit certain subtypes of nicotinic AChR currents. Our data calls for caution when interpreting many of the results obtained in neurons and other cell types, where nicotinic receptor and Ca^{2+} channels coexist.

Keywords: ω-conotoxin MVIIC; ω-conotoxin GVIA; ω-agatoxin IVA; diltiazem; $\alpha_3\beta_4$ nicotinic AChRs; α_7 nicotinic AChRs; *Xenopus* oocytes

Abbreviations: ACh, acetylcholine; AChRs, acethylcholine receptors; DMPP, dimethylphenylpiperazinium; DMSO, dimethyl sulphoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

Introduction

In addition to specific interactions with the α_1 subunit of the voltage-dependent L-type Ca^{2+} channels, an increasing number of other molecular targets for the different subgroups of organic Ca^{2+} channel antagonist drugs have been recognized (Zernig, 1990). Thus, blocking effects exerted by dihydropyridines, verapamil and diltiazem on $^{45}Ca^{2+}$ uptake, intracellular Ca^{2+} signal, catecholamine secretion, whole-cell inward currents, and $^{86}Rb^{+}$ efflux, upon neuronal nicotinic acetylcholine receptor (nicotinic AChR) stimulation have been described in bovine chromaffin cells (López *et al*., 1993; Gandía *et al*., 1996; Villarroya *et al*., 1997) and in a human neuroblastoma cell line (Donnelly-Roberts *et al*., 1995). However, contradictory data have been obtained with ω-toxins traditionally employed as selective blockers of non L-type Ca^{2+} channels. For instance, the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (1 μM) and the P-type Ca^{2+} channel blocker ω-agatoxin IVA (100 nM) reduce by 80 and 70% respectively, the nicotinic currents in bovine chromaffin cells (Fernández *et al*., 1995; Granja *et al*., 1995). In contrast, other authors found no significant effects of these two toxins on $^{45}Ca^{2+}$ uptake (Villarroya *et al*., 1997) or $^{86}Rb^{+}$ efflux (Donnelly-Roberts *et al*., 1995) induced by nicotinic activation of chromaffin cells and human neuroblastoma cells. No data on nicotinic receptors are available with the N/P/Q-type Ca^{2+} channel blocker ω-conotoxin MVIIC.

Recently, several nicotinic subunits from bovine chromaffin cells which resemble the brain α_3, α_5, α_7 and β_4 neuronal nicotinic AChR subunits with homologies above 90%, have been cloned (Criado *et al*., 1992; García-Guzmán *et al*., 1995; Campos-Caro *et al*., 1997). Moreover, α_3, α_5 and β_4 subunits are expressed both in adrenergic and noradrenergic chromaffin cells, while the α_7 subunit is preferentially expressed in adrenergic cells (Criado *et al*., 1997). Therefore, it is possible that a wide variety of nicotinic AChR subtypes could be expressed in each cell type. Since previous experiments were not able to distinguish between the blockade exerted by Ca^{2+} channel blockers on different subtypes of nicotinic AChRs, discrepancies concerning the effects of ω-toxins on nicotinic-mediated responses could be explained by differences in the chromaffin cell type tested and/or the nicotinic receptor subtype(s) expressed in each cell assayed.

In this study we have taken advantage of the oocyte as a receptor expression model, to determine the sensitivity of pure populations of $\alpha_3\beta_4$ or α_7 neuronal nicotinic AChRs to ω-toxins and non-peptide molecules, at concentrations in the range of those used as Ca^{2+} channel blockers. Our results show that whereas α_7 nicotinic AChRs are scarcely affected by most of the compounds tested, $\alpha_3\beta_4$ nicotinic AChRs are sensitive to all of them, particularly to ω-conotoxin MVIIC and diltiazem.

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We present here a thorough study on the differential inhibitory effects of peptide and non-peptide Ca^{2+} channel blockers, on rat brain nicotinic AChRs of the $\alpha_3\beta_4$ and α_7 subtypes expressed in *Xenopus laevis* oocytes. Additionally, we present a study on the mechanism of blockade exerted by ω-conotoxin MVIIC and by diltiazem, the two most potent blockers of $\alpha_3\beta_4$ receptors.

Methods

Techniques for the *in vitro* transcription of nicotinic AChR subunits cDNAs, oocytes injection and electrophysiological recordings of the expressed foreign receptors have been described previously (Miledi *et al*., 1989; Montiel *et al*., 1997; López *et al*., 1998).

Preparation of RNA and injection of Xenopus *oocytes*

The plasmids pPCA48E, pZPC13, PCX49 and pHIP306 containing the entire coding regions of rat brain nicotinic AChR α_3, β_4, β_2 and α_7 subunits were linearized with the restriction enzymes *Eco*RI, *Xho*I, *Bam*HI and *Sma*I, respectively. Linearized plasmids were transcribed with SP6 (α_3), T3 (β_4) and T7 (β_2, α_7) RNA polymerases using a mCAP RNA capping Kit (Stratagene C.S. La Jolla, CA, U.S.A.).

Mature female *Xenopus laevis* frogs obtained from a commercial supplier (CRBM du CNRS, Montpellier, France) were anaesthetized with tricaine solution (0.125%) and ovarian lobes were dissected out. Then, follicle-enclosed oocytes were manually stripped from the ovary membranes and incubated overnight at 16°C in a modified Barth's solution containing (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) 10, buffered to pH 7.4 and supplemented with gentamycin (0.1 mg ml^{-1}) and sodium pyruvate (5 mM). Next day, healthy follicle-enclosed oocytes were injected with 50 nl (50 ng) of α_7 RNA or 50 nl (25 : 25 ng) of $\alpha_3 : \beta_4$ or $\alpha_3 : \beta_2$ RNAs using a nanoject automatic injector (Drummond Scientific Co., Broomall, PA, U.S.A.). Electrophysiological recordings were made 2–5 days after RNA injections.

Electrophysiological recordings

Experiments were carried out at room temperature (22–25°C) in Ringer's solution containing (in mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, HEPES 5, buffered to pH 7.4 with NaOH. Membrane currents were recorded with a two-electrode voltage clamp amplifier (OC-725-B Warner Instrument Corporation, Hamden, CT, U.S.A.) using microelectrodes with resistances of 0.5–5 MΩ made from borosilicate glass (GC100TF-15, Clark Electromedical, Pangbourne, U.K.) and filled with KCl (3 M). The holding potential in all experiments was −60 mV, except in those carried out to study the voltage-dependent effects of Ca^{2+} antagonist compounds (see Results). Single oocytes were held in a 0.3 ml volume chamber and constantly superfused with Ringer's solution by gravity (4 $ml.min^{-1}$). The volume in the chamber was maintained constant using the reverse suction of one air pump. Solutions containing the nicotinic agonist dimethylphenylpiperazinium (DMPP), or the nicotinic blockers were applied with the use of a set of 2-mm diameter glass tubes located close to the oocyte. Voltage protocols, DMPP pulses and data adquisition were controlled using a Digidata 1200 Interface and CLAMPEX software (Axon Instruments, Foster City, CA, U.S.A.).

Materials and solutions

All products not specified were purchased from SIGMA (Madrid, Spain). Furnidipine was supplied by Laboratorios Alter (Madrid, Spain). Diltiazem and verapamil were purchased from Research Biochemical International (Natick, MA, U.S.A.). ω-Conotoxin MVIIC and ω-agatoxin IVA were purchased from Peptide Institute (Osaka, Japan) and ω-conotoxin GVIA from Bachem Feinchemikalien (Bubendorf, Switzerland). Furnidipine was dissolved in dimethylsulphoxide (DMSO) at 10^{-2} M and diluted in Ringer's solution to the desired concentrations. Toxins were prepared in distilled water at 10^{-4} M. Concentrate stock solutions of toxins were aliquoted and stored at −20°C until use. Final concentrations of toxins were prepared in Ringer's solution.

Statistical analysis

Values of agonist concentration eliciting half maximal current EC_{50} and antagonist concentration eliciting 50% blockade of maximal current IC_{50} were estimated through non-linear regression analysis of ISI software, for a PC computer from the concentration-response curves for agonist (DMPP) and antagonists (diltiazem, ω-conotoxin MVIIC). To calculate the time constant (τ) for blockade and recovery of nicotinic currents, records were fitted to a single exponential curve. Differences between groups were compared by Student's *t*-test with the statistical program Statworks TM; a value of $P \leqslant 0.05$ was taken as the limit of statistical significance.

Results

Blockade by ω-toxins of $\alpha_3\beta_4$ and α_7 currents

Oocytes expressing $\alpha_3\beta_4$ or α_7 nicotinic AChRs were stimulated with DMPP (100 μM, 20 s) at a holding potential of −60 mV. As previously described (Papke & Heinemann, 1991; López *et al*., 1998), important differences in the kinetics of currents (I_{DMPP}) between both receptor subtypes were seen. Figure 1a shows normalized I_{DMPP} for $\alpha_3\beta_4$ or α_7 nicotinic AChRs; α_7 current exhibited a faster activation and inactivation kinetics. With the purpose of studying the effect of ω-toxins on $\alpha_3\beta_4$ or α_7-activated currents, protocols with brief pulses of DMPP (100 μM, 1 s) applied at 1 min intervals were selected; this interval was chosen in order to avoid nicotinic receptor desensitization.

After a few initial DMPP pulses, $\alpha_3\beta_4$ and α_7-mediated peak currents were quite reproducible over a 30 min period. The peak amplitude of the stabilized agonist-induced current, just preceding the addition of toxin, was used as control response (100%); then toxin was added 1 min before the next DMPP pulse. Figure 1 (b and c) show two examples of original traces of control currents obtained in two different oocytes expressing α_7 and $\alpha_3\beta_4$ nicotinic AChRs, and the inhibitory effects exerted by 1 μM ω-conotoxin MVIIC. Whereas α_7 nicotinic AChRs were unaffected, ω-conotoxin MVIIC blocked by 50% the $\alpha_3\beta_4$ current. This protocol was repeated but using increasing concentrations of either ω-conotoxin MVIIC or ω-conotoxin GVIA, in different oocytes expressing $\alpha_3\beta_4$ or α_7 nicotinic AChRs. Figure 2 (a and b) shows averaged results of the inhibition curve obtained using these two toxins. ω-Conotoxin MVIIC had little effect on α_7 current, whereas $\alpha_3\beta_4$ currents were very sensitive to blockade (IC_{50}, 1.3 μM). Thus, at a concentration as low as 0.3 μM, this toxin produced a significant inhibition of I_{DMPP} evoked by activation of $\alpha_3\beta_4$

nicotinic AChRs (20±2%; $P \leqslant 0.01$). Moreover, concentrations of ω-conotoxin MVIIC usually employed to block N/P/Q-type Ca^{2+} channels (1 and 3 μM), inhibited significantly ($P \leqslant 0.001$) by 44±3 and 55±5% respectively, the $\alpha_3\beta_4$ currents. At 10 μM, the α_7 current was inhibited by 17±6% ($P \leqslant 0.05$) whereas the $\alpha_3\beta_4$ current was blocked by 75±6% ($P \leqslant 0.001$). In all cases, the reversibility of blockade upon washout of the toxin was fast and complete (not shown). This contrasts with the long-lasting blockade exerted by this toxin on N/P/Q-type Ca^{2+} channels (Albillos *et al*., 1996; Gandía *et al*., 1997).

Figure 2b shows the effects of increasing concentrations of ω-conotoxin GVIA; at the concentration usually employed as Ca^{2+} channel blocker (1 μM), the toxin inhibited $\alpha_3\beta_4$ and α_7 currents by 24% ($P \leqslant 0.05$) and 20% ($P \leqslant 0.05$), respectively. This inhibition was fully reversible upon washout (not shown). Once again, this finding contrasts with the long-lasting blockade of N-type Ca^{2+} channels exerted by this toxin (Albillos *et al*., 1996; Gandía *et al*., 1997).

ω-Agatoxin IVA was unable to block significantly α_7 currents in spite of the use of 1 μM, a concentration higher than that considered selective to block P-type Ca^{2+} channels

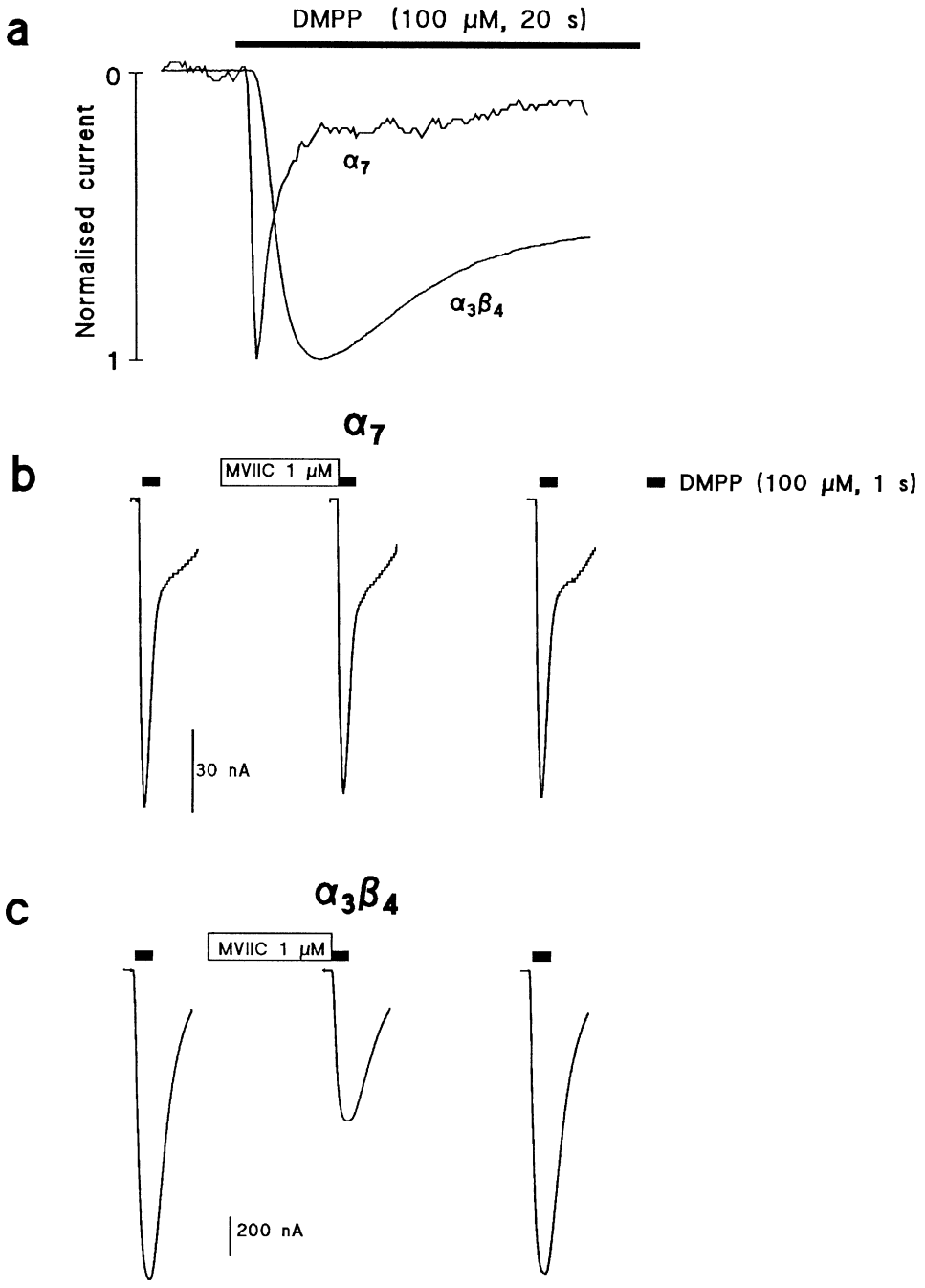

Figure 1 Effects of ω-conotoxin MVIIC on α_7 and $\alpha_3\beta_4$ nicotinic AChRs expressed in oocytes. Oocytes were voltage-clamped at a holding potential of −60 mV and stimulated with 100 μM DMPP. (a) Normalized currents induced by a prolonged DMPP pulse (20 s) applied to two different oocytes expressing α_7 or $\alpha_3\beta_4$ nicotinic AChRs. (b and c) show original traces of currents induced by brief pulses of DMPP (1 s), applied at 1 min intervals, to an oocyte expressing α_7 or $\alpha_3\beta_4$ receptors. Traces in each panel represent control stable currents after a few initial pulses, the current in the presence of ω-conotoxin MVIIC (1 μM) and the current upon washout of the toxin.

(low nanomolar range) and in the same range of that used to block Q-type channels (Figure 2c). Due to the expense of this toxin, it was not used at concentrations above 1 μM. At this concentration, ω-agatoxin IVA blocked the $\alpha_3\beta_4$ current by $15\pm4\%$ ($P\leqslant0.05$).

Inhibition by diltiazem, furnidipine and verapamil of $\alpha_3\beta_4$ or α_7 currents

The same experimental protocol described for ω-toxins was employed to assay the effects of diltiazem (a benzothiazepine derivative), furnidipine (a 1,4-dihydropyridine and verapamil (a phenylalkylamine) on nicotinic AChR currents. The α_7 current was the most resistant to blockade in all cases. Diltiazem was the most potent on $\alpha_3\beta_4$ nicotinic AChRs (IC_{50} 3 μM; Figure 2d). At the higher concentration tested (10 μM), diltiazem blocked by 75 ± 4 and $32\pm3\%$ ($P\leqslant0.001$) the I_{DMPP} induced by activation of $\alpha_3\beta_4$ and α_7 nicotinic AChRs, respectively. Figure 2d also shows the $\alpha_3\beta_4$ or α_7 current inhibition by furnidipine and verapamil at the highest concentration used (10 μM). At this concentration, the inhibition by furnidipine of $\alpha_3\beta_4$ and α_7 currents was $62\pm4\%$ ($P\leqslant0.001$) and $24\pm3\%$ ($P\leqslant0.05$), respectively; whereas verapamil reduced by $55\pm2\%$ ($P\leqslant0.001$) and $32\pm3\%$ ($P\leqslant0.01$) the currents elicited by the activation of these two receptors. At 3 μM, furnidipine inhibited significantly ($P\leqslant0.01$) $\alpha_3\beta_4$ currents by $39\pm4\%$ and α_7 currents by $21\pm3\%$; while verapamil reduced $\alpha_3\beta_4$ and α_7 currents by $32\pm3\%$ ($P\leqslant0.001$) and $26\pm2\%$ ($P\leqslant0.01$), respectively.

Nicotinic currents measured in oocytes clamped at a holding potential of -60 mV, were a combination of a direct Na^+ influx current through the nicotinic ionophore plus an indirect chloride efflux generated upon the activation of chloride channels by Ca^{2+} entry through the nicotinic pore; hence, the blockade of the current by peptide and non-peptide compounds in this study could be attributed to a direct effect on these chloride channels more than a nicotinic receptor inhibition. However, this did not seem to be the case since when chloride channels were directly recruited by photo-released Ca^{2+}, currents were not affected by any of the non-peptide (data not shown) and peptide blockers used in this study (Lomax *et al*., 1998). Furthermore, diltiazem inhibited in the same extent $\alpha_3\beta_4$ or α_7 currents in the presence of external Ca^{2+} (current experiments) or 1.8 mM Ba^{2+} (not shown). Additionally, it is well known that α_7 nicotinic AChRs are highly permeable to Ca^{2+} (Seguela *et al*., 1993), which imply a higher component of Ca^{2+}-activated chloride current in the α_7 mediated response. Since most of the compounds used in this study had little or no effect on the α_7 current, it seems unlikely that they were affecting the chloride current itself.

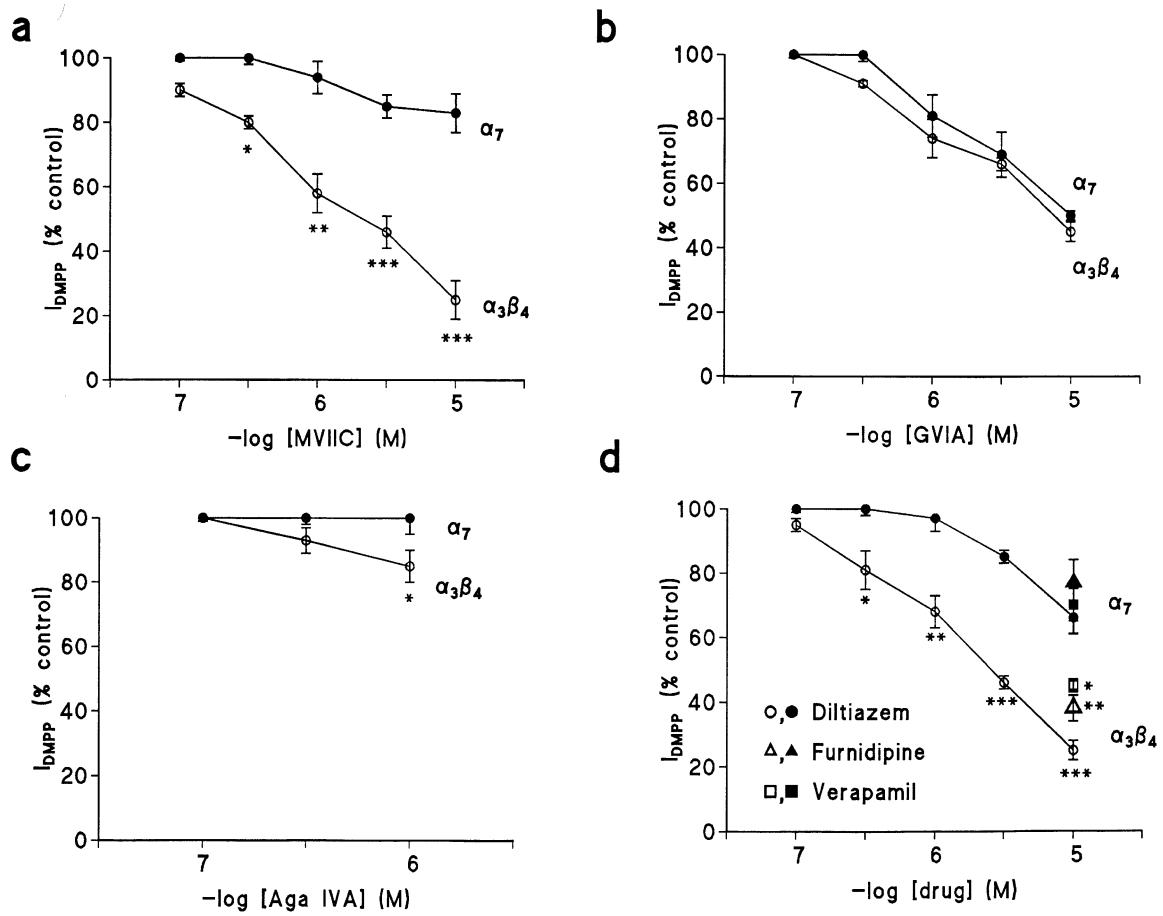

Figure 2 Concentration-response blockade of $\alpha_3\beta_4$ and α_7 nicotinic AChRs by different peptides and non-peptide Ca^{2+} channel antagonists. Currents (I_{DMPP}) were induced by DMPP pulses (100 μM, 1 s) applied at 1 min intervals. The peak amplitude of the stabilized I_{DMPP}, just preceding the addition of the drug, was used as control response (100%). (a, b, c and d) show the effects of increasing concentrations of ω-conotoxin MVIIC (MVIIC), ω-conotoxin GVIA (GVIA), ω-agatoxin IVA (Aga IVA) and diltiazem on the two receptor subtypes expressed. The effect of furnidipine and verapamil, assayed at 10 μM, on $\alpha_3\beta_4$ and α_7 nicotinic AChRs are also shown in (d). Data are means$\pm$s.e.mean of the currents obtained in pooled 5–10 oocytes. Effects of blockers were expressed as percentage of control I_{DMPP}. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ comparing $\alpha_3\beta_4$ with α_7 blockade.

Effect of ω-toxins and diltiazem on the kinetics of nicotinic receptor currents: relevance of the β-subunit

Diltiazem, the most potent blocker among all the organic Ca^{2+} channel antagonists assayed, was selected for further comparative studies with ω-toxins. To study current kinetics, oocytes expressing $\alpha_3\beta_4$ nicotinic AChRs were stimulated with DMPP for 20 s. To avoid receptor desensitization and current inactivation, the concentration of DMPP was reduced to 10 μM, and the pulses applied every 3 min. Under these experimental conditions, the control currents were quite reproducible and they exhibited practically no desensitization.

Figure 3 shows typical records of $\alpha_3\beta_4$ control currents, and their inhibition by ω-conotoxin MVIIC (1 μM), ω-conotoxin GVIA (1 μM), ω-agatoxin IVA (1 μM) and diltiazem (3 μM), added 1 min before and during the DMPP pulse. Whereas the three toxins blocked I_{DMPP} without affecting the kinetics of the current (a–c), diltiazem promoted a clear current inactivation (see different blockade of peak and late I_{DMPP} in Figure 3d). This inactivating effect exerted by diltiazem did not occur in oocytes expressing $\alpha_3\beta_2$ receptors. The blocking effects of diltiazem and ω-conotoxin MVIIC on $\alpha_3\beta_2$ currents are shown in Figure 4. Note the specially marked blockade induced by the toxin on this receptor subtype. Because desensitization of control $\alpha_3\beta_2$ currents was higher than that obtained with $\alpha_3\beta_4$ receptors, a lower concentration of DMPP (3 μM) was employed for $\alpha_3\beta_2$ experiments.

Quantitative averaged blockade of peak and late I_{DMPP} upon the activation of $\alpha_3\beta_4$ and $\alpha_3\beta_2$ nicotinic AChRs exerted by diltiazem and ω-conotoxin MVIIC are plotted in Figure 5. Diltiazem blocked significantly more the late I_{DMPP} than the peak $\alpha_3\beta_4$ currents (63 *versus* 43%). However, a similar degree of blockade of peak and late I_{DMPP} by diltiazem, in oocytes expressing $\alpha_3\beta_2$ nicotinic AChRs, was observed (34 *versus* 33%). Note the significant higher blockade by diltiazem of late I_{DMPP} in oocytes expressing $\alpha_3\beta_4$ receptors compared with those

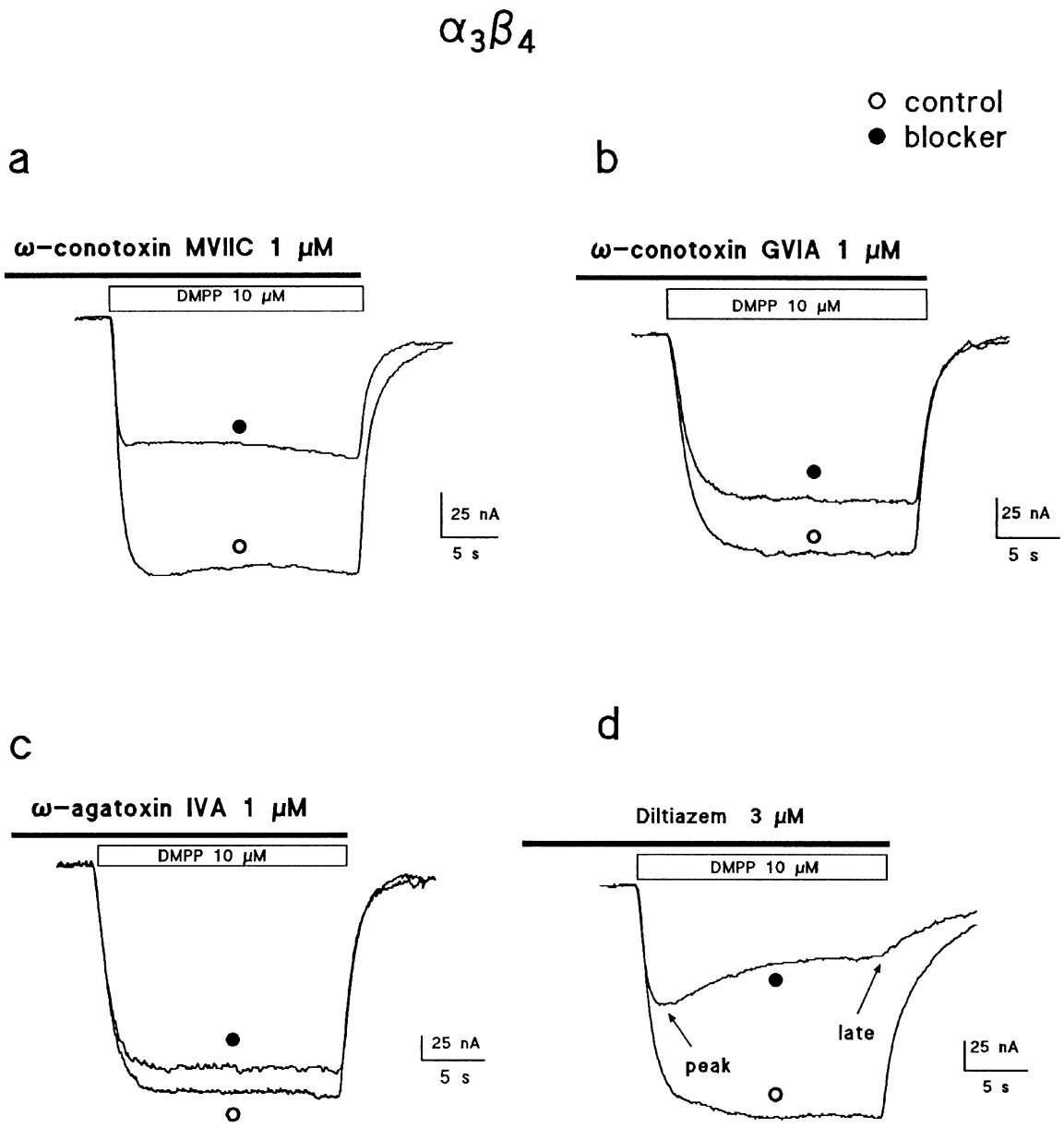

Figure 3 Effects of ω-toxins and diltiazem on the kinetics of $\alpha_3\beta_4$ currents induced by long DMPP pulses. Currents (I_{DMPP}) were elicited by pulses of DMPP (10 μM, 20 s) applied every 3 min in oocytes expressing $\alpha_3\beta_4$ nicotinic AChRs. Blockers were added 1 min before and during the DMPP pulse. Original records of the currents obtained in four different oocytes expressing $\alpha_3\beta_4$ receptors are shown in (a–d). Each panel represents a typical record of the current induced by DMPP in the absence or the presence of blockers. Peak and late I_{DMPP} were measured where indicated by the arrows in (d).

expressing $\alpha_3\beta_2$ receptors. ω-Conotoxin MVIIC, at a concentration of 1 μM, blocked to a similar extent the peak and late I_{DMPP} in oocytes expressing $\alpha_3\beta_4$ and $\alpha_3\beta_2$ nicotinic AChRs; however, this time the β_2 subunit conferred the receptor a higher sensitivity to the toxin (64±3% blockade of peak I_{DMPP} in oocytes expressing $\alpha_3\beta_2$ nicotinic AChRs *versus* 47±3% inhibition of peak I_{DMPP} in oocytes expressing $\alpha_3\beta_4$ receptors; $P \leqslant 0.01$).

Time-course of $\alpha_3\beta_4$ current blockade and recovery induced by ω-conotoxin MVIIC, ω-conotoxin GVIA and diltiazem

These experiments were designed to study the rates of blockade and recovery of nicotinic $\alpha_3\beta_4$ currents following the application of ω-toxins and diltiazem. Two different experimental protocols were used. In the first, two DMPP pulses (10 μM, 80 s), 5 min apart, were applied to the same oocyte. This concentration and time interval were selected because currents induced in this way were reproducible (not shown) and do not present run-down upon successive pulses. After a first DMPP pulse, in which a stable current response was obtained in most of the oocytes tested, a second DMPP pulse was applied 5 min later. During this second pulse, when current stabilized (20 s after starting DMPP stimulation), ω-conotoxin MVIIC (1 μM), ω-conotoxin GVIA (3 μM) or diltiazem (3 μM) were added along with DMPP. Typical examples of this protocol are illustrated in Figure 6 (a–c). ω-Conotoxin MVIIC produced an inhibition of the current following a time-course curve that could be fitted to a single exponential with a τ_{on} of 3±1 s ($n=6$); blockade of current amounted to 40–50%. The same was observed with ω-conotoxin GVIA assayed at a higher concentration (3 μM) to observe better the blockade; this blockade also fitted to a single exponential with a τ_{on} of 1.9±0.1 s ($n=4$). Diltiazem (3 μM) inhibited the $\alpha_3\beta_4$ current, although with a slower time-course than ω-toxins (τ_{on}, 10±1 s; $n=4$; $P \leqslant 0.05$). Differences in the time required to remove the blockade were also found between toxins and diltiazem using a second experimental protocol. Now, oocytes were stimulated with two DMPP pulses (80 s), 5 min apart; toxins or diltiazem were superfused during the first 40 s of the second DMPP pulse, and then the blockers

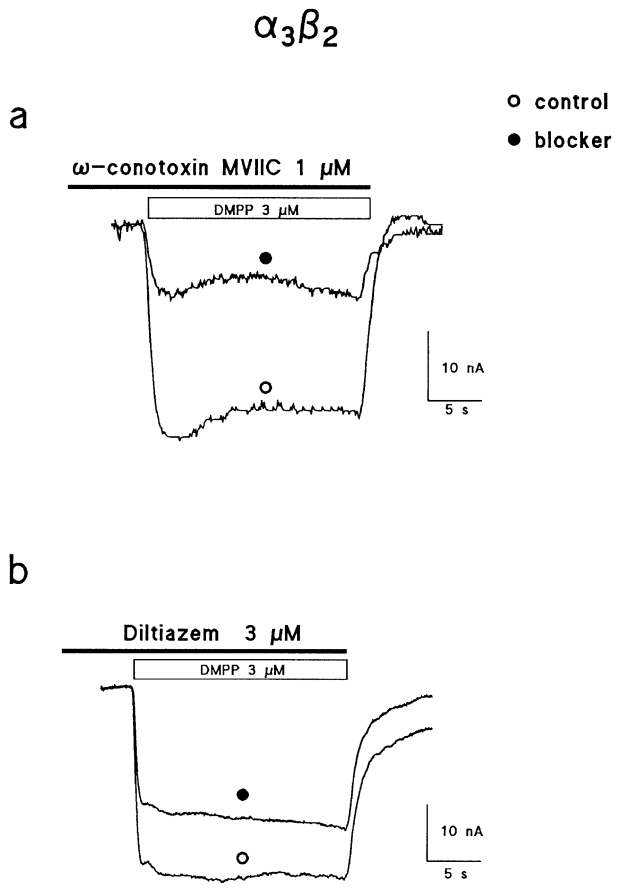

Figure 4 Effects of ω-conotoxin MVIIC and diltiazem on the kinetics of $\alpha_3\beta_2$ currents induced by long DMPP pulses. Currents (I_{DMPP}) were induced by 3 μM DMPP applied during 20 s every 3 min. Blockers were added 1 min before and during the DMPP pulse. Original records of the currents obtained in two different oocytes expressing $\alpha_3\beta_2$ nicotinic AChRs are shown in (a and b). Each panel represents a typical record of the current induced by DMPP in the absence or the presence of blockers.

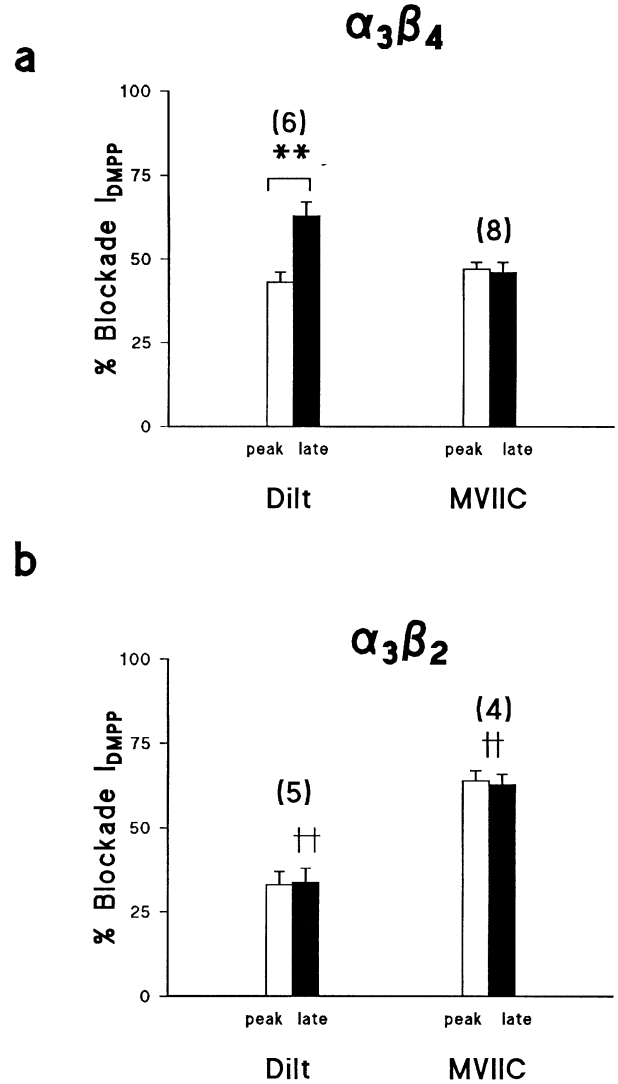

Figure 5 Blockade by ω-conotoxin MVIIC and diltiazem of peak and late DMPP currents in oocytes expressing $\alpha_3\beta_4$ or $\alpha_3\beta_2$ nicotinic AChRs. Protocols and concentrations were similar to those described in Figures 3 and 4. Data were expressed as percentage of blockade of control peak and late I_{DMPP} (100%), in the absence of the drug. Values represent means±s.e.mean of the results obtained in different oocytes expressing $\alpha_3\beta_4$ (a) or $\alpha_3\beta_2$ nicotinic AChRs (b). In parentheses, number of oocytes tested for each blocker and receptor subtype. $^{**}P \leqslant 0.01$ comparing blockade by diltiazem of peak and late $\alpha_3\beta_4$ current. $\dagger\dagger P \leqslant 0.01$ comparing blockade by diltiazem of late $\alpha_3\beta_2$ current *versus* late $\alpha_3\beta_4$ current; and the inhibition by ω-conotoxin MVIIC of $\alpha_3\beta_2$ current *versus* $\alpha_3\beta_4$ current.

were removed. Typical records corresponding to ω-conotoxin MVIIC, ω-conotoxin GVIA or diltiazem washouts are shown in Figure 6 (d–f). After removing ω-conotoxin MVIIC or ω-conotoxin GVIA, I_{DMPP} blockade recovered faster (τ_{off}, 1.6±0.2 and 2.4±0.3 s, respectively; $n=4$) than diltiazem (τ_{off} 9.3±0.9 s; $n=4$; $P\leqslant 0.01$).

Voltage-dependence and use-dependence of the blockade of $\alpha_3\beta_4$ currents exerted by ω-toxins and diltiazem

The possible voltage-dependence of the blocking effects of ω-conotoxin MVIIC, ω-conotoxin GVIA and diltiazem on $\alpha_3\beta_4$ currents were explored at different holding potentials (from

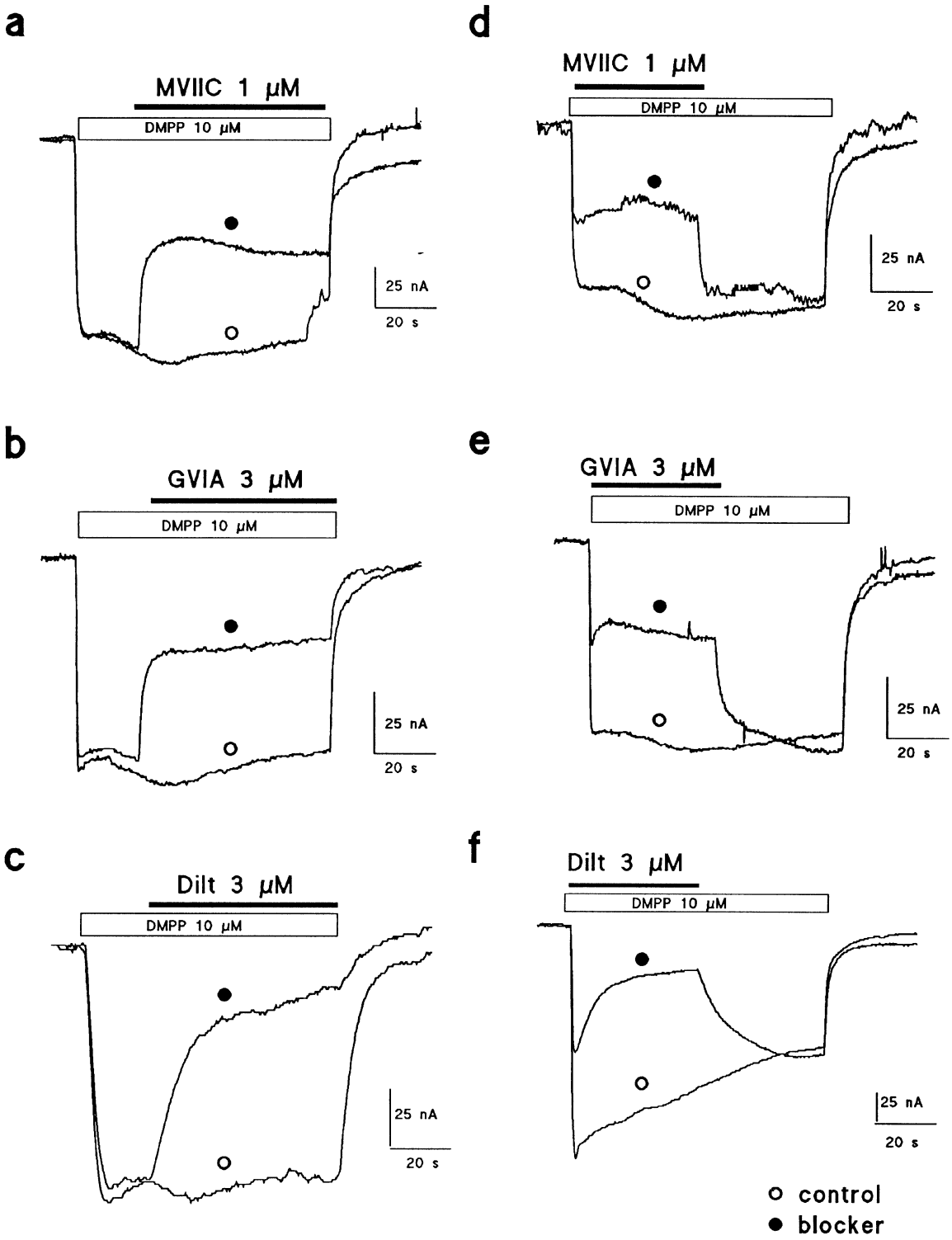

Figure 6 Onset and offset of blockade produced by ω-conotoxin MVIIC, ω-conotoxin GVIA or diltiazem on $\alpha_3\beta_4$ currents. (a, b and c) show original records of control currents obtained in three different oocytes (each out of 4–6) stimulated with 10 μM of DMPP during 80 s. Five min later, the DMPP stimulation was repeated in the same oocyte but adding, 20 s after starting the pulse, the blocker along with DMPP. (d, e and f) show the control currents evoked by a pulse of DMPP (10 μM, 80 s) in another three different oocytes (each out of four), and 5 min later the currents induced by a new DMPP pulse, but this time adding simultaneously, during the first 40 s of the pulse, ω-conotoxin MVIIC, ω-conotoxin GVIA or diltiazem. Blockers were washed out just in the middle of the DMPP pulse.

−100 to +20 mV), using 20 mV steps. At each holding potential, DMPP current (I_{DMPP}) was activated by two pulses of DMPP (100 μM 1 s, 1 min apart). When all potentials were tested, the protocol was repeated in the same oocyte, but this time in the presence of ω-conotoxin MVIIC (1 μM), ω-conotoxin GVIA (3 μM) or diltiazem (3 μM); blockers were

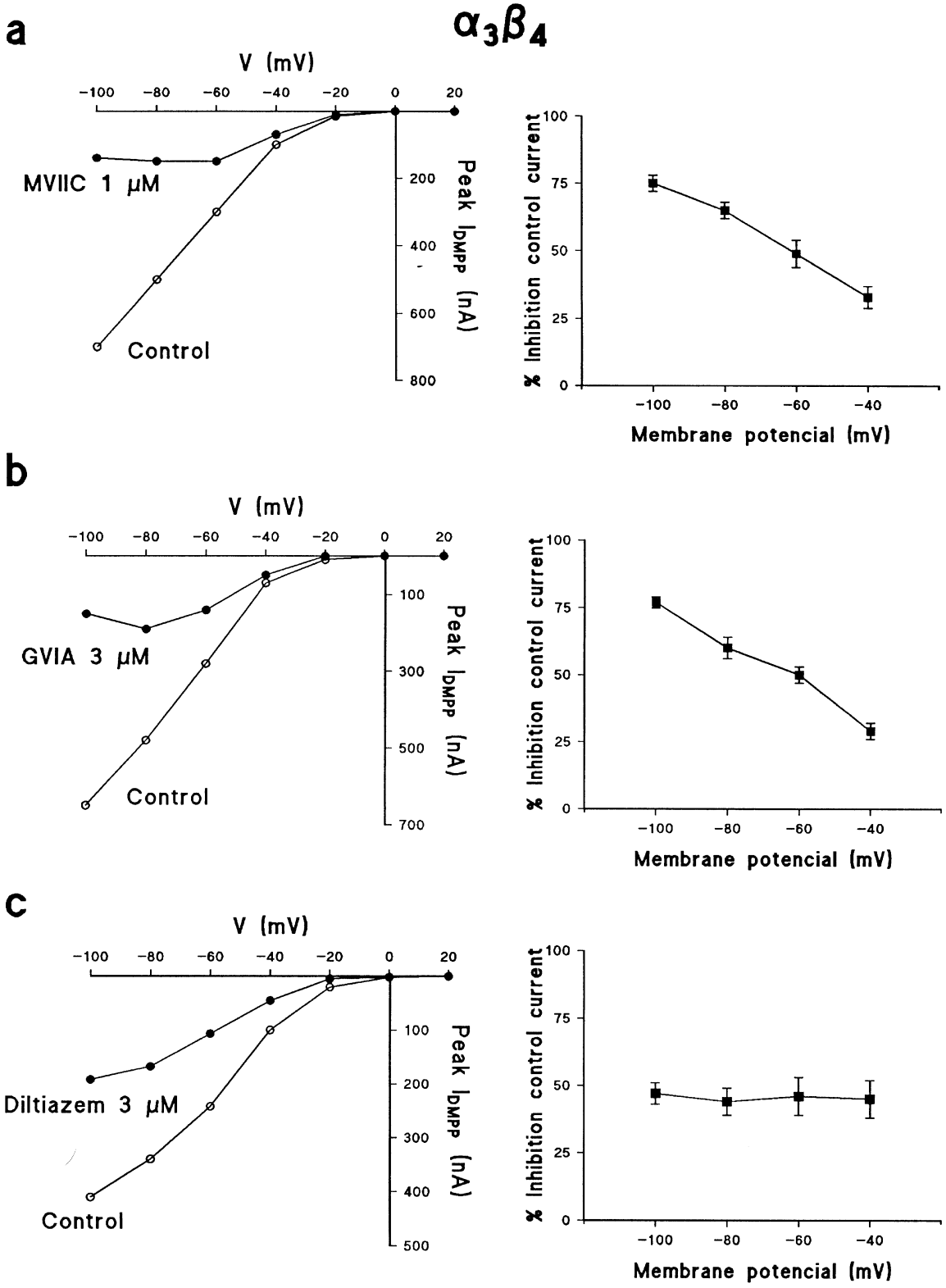

Figure 7 Effects of membrane potential on the blockade of I_{DMPP} elicited by ω-conotoxin MVIIC (MVIIC), ω-conotoxin GVIA (GVIA) and diltiazem in $\alpha_3\beta_4$-injected oocytes. Oocytes were clamped at different holding potentials (from −100 to +20 mV) using 20 mV steps. At each holding potential, I_{DMPP} was activated by two pulses of DMPP (100 μM, 1 s), 1 min apart. The whole experimental protocol was repeated in the same oocyte, but this time in the presence of ω-conotoxin MVIIC, ω-conotoxin GVIA or diltiazem. Blockers were present 1 min before the DMPP pulse and throughout all the experiment, including the DMPP pulse. Ordinates in left panels show Peak I_{DMPP} induced by DMPP pulses at different holding potentials in the absence or the presence of blocker; data of each figure were obtained from a typical oocyte. Right panels show the mean ± s.e.mean inhibition values obtained from four different oocytes tested for each blocker (data were expressed as percentage of control current inhibition).

present throughout the experiment, including the minute before and during the DMPP pulse. Figure 7 shows that whereas the blockade by diltiazem did not exhibit voltage-dependence (Figure 7c), the inhibition by ω-toxins was higher at hyperpolarizing potentials. For instance, at −100 mV ω-conotoxin MVIIC blocked I_{DMPP} by 74±2%, while at

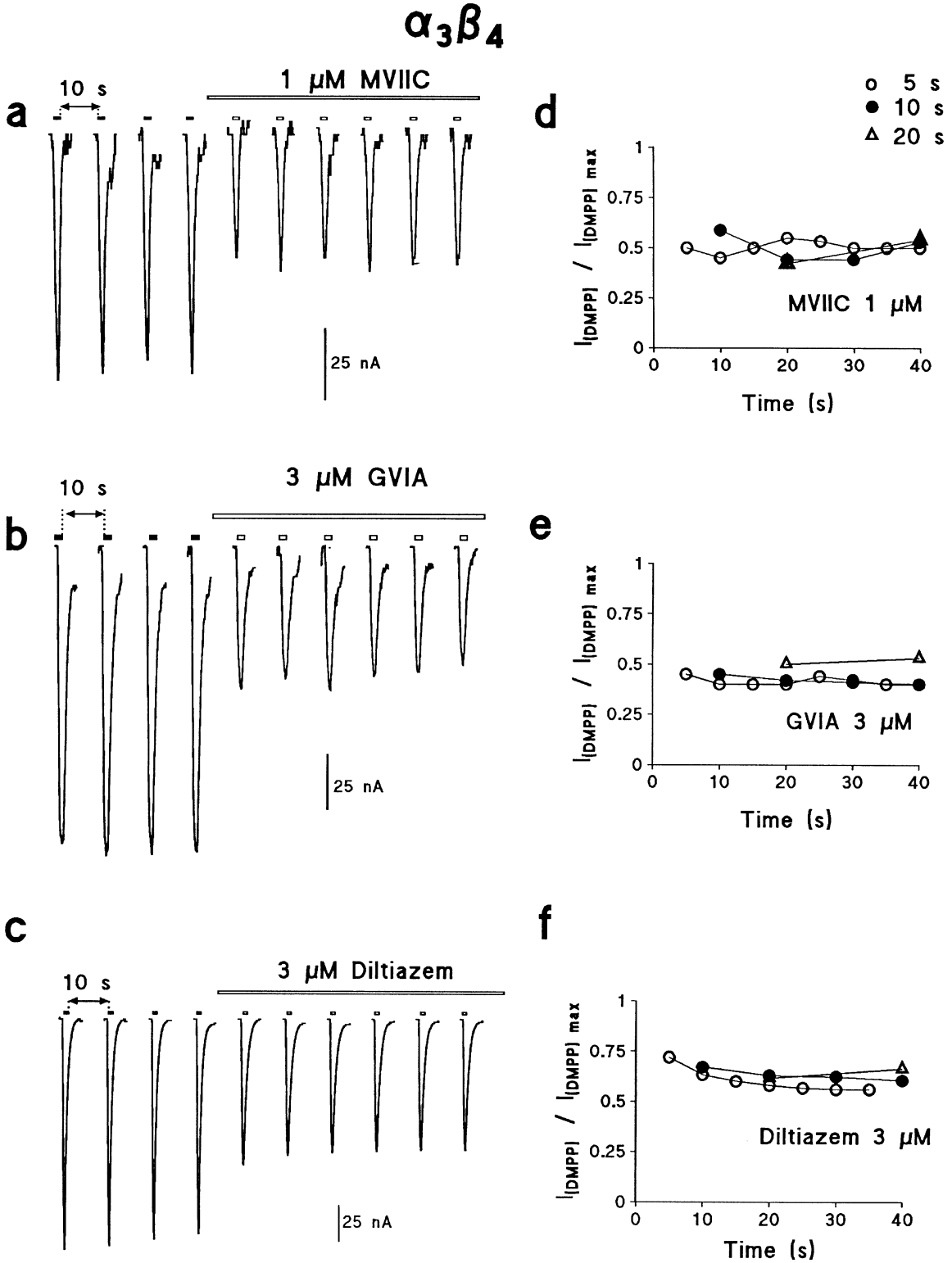

Figure 8 Blocking effects of ω-conotoxin MVIIC, ω-conotoxin GVIA and diltiazem on $\alpha_3\beta_4$ currents induced by DMPP pulses applied at different frequencies. Oocytes expressing $\alpha_3\beta_4$ nicotinic AChRs were stimulated with DMPP pulses (10 μM, 500 ms) applied every 20 s. After a few initial pulses, when the current stabilized ($I_{DMPPmax}$), the blockers were added before and during the successive DMPP pulses. After washout of the blocker, new DMPP pulses were applied to the same oocyte, but this time at 10 s intervals. Once again, currents were measured in the absence or presence of toxins or diltiazem. At the end, upon washout, the protocol was repeated in the same oocyte, but at a higher frequency of agonist stimulation (5 s). Left panels in the figure (a–c) show typical records of the currents obtained in three different oocytes stimulated with DMPP, at a frequency of 10 s, in the absence or presence of blockers. On the right part (d–f) the ratios of I_{DMPP} in the presence of blockers *versus* control $I_{DMPPmax}$ in three oocytes stimulated with DMPP pulses at different frequency.

−40 mV blockade was significantly lower ($P \leqslant 0.001$) and amounted to 37±3% ($n=4$). Similar difference in the blockade ($P \leqslant 0.001$) were obtained in the case of ω-conotoxin GVIA, 77±4 and 30±2% blockade ($n=4$) at −100 mV and −40 mV, respectively (Figure 7, right parts of a and b).

To address the question of whether toxins and diltiazem produce a use-dependent receptor blockade, the effects of these compounds on successive DMPP pulses, applied at different frequencies, were assayed. Oocytes expressing $\alpha_3\beta_4$ nicotinic AChRs were stimulated with DMPP pulses (10 μM, 500 ms) every 20 s. Once a stable current was reached ($I_{DMPPmax}$), ω-conotoxin MVIIC (1 μM), ω-conotoxin GVIA (3 μM), or diltiazem (3 μM) were superfused during the next 5–6 DMPP pulses. After washing out the blocker and a complete recovery of current was obtained, the protocol was repeated in the same oocyte, but using 10 s intervals between DMPP pulses (up to ten pulses). Once more, upon washout of the blockers, a third stimulation train with 15 successive DMPP pulses, at 5 s

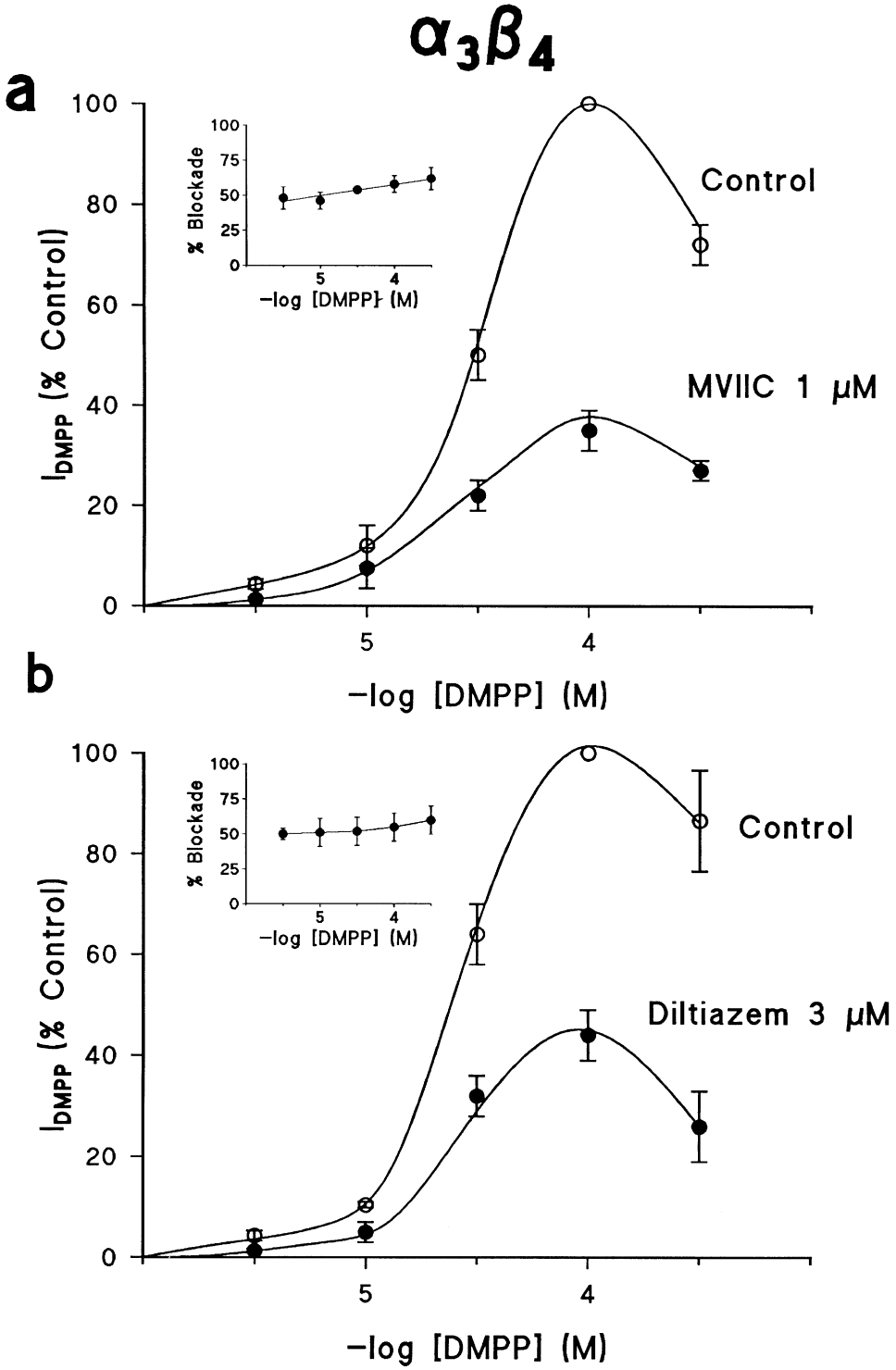

Figure 9 Non-competitive effect of ω-conotoxin MVIIC and diltiazem on $\alpha_3\beta_4$ nicotinic currents. Currents were evoked by successive pulses (1 s) of increasing concentrations of DMPP, applied every 1 min, in the absence or the presence of blockers. Values are means±s.e.mean of the results obtained in six oocytes (a) and five oocytes (b) expressing $\alpha_3\beta_4$ nicotinic AChRs. Inserts the blockade of control current by ω-conotoxin MVIIC and diltiazem (expressed as percentage) *versus* DMPP concentration.

intervals, was applied in the absence or presence of the blocker. Figure 8 shows the results obtained from a typical oocyte (out of three) for each blocker Figure 8a–c show the experimental protocol for ω-conotoxin MVIIC, ω-conotoxin GVIA, or diltiazem, using 10 s intervals between DMPP pulses. Figure 8 (d–f) summarizes the $I_{DMPP}/I_{DMPPmax}$ results obtained with the blockers during the first 40 s, using the three frequencies. Both ω-toxins and diltiazem inhibited the current equally well, and in a single step. No statistical differences in the blockade exerted by each of the compounds, at the three frequencies studied, were found. Therefore, $I_{DMPP}/I_{DMPPmax}$ values obtained during the last DMPP pulse, for the three frequencies assayed (5, 10 and 20 s) were respectively, 0.45 ± 0.04, 0.48 ± 0.05 and 0.46 ± 0.05 with ω-conotoxin MVIIC; 0.38 ± 0.02, 0.39 ± 0.03 and 0.44 ± 0.04 with ω-conotoxin GVIA; and 0.56 ± 0.05, 0.60 ± 0.04 and 0.59 ± 0.04 with diltiazem.

Blockade by ω-conotoxin MVIIC and diltiazem of $\alpha_3\beta_4$ currents generated by increasing DMPP concentrations

To know more about the nature of blockers interaction with the neuronal nicotinic AChR, attempts were made to define whether the blockade of I_{DMPP} was competitive or non-competitive. Oocytes expressing $\alpha_3\beta_4$ receptors were stimulated with increasing concentrations of DMPP applied as brief pulses (1 s) every 1 min, in the absence and later on, in the presence of ω-conotoxin MVIIC (1 μM) or diltiazem (3 μM). Figure 9 shows that a maximal current amplitude was evoked by 100 μM of DMPP; higher concentrations of the nicotinic agonist produced an important desensitization of the receptor. The inhibition produced by ω-conotoxin MVIIC or diltiazem could not be overcome by increasing concentrations of DMPP as it is clearly shown in the two inserts of the figure. The calculated EC_{50} values for DMPP in the absence or presence of ω-conotoxin MVIIC were 17 and 12 μM respectively; and 26 and 17 μM in the absence or the presence of diltiazem. All these results suggest a non-competitive mechanism of action between both blockers and the nicotinic agonist for the receptor.

Discussion

The results of this study are relevant in two aspects: (i) an emerging pharmacology of nicotinic AChR subtypes; (ii) the limited selectivity of agents available to block L-, N- or P/Q-subtypes of Ca^{2+} channels. We report, for the first time, that ω-conotoxin MVIIC is a selective blocker of heteromeric $\alpha_3\beta_2$ and $\alpha_3\beta_4$ nicotinic AChRs, but did not recognize homomeric α_7 receptors. This finding contrasts with the fact that α_7 currents are highly sensitive to other toxins such as α-conotoxin ImI, methyllycaconitine or α-bungarotoxin (López *et al*., 1998). Another interesting finding is the scarce activity of diltiazem and other non-peptide Ca^{2+} channel antagonists on α_7 receptors in contrast to their blocking effects on $\alpha_3\beta_4$ nicotinic AChRs.

Blockade of neuronal nicotinic AChR by ω-conotoxins and non-peptide compounds described above does not seem to be the result of a non-specific indiscriminate interaction with ion-activated or ligand-gated ion channels. Thus, ω-toxins do not inhibit the Ca^{2+}-activated chloride channels in oocytes (Lomax *et al*., 1998) and present results show that both organic and peptide molecules affected little if at all, α_7 nicotinic AChRs. Moreover, this study point out that these compounds were capable of discriminating between nicotinic AChRs containing β_2 or β_4 subunits; i.e. ω-conotoxin MVIIC blocked more $\alpha_3\beta_2$ than $\alpha_3\beta_4$ receptors whereas diltiazem promoted the inactivation of I_{DMPP} elicited by the activation of $\alpha_3\beta_4$ but not by $\alpha_3\beta_2$ receptors. These findings agree with the recent view that β subunits are involved in determining the physical structure and the pharmacological and kinetic properties of nicotinic AChRs (Duvoisin *et al*., 1989; Cachelin & Jaggi, 1991; Luetje & Patrick, 1991, Papke & Heinemann, 1991; Harvey & Luetje, 1996).

Our data shows that we are dealing with selective blocking effects of peptide and non-peptide molecules, traditionally considered as Ca^{2+} channel antagonists, on nicotinic AChRs. Also we have observed, at least, three pronounced differences between non-peptide drugs and ω-conotoxins regarding their mechanism of nicotinic blockade. Firstly, the onset and offset of $\alpha_3\beta_4$ current blockade are quite different. So, ω-conotoxins MVIIC and GVIA had a τ_{on} for blockade of only 2–3 s, while that of diltiazem was 10 s. Also, the τ_{off} for reversal of blockade exhibited a similar pattern. These effects might simply reflect the different degrees of hydrophylicity of Ca^{2+} channels ω-toxins blockers (water soluble, polar compounds) and diltiazem (a lipophilic molecule); but it is also plausible that more selective mechanisms (i.e. different dissociation equilibrium constants, K_D, to specific receptor sites) might also contribute to the observed differences. Secondly, another interesting difference is the promotion of $\alpha_3\beta_4$ current inactivation by diltiazem, but not by ω-conotoxins. This keeps pace with previous observations that organic Ca^{2+} antagonist molecules directly interact with the muscle nicotinic receptor channel to enhance its autodesensitization (Chang *et al*., 1990). Thirdly, we have observed differences in the voltage-dependence of blockade between ω-conotoxins and diltiazem. Thus, whereas diltiazem blocked in a similar extent $\alpha_3\beta_4$ currents at all membrane potentials, ω-conotoxins exerted stronger inhibition of the current at hyperpolarized potentials. This finding, along with the non-competitive blockade, would indicate that ω-conotoxins behave as open-channel blockers of $\alpha_3\beta_4$ nicotinic AChRs. However, the lack of use-dependent blockade does not agree with such mechanism (Buisson & Bertrand, 1998). Our results suggest that ω-conotoxins and diltiazem should bind to a different receptor site; in the case of toxins such binding-site should be in a receptor region located deeply enough in the membrane to detect the changes of potential.

The findings of this study have clinical and methodological implications. From the clinical point of view, concerning the wide use of L-type Ca^{2+} channel blockers in the therapy of cardiovascular diseases, it might very well be that, at therapeutic doses, when plasma levels around 0.5–1 μM could be reached (Yeung *et al*., 1996), a mild blockade of nicotinic receptors in adrenal medulla and in sympathetic ganglia (where nicotinic AChRs containing α_3 and β_2 subunits are present) might mitigate the surge of catecholamines to the circulation during stressful conflicts. Interestingly, the methodological implications of this study are even more relevant since the action of these compounds on nicotinic AChRs might obscure the conclusions related to the involvement of certain Ca^{2+} channel subtypes in the regulation of various central and peripheral functions. This is the case for the physiologically mediated catecholamine release response to ACh stimulation of adrenal chromaffin cells. We have recently demonstrated that nicotinic receptors containing $\alpha_3\beta_4$ or α_7 subunits participate in the ACh-mediated catecholamine release responses in chromaffin cells (López *et al*., 1998). Therefore, in trying to determine the Ca^{2+} channel subtypes that control the ACh-evoked catecholamine release, a judicious use of Ca^{2+} channel blockers and activators should be made.

From the results of the present study it seems that ω-agatoxin IVA can be safely used to irreversibly block the P/Q-type Ca^{2+} channels (Olivera *et al*., 1994; García *et al*., 1996), without much interference with nicotinic AChR functions. This is not the case for ω-conotoxin MVIIC, that caused a pronounced blockade of $\alpha_3\beta_2$ and $\alpha_3\beta_4$ currents at concentrations currently used to block the N- and P/Q-type channels. However, while blockade of nicotinic AChR currents was fully reversible in only a few seconds after toxin washout, blockade by this toxin of P/Q-type Ca^{2+} channels in chromaffin cells and in neurons is long-lasting (Albillos *et al*., 1996; Gandía *et al*., 1997; Lara *et al*., 1998; McDonough *et al*., 1996). Thus, a selective stable blockade of non- L-type Ca^{2+} channels can be achieved by preincubation of the cells with ω-conotoxin MVIIC, followed by a few minutes washout; under these conditions the nicotinic AChRs are unlikely to be affected. Although to a smaller extent, caution should also be taken with ω-conotoxin GVIA when using it to block N-type Ca^{2+} channels; $\alpha_3\beta_4$ and α_7 nicotinic AChRs were moderately affected by this toxin at concentrations currently employed for Ca^{2+} channel blockade. Once again, differences in the duration of blockade of neuronal Ca^{2+} channels (Olivera *et al*., 1994; Kasai *et al*., 1987) and nicotinic AChRs (present study) by ω-conotoxin GVIA, might discriminate between both potential toxin targets. Concerning L-channels, it seems that all non-peptide blockers will affect to some extent the nicotinic AChRs, particularly diltiazem. It could be that peptide blockers of L-type channels recently available (i.e. calcicludine, calciseptine; Schweitz *et al*., 1994; De Weille *et al*., 1991) might preserve the functionality of nicotinic AChRs and, hence, can be more adequate tools than organic molecules. This hypothesis needs experimental testing.

In conclusion, our data calls for caution when interpreting data related with the regulation of various central and peripheral functions in neurons and other cell types, where nicotinic receptor and Ca^{2+} channels coexist.

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