Zinc permeates mouse muscle ACh receptor channels expressed in BOSC 23 cells and affects channel function

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(Received 5 May 2000; accepted after revision 8 August 2000)

1. The influx of Zn^{2+} through the channels of fetal and adult mouse muscle nicotinic acetylcholine receptors (γ- and ϵ-AChRs) and its effects on receptor function were studied in transiently transfected human BOSC 23 cells, by combining patch-clamp recordings with digital fluorescence microscopy.

2. ACh-induced whole-cell currents were reversibly reduced by external $ZnCl_2$, with half-maximal inhibitory concentrations of 3 and 1 mM for γ- and ϵ-AChRs, respectively.

3. Both γ- and ϵ-AChR channels were permeable to Zn^{2+}, as shown by fluorescence measurements using Zn^{2+}-sensitive dyes. The fractional current carried by Zn^{2+} ($P_{f,Zn}$; 0.5 mM Zn^{2+} in Ca^{2+}- and Mg^{2+}-free medium) through γ- and ϵ-AChR channels was 1.7 and 4 %, respectively.

4. $P_{f,Zn}$ increased with the concentration of $ZnCl_2$, but was little affected by physiological concentrations of Ca^{2+} and Mg^{2+} in the external medium.

5. The conductance of ACh-evoked unitary events, measured by cell-attached or outside-out recordings, decreased when the patched membrane was exposed to $ZnCl_2$ (1 or 3 mM). Simultaneous application of ACh and Zn^{2+} to the extra-patch membrane lengthened channel open duration (τ_{op}) by 50%. No obvious increment of τ_{op} was observed following exposure of inside-out patches to Zn^{2+}.

6. The possible physiological relevance of zinc-induced modulation of AChR channels is discussed.

Considerable quantities of Zn^{2+} are present in mammalian nerve cells, about 90% of it bound to zinc-fingers of regulatory proteins (Huang, 1997), the remainder stored in synaptic vesicles and released during synaptic activity, reaching extracellular concentrations estimated to be in the range of hundreds of micromolar at the synapses (Assaf & Chung, 1984). At these concentrations, Zn^{2+} influences transmitter release and modulates post-synaptic receptors for glutamate, GABA and glycine (as reviewed by Huang, 1997; Choi & Koh, 1998), with possible consequences for synaptic transmission. Outside the physiological range, both the excess and deficiency of Zn^{2+} affect cellular metabolic processes, inducing neurotoxic symptoms (Choi & Koh, 1998). Intracellular Zn^{2+} excess, which may result in cell death, can be attained during intense synaptic activity, when Zn^{2+} enters postsynaptic cells through a variety of pathways, such as voltage-activated Ca^{2+} channels, Zn^{2+}-permeable receptors or the Na^+–Ca^{2+} exchanger (Koh & Choi, 1994; Yin & Weiss, 1995; Yu & Choi, 1997; Sensi *et al.* 1997).

At the neuromuscular junction, Zn^{2+} inhibits the spontaneous transmitter release in the presence of Ca^{2+}, while transiently increasing the spontaneous transmitter release in the absence of Ca^{2+} (Benoit & Mambrini, 1970; Nishimura, 1987; Wang & Quastel, 1990). Frog endplate AChRs are permeable to Zn^{2+}, as estimated from reversal potential measurements (Adams *et al.* 1980). Thus, during synaptic activity, Zn^{2+} may enter into muscle cells, possibly modulating AChR function. However, it is not known whether muscle AChRs are directly modulated by Zn^{2+}. The aim of the present study was to measure the Zn^{2+} permeability of muscle AChR channels, by combining electrophysiological and fluorescence techniques, and to investigate the influence of Zn^{2+} on AChR function.

METHODS

Expression of AChR subunits in BOSC 23 cells

Full-length cDNAs encoding α (P04756), β (P09690), γ (P04760), ϵ (P20782) and δ (P02716) subunits of mouse muscle nicotinic AChR in the SV40-based pSM expression vector were kindly provided by Dr J. Patrick (Baylor College of Medicine, Houston, TX, USA). The cDNAs coding for the α, β, γ and δ (γ-AChR) or the α, β, ϵ and δ (ϵ-AChR) subunits (0.2 μg each per 35 mm dish) were transiently transfected

into the human cell line BOSC 23 using a Ca^{2+} phosphate method, as previously described (Fucile *et al.* 1996). Cells were washed twice 8–12 h after the start of transfection and used for experiments 36–48 h after transfection. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM; Gibco, USA), supplemented with 10% calf serum (Hyclone, USA).

Solutions

The standard external solution had the following composition (mM): NaCl 140, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 2, Hepes-NaOH 10 and glucose 10, pH 7.3. Fluorescence determination of Zn^{2+} influx through AChRs was performed using an external solution containing (mM): NaCl 140, KCl 2.5, Hepes-NaOH 10 and glucose 10, pH 7.3, plus various concentrations of Zn^{2+}. Patch pipettes were filled with internal solution containing (mM): CsCl 150 and Hepes-CsOH 10, pH 7.3, for combined fluorescence and whole-cell current measurements; or CsCl 140, $MgCl_2$ 2, Hepes-CsOH 10, EGTA 0.5 and Na-ATP 4, pH 7.3, for whole-cell recordings. For calibration of fluorescence measurements, intra- and extracellular solutions were prepared by adding 50 μM Mg^{2+}-Green or 2 mM $ZnCl_2$, respectively, to a solution composed of (mM): *N*-methyl-D-glucamine (NMGA) 140 and Hepes-HCl 10, pH 7.3. For outside-out recordings, patch pipettes were filled with the following solution (mM): KCl 140, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 11, Mg-ATP 2 and Hepes-KOH 10, pH 7.3. Inside-out patches were bathed with the following solution (mM): KCl 155, $MgCl_2$ 1 and Hepes-KOH 10, pH 7.3, supplemented with 0.2 mM $ZnCl_2$ where indicated. Mg^{2+}-Green and Newport Green were purchased from Molecular Probes, TPEN from Sigma and $ZnCl_2$ from Fluka or Sigma. All other chemicals were of analytical grade.

Whole-cell recordings

Whole-cell recordings of ACh- and nicotine-induced currents were performed at room temperature (24–26°C) using borosilicate glass patch pipettes (2–4 MΩ tip resistance) connected to an Axopatch 200A amplifier (Axon Instruments). The series resistance, estimated from slow transient cancellation, was compensated by 80–90%. Currents were digitized at 500 Hz and analysed with pCLAMP programs (pCLAMP 6, Axon Instruments). Unless otherwise indicated, recordings were performed at a membrane holding potential of −50 mV. Agonists were applied by a gravity-driven perfusion system (for details see Ragozzino *et al.* 1998). The concentration of Zn^{2+} yielding half-maximal inhibition (x_0) was obtained by non-linear fitting of the data to eqn (1):

$$\frac{I}{I_{max}} = \frac{1-K}{1+([Zn^{2+}]/x_0)^{n_H}} + K, \quad (1)$$

where $[Zn^{2+}]$ represents the concentration of Zn^{2+}, n_H is the Hill coefficient, I_{max} is the maximum current response and K is a constant that takes into account the residual, non-blocked current observed at high concentrations of Zn^{2+}.

Single-channel recordings

Single-channel currents were recorded at room temperature (23–26°C) in the cell-attached, outside-out or inside-out configuration using a low-noise Axopatch 200B amplifier (Axon Instruments). For cell-attached recordings, Sylgard-coated borosilicate patch pipettes (3–5 MΩ resistance) were filled with standard external solution plus ACh (100 nM). In one set of experiments, $ZnCl_2$ (0.05 to 0.1 or 1 mM) was included in the patch pipette. Since control channel open duration (τ_{op}) varied in different cell preparations, ranging from 2.8 to 5.5 ms, the values of τ_{op} in the presence of Zn^{2+} were compared to control values obtained in the same experimental session. In another set of experiments, the effects on single-channel activity of ACh (20 μM), ACh + $ZnCl_2$ (200 μM) and TPEN (50 μM) applied to the extra-patch membrane, using a gravity-driven fast perfusion system (RSC-200, Biologic, France), were studied. Control ACh-evoked unitary events were recorded in each cell at the beginning of the experiment, while superfusing with standard external solution, then test solutions were applied for 2–4 min, washed out and channel activity recorded again. This protocol ensured that recordings were performed at stable membrane potentials, since during ACh application cells depolarized, then repolarized due to AChR desensitization, as revealed by changes in channel amplitude. Once exposed to $ZnCl_2$, Petri dishes were discarded. Data were sampled at 10 kHz and analysed after Gaussian digital filtering at 2 kHz, using a threshold-crossing method by pCLAMP 6 software (Axon Instruments), as previously detailed (Fucile *et al.* 1996). Channel slope conductance was calculated by least-squares linear fitting of unitary *i–V* relations. Cell resting potential was estimated from these fits, assuming a reversal potential of 0 mV (see Fucile *et al.* 1996) and the kinetic properties of ACh-evoked events were compared at an estimated membrane potential of about −60 mV. Results are given as means ± S.E.M. To ascertain that the lengthening of τ_{op} caused by extra-patch $ZnCl_2$ was significant, in each patch τ_{op} values recorded under control conditions and after application of ACh plus Zn^{2+} were compared by means of a one-way ANOVA, using Origin 4 (Microcal, USA). Statistical significance was accepted for $P < 0.05$. For outside-out recordings, ACh (200 nM) or ACh plus $ZnCl_2$ (3 mM) was applied to the excised membrane patch using the fast perfusion system as above. For inside-out recordings, patch pipettes were filled with normal external solution plus ACh (100 nM), while cells were equilibrated in a nominally Ca^{2+}-free, KCl-containing solution. After patch excision, channel activity was recorded for 2–20 min, Zn^{2+}-containing solution was then added and channel activity recorded for 2–5 min. For all the excised patches, recordings were performed at potentials ranging between −50 and −90 mV. Data were collected and analysed as above.

Fluorescence measurements

Fluorescence determinations were made using real-time confocal laser microscopy (Odyssey, Noran Instruments, CA, USA), as previously described in detail (Ragozzino *et al.* 1998). In these experiments, ACh was routinely replaced by nicotine (except where otherwise indicated), to avoid possible artefacts caused by the stimulation of muscarinic receptors. The nicotine-induced rise of cytosolic Zn^{2+} concentration was expressed as the ratio of fluorescence increase over basal fluorescence ($\Delta F/F_0$). Fluorescence signals were measured as averages over square domains approximating cell shape, assuming a homogeneous receptor density. All determinations were performed with the confocal slit set at 100 μm, to detect fluorescence changes over the entire cell depth. Identical conditions of illumination and detection were maintained, taking care that the basal fluorescence of each cell was similar. Loading with cell-permeant dyes (Newport Green diacetate, 5 μM, or Mg^{2+}-Green acetoxymethyl ester, 4 μM) was achieved by incubating transfected cells with the dye at 37°C for 20–40 min in serum-free DMEM, then extensively washing with standard medium. When fluorescence and current responses were to be measured simultaneously, cells responding with a large fluorescence increase to a preliminary nicotine application were selected. After rupture of the membrane patch and establishment of the whole-cell configuration, the cell-impermeant dye (Mg^{2+}-Green, 50 μM; Newport Green, 10 μM; Fluo-3, 250 μM) contained in the patch pipette diffused into the cell, leading to a slow increase in the basal fluorescence that reached a stable level (F_0) within 5–6 min. Only cells that exhibited stable F_0 during experiments were considered for analysis. To rule out artefacts due to fluorescence run-up (see Sensi *et al.* 1997), F_0 was measured immediately before agonist application. The permeability of γ- and ϵ-AChR channels to Zn^{2+} was evaluated by estimating the fractional Zn^{2+} current ($P_{f,Zn}$) of AChR subtypes using the procedure

previously described for Ca^{2+} (Schneggenburger *et al.* 1993; Zhou & Neher, 1993; Ragozzino *et al.* 1998). Briefly, during the first 1–3 s of agonist application, the fluorescence increase ($F = \Delta F/F_0$) and the current integral (Q) were measured at 50 ms intervals and their point ratio (F/Q) calculated. To determine $P_{f,Zn}$, the F/Q ratio at a given Zn^{2+} concentration was normalized to the F/Q ratio of a pure Zn^{2+} current (5–50 pA peak amplitude), obtained using an extracellular solution with Zn^{2+} as the only charge carrier (see 'Solutions'). Measurements performed using patch pipettes filled with CsCl or NMGA solutions yielded comparable values for $P_{f,Zn}$. All the results are expressed as means ± S.E.M.

RESULTS

Mouse muscle AChRs are permeable to Zn^{2+}

To determine the optimal Zn^{2+} concentration for fluorescence measurements, we tested the effects of Zn^{2+} on the amplitude of whole-cell currents evoked by ACh (10 μM; I_{ACh}) at the γ- and ε-AChRs. Zn^{2+} reduced I_{ACh} amplitude at both receptors in a concentration-dependent manner (Fig. 1*A* and *B*). This effect was fully reversible by wash in Zn^{2+}-free medium (2 min if Zn^{2+} concentration was < 1 mM; 8–10 min at higher concentrations). Fitting the relationship between I_{ACh} amplitude and Zn^{2+} dose with eqn (1) (Fig. 1*C*) showed that the ε-AChR was blocked by Zn^{2+} with higher potency than the γ-AChR. However, even at high Zn^{2+} concentrations the block was not complete. For both the γ- and ε-AChRs, the block of I_{ACh} by Zn^{2+} was voltage independent for membrane potentials ranging from −150 to +50 mV (not shown). Thus, we studied the Zn^{2+} permeability of the AChR channels using a Zn^{2+} concentration of 0.5 mM, which induces only a minor block of the γ- and ε-AChRs. The Zn^{2+}-induced block of I_{ACh} was investigated in more detail by single-channel recordings (see below).

The Zn^{2+} influx in standard medium, containing Ca^{2+} and Mg^{2+}, was studied using the specific low-sensitivity dye Newport Green diacetate ($K_d = 1.5$ μM, as indicated by the manufacturer). When $ZnCl_2$ was added to the standard external solution, prolonged application of nicotine (100 μM) evoked tiny but sustained fluorescence changes ($\Delta F/F_0 = 0.16 \pm 0.03$ for γ-AChR, $n = 23$), which did not recover to basal even 3–4 min after nicotine and/or $ZnCl_2$ washout, unless the cell-permeant Zn^{2+} chelator TPEN (40 μM) was added to the standard solution (Fig. 2*A*). The fluorescence change developed very slowly, presumably as Zn^{2+} accumulated within the cell to a level detectable by this low-affinity dye. No fluorescence increase was elicited by nicotine in the absence of Zn^{2+}, confirming that Newport Green specifically recognizes Zn^{2+} (Canzoniero *et al.* 1999), but the small size and slow rise of the responses observed limited the reliability of the Zn^{2+} influx measurements performed with this fluorescent dye. We therefore turned to Mg^{2+}-Green, a dye with a higher affinity for Zn^{2+} but which is sensitive to other divalent cations. In a Ca^{2+}-, Mg^{2+}-free solution containing $ZnCl_2$, nicotine promptly elicited a long-lasting increase of fluorescence ($\Delta F/F_0 = 0.46 \pm 0.06$ for γ-AChR, $n = 20$; 0.35 ± 0.05 for ε-AChR, $n = 36$), which was insensitive to the removal of the transmitter or external Zn^{2+}, but rapidly recovered to basal level (time for half-recovery, $T_{0.5} = 10 \pm 5$ s) after application of TPEN (Fig. 2*B*). After fluorescence recovery and TPEN washout, a second nicotine application elicited a comparable response (data not shown). Thus, Mg^{2+}-Green appeared to be sensitive enough to measure Zn^{2+} influx in our cells. We also compared the results obtained in the absence of external Ca^{2+} and Mg^{2+} to those obtained under physiological

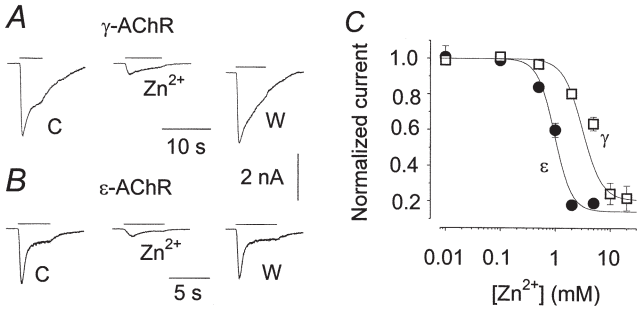

Figure 1. Zn^{2+} reversibly blocks nicotine-evoked currents in BOSC 23 cells expressing muscle AChRs

A, typical inward whole-cell currents (downward deflections) evoked by ACh (10 μM, indicated by bars) before (control, C), during (Zn^{2+}) and after (wash, W) the simultaneous application of $ZnCl_2$ (10 mM) to a cell expressing γ-AChR. Holding potential, −50 mV. Note the full reversibility of the block with wash (8 min). *B*, in a cell expressing ε-AChR, 2 mM $ZnCl_2$ produced a comparable block of the ACh-evoked current. *C*, inhibition curves at a holding potential of −50 mV. The amplitude of the current recorded in the presence of various concentrations of Zn^{2+} was normalized to the control response in each cell and plotted *vs.* Zn^{2+} dose. Each point represents the mean of 4–8 values, with error bars (representing S.E.M.) omitted when smaller than the symbols (□ for γ-AChR, ● for ε-AChR). The continuous lines represent the best fit of the data with eqn (1), yielding: $x_0 = 3.1$ mM, $n_H = 2.46$ and $K = 0.2$ for γ-AChR; $x_0 = 0.97$ mM, $n_H = 2.84$ and $K = 0.14$ for ε-AChR.

conditions as, in neurones, Zn^{2+} influx is reduced by the addition of Ca^{2+} and Mg^{2+} to the external medium (Sensi *et al.* 1997). Using standard external solution plus $ZnCl_2$, the response to nicotine showed an initial transient followed by a sustained plateau, terminated by TPEN addition but not by agonist or Zn^{2+} withdrawal (Fig. 2*Ca*). In each of the eight cells tested (four loaded with cell-impermeant Mg^{2+}-Green under the whole-cell recording configuration, the others with the cell-permeant dye), this response virtually overlapped (e.g. Fig. 2*Ca*) the sum of the transient fluorescence increase (Fig. 2*Cb*) recorded in standard external medium (no $ZnCl_2$ added) and the sustained fluorescence signal due to the influx of Zn^{2+} in Ca^{2+}-, Mg^{2+}-free medium (Fig. 2*Cc*). No fluorescence increase was detected when nicotine was applied in the absence of Ca^{2+}, Mg^{2+} and Zn^{2+} ($n = 21$), or when $ZnCl_2$ (0.1–5 mM) was applied in the absence of nicotine (data not shown).

Thus, under our experimental conditions, Zn^{2+} influx appeared to be little influenced by Ca^{2+} and Mg^{2+} at physiological concentrations. These observations indicate that Zn^{2+} permeation through γ- and ϵ-AChR channels may be adequately studied in BOSC 23 cells loaded with Mg^{2+}-Green using a Ca^{2+}-, Mg^{2+}-free medium.

Fractional Zn^{2+} current through γ- and ϵ-AChRs

The fractional current carried by Zn^{2+} ($P_{f,Zn}$) through γ- and ϵ-AChR channels was determined by simultaneously recording, in the presence of Zn^{2+} (0.5 mM), agonist-evoked fluorescence and whole-cell current responses, in cells loaded with the cell-impermeant dye Mg^{2+}-Green (50 μM) via the patch pipette (e.g. Fig. 3*A*). Application of nicotine elicited a current that was terminated by agonist washout, whereas the parallel fluorescence increase was maintained until TPEN was added to the external medium (see Fig. 3*A*). To estimate $P_{f,Zn}$, we determined the ratio of nicotine-elicited fluorescence to charge responses (F/Q) and normalized this to the F/Q ratio obtained when Zn^{2+} was the only charge carrier (Fig. 3*B*), as described previously (Vernino *et al.* 1994; Ragozzino *et al.* 1998). The fetal and adult AChR channels showed a significant difference in their permeability to Zn^{2+}, as $P_{f,Zn}$ was 1.7 ± 0.3% for γ-AChR ($n = 15$) and 3.9 ± 0.9% ($n = 11$) for ϵ-AChR (one-way ANOVA, $P = 0.014$). The same $P_{f,Zn}$ (1.7 ± 0.4%, $n = 11$ for γ-AChR) was obtained for ACh-induced responses, indicating that the agonist used did not influence Zn^{2+} influx. Similar values for $P_{f,Zn}$ of γ-AChR were obtained when the intracellular solution contained NMGA instead of CsCl.

The voltage dependence of Zn^{2+} influx was studied for membrane potentials ranging between −80 and +50 mV. Current–voltage relations were linear for γ- and ϵ-AChRs, both reversing close to 0 mV (not shown). At hyperpolarized potentials (−80 to −30 mV), $P_{f,Zn}$ was voltage independent, but it abruptly rose when the membrane potential approached the equilibrium potential of monovalent cations (0 mV) and most of the current was carried by Zn^{2+} ions (not shown). At positive membrane potentials, fluorescence increases were observed (not shown), indicating that Zn^{2+} enters the cell even when the net current is outward.

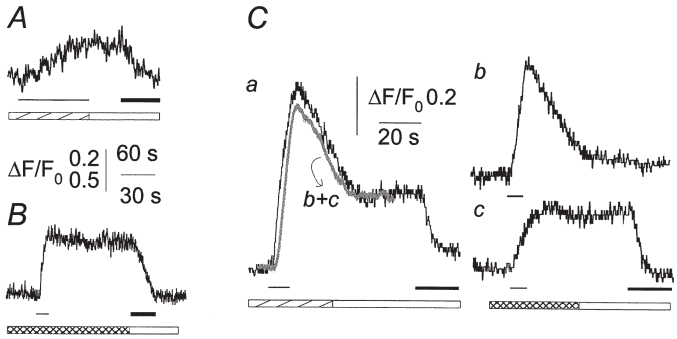

Figure 2. Zn^{2+} influx through mouse muscle AChR channels is independent of Ca^{2+} and Mg^{2+}

Representative examples of fluorescence responses to nicotine (100 μM, indicated by the thin bar) obtained, under different conditions, in three cells expressing γ-AChR. *A*, the small and slow fluorescence increase in a cell loaded with cell-permeant Newport Green diacetate and equilibrated in standard external solution plus $ZnCl_2$ (0.5 mM, hatched bar) was unaffected by Zn^{2+} washout with a Ca^{2+}-, Mg^{2+}-free solution (open bar), but recovered to basal when TPEN (40 μM, filled bar) was applied. *B*, in cells loaded with Mg^{2+}-Green acetate and equilibrated in Ca^{2+}-, Mg^{2+}-free solution containing Zn^{2+} (cross-hatched bar), the sustained fluorescence response was prompt and larger than those observed using Newport Green, and recovered to basal only upon TPEN application (in Ca^{2+}-, Mg^{2+}-free solution). *C*, in a cell loaded with Mg^{2+}-Green via the patch pipette, the fluorescence response in standard external solution plus $ZnCl_2$ (*a*) was matched by the sum (trace labelled $b + c$) of the transient responses obtained in standard external solution in the absence of Zn^{2+} (*b*), and the sustained response observed in Ca^{2+}-, Mg^{2+}-free solution containing Zn^{2+} (*c*). TPEN application caused recovery in both *a* and *c*. Membrane holding potential, −50 mV.

Since the synaptic Zn^{2+} concentration may increase up to 100-fold during intense synaptic activity (Assaf & Chung, 1984), we determined $P_{f,Zn}$ for Zn^{2+} concentrations between 0.1 and 3.5 mM. In cells expressing the γ-AChR, $P_{f,Zn}$ became larger with increasing Zn^{2+} concentration, reaching a plateau at millimolar concentrations (Fig. 3*C*).

It remains to be confirmed whether the measurements of Zn^{2+} influx performed in the absence of Ca^{2+} and Mg^{2+} are representative of more physiological conditions. As shown in Fig. 2*C*, the late phase of the nicotine-evoked fluorescence response in Mg^{2+}-Green-loaded cells (attributable to Zn^{2+}) was not influenced by the addition of Ca^{2+} and Mg^{2+} at physiological concentrations. The observation that $P_{f,Zn}$ increased about 2-fold when the concentration of external Zn^{2+} was raised from 0.1 to 0.5 mM (Fig. 3*C*), which causes only a small (if any) reduction of current amplitude (see Fig. 1*C*), supports the notion that fluorescence signals are adequately sensitive to changes in Zn^{2+} influx. This point was further investigated by directly measuring the *F*/*Q* ratio of nicotine-induced responses in cells loaded with the Zn^{2+}-specific dye Newport Green (Fig. 3*D*). As tested in cells expressing the γ-AChR at an extracellular Zn^{2+} concentration of 0.5 mM, the *F*/*Q* ratio did not change when Ca^{2+} and Mg^{2+} (2 mM each) were added to the external medium (0.009 ± 0.002 *vs.* 0.008 ± 0.002, $n = 7$). Given the low sensitivity of Newport Green, we also examined the effect of Zn^{2+} on the nicotine-evoked Ca^{2+} responses of five cells loaded with the Ca^{2+}-sensitive, Zn^{2+}-insensitive dye Fluo-3 (Fig. 3*E*). The *F*/*Q* ratio was unaffected (ANOVA test, $P > 0.75$) by addition to the standard external medium of 0.5 or 5 mM $ZnCl_2$, the values being 0.0354 ± 0.006 (normal external solution), 0.0322 ± 0.007 (0.5 mM Zn^{2+}) and 0.034 ± 0.009 (5 mM Zn^{2+}). These data confirm that divalent cations at physiological concentrations interfere little with each other's influx through AChR channels.

Effects of Zn^{2+} on unitary γ-AChR channel function

To analyse the effects of Zn^{2+} on AChR channel behaviour, cell-attached and outside-out recordings of unitary events evoked by ACh were performed in cells expressing the γ-AChR. Under control conditions, cell-attached channel conductance and mean open time were 34.7 ± 0.6 pS and 4.4 ± 0.2 ms ($n = 5$), respectively, in line with previous observations in the same cell system (Fucile *et al.* 1996). When $ZnCl_2$ (50 or 100 μM) was included in the pipette-filling solution (3 cells), neither the single-channel slope conductance (35.6 ± 0.7 pS), nor

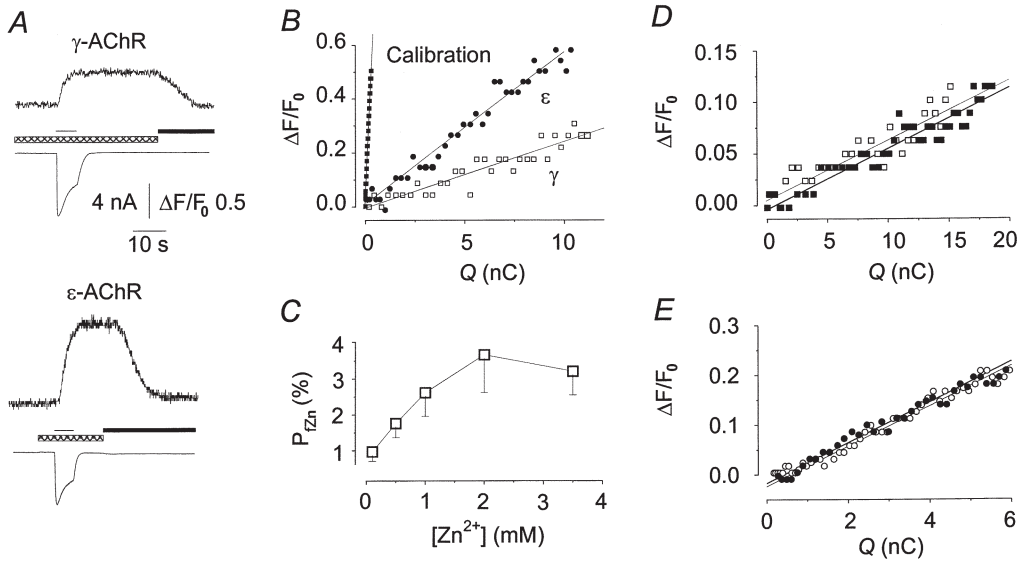

Figure 3. Fractional Zn^{2+} current through muscle AChRs

A, typical fluorescence and current responses evoked by nicotine (thin bar), simultaneously recorded in two cells expressing γ-AChR (top) and ϵ-AChR (bottom), equilibrated in Ca^{2+}-, Mg^{2+}-free solution containing Zn^{2+} (0.5 mM, cross-hatched bar). Note that the current response was terminated by nicotine washout, while fluorescence recovered to basal only upon TPEN application (filled bar). Membrane holding potential, −50 mV. *B*, plot of $\Delta F/F_0$ *vs.* *Q* in the same cells as in *A* (□, γ-AChR; ●, ϵ-AChR) and in a third cell used for calibration measurements (■). Data points were calculated every 50 ms. The slopes of the straight lines, obtained by best fitting the data, represent the *F*/*Q* ratios of the responses. *C*, in cells expressing γ-AChR, $P_{f,Zn}$ increased with Zn^{2+} concentration in the extracellular medium. Each point represents the mean ± S.E.M. of 6–11 cells. *D*, plot of $\Delta F/F_0$ *vs.* *Q* in a cell loaded with Newport Green, with nicotine applied in the presence of $ZnCl_2$ (0.5 mM, □), or $ZnCl_2$ plus $CaCl_2$ and $MgCl_2$ (2 mM each, ■). Note that the values virtually overlap. *E*, plot of $\Delta F/F_0$ *vs.* *Q* in a cell loaded with Fluo-3, and superfused with nicotine in standard external solution (○) and in the presence of $ZnCl_2$ (0.5 mM, ●). Data points were obtained and fitted as in *B*.

the channel open time (3.8 ± 0.4 ms) was different from the control values. At millimolar concentrations, $ZnCl_2$ clearly reduced channel conductance, with no significant effect on mean channel open time, the values being 24.9 ± 1.4 pS and 4.8 ± 0.5 ms ($n = 6$) at 1 mM. However, patches were very unstable and recordings using $ZnCl_2$ at its half-maximal inhibitory concentration (3 mM for γ-AChR) could only be performed in the outside-out configuration (Fig. 4*A*). In the three patches examined, the unitary conductance of ACh-evoked channels (at −70 mV) in the presence of $ZnCl_2$ was 65–76% (mean, 70.4%) of the control conductance (26.5 *vs.* 37.4 pS). In contrast, channel open duration was essentially unaltered in the presence of Zn^{2+} (4.3 ms for the control, 3.9 ms in the presence of Zn^{2+}). Following patch excision, a slight decrease in channel opening frequency was observed, independent of Zn^{2+} addition. Together, these data show that in the presence of 1–3 mM $ZnCl_2$, unitary channel conductance was reduced by about 70%, in agreement with the whole-cell data. No flickering behaviour was ever observed in the presence or absence of Zn^{2+} at these low ACh concentrations (data not shown).

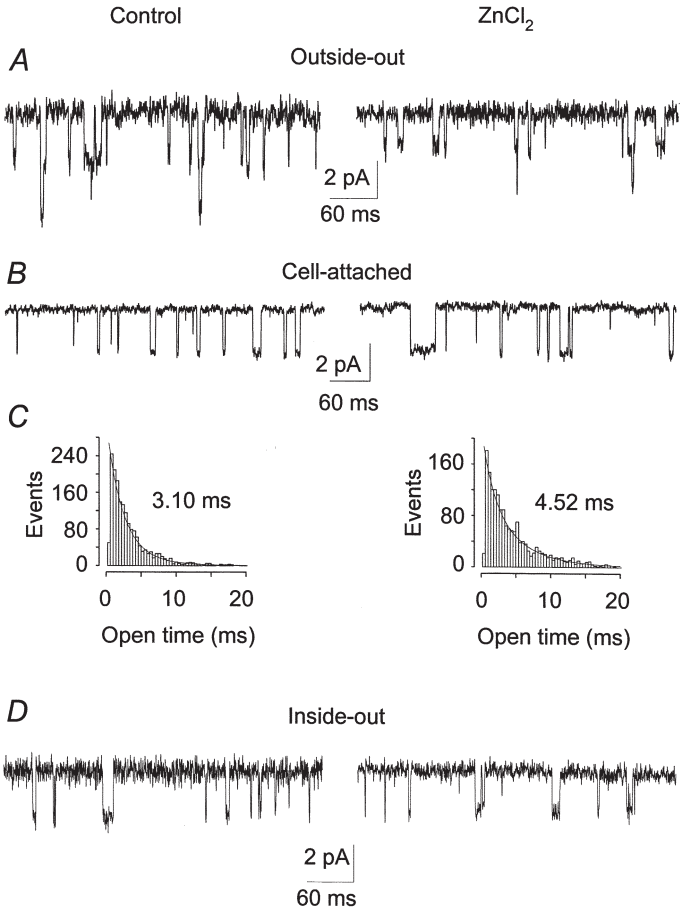

Figure 4. Effect of $ZnCl_2$ on γ-AChR channel behaviour

A, typical traces recorded in an outside-out patch (pipette potential, −70 mV), superfused with ACh (200 nM, left), then with ACh plus $ZnCl_2$ (3 mM, right). Downward deflections represent inward currents. Conductances and mean open times were: Control, 37.5 pS and 3.9 ms; $ZnCl_2$, 26.6 pS and 3.9 ms. Note the slight reduction in opening frequency, due to channel run-down. *B*, representative examples of cell-attached currents recorded in a BOSC 23 cell before (Control) and after a 4 min application to the extra-patch membrane of ACh (20 μM) plus $ZnCl_2$ (200 μM), as indicated. Unitary slope conductances were 38.4 pS (Control) and 37.9 pS ($ZnCl_2$). Traces were recorded at an estimated transmembrane potential of about −60 mV, with 100 nM ACh in the patch pipette. *C*, histograms of the channel open times for the recordings shown in *B*, with the indicated mean open times. Superimposed are the best fits of the data with the sum of two exponentials, with the following time constants (weight): Control, $\tau_1 = 2.1$ ms (66%), $\tau_2 = 3.9$ ms (34%); $ZnCl_2$, $\tau_1 = 1.7$ ms (24%), $\tau_2 = 4.9$ ms (76%). Note the increase of τ_{op} following administration of $ZnCl_2$. *D*, typical events recorded in an inside-out patch before (Control) and 1 min after patch exposure to $ZnCl_2$ (200 μM), as indicated. Recordings were performed with a pipette potential of −80 mV. Conductance and mean open time were: Control, 31 pS and 2.97 ms; $ZnCl_2$, 29 pS and 3.01 ms. Note the ineffectiveness of Zn^{2+} application.

To examine whether the increase of cytosolic Zn^{2+} concentration caused by its influx through AChR channels influences channel activity, cell-attached recordings were performed before and after (or during) application of ACh (20 μM) plus $ZnCl_2$ (200 μM) to the extra-patch membrane (Fig. 4*B*). Under control conditions, unitary events showed a conductance of 39 ± 1 pS, slightly higher than in the absence of continuous superfusion, and a mean τ_{op} of 3.2 ± 0.3 ms ($n = 9$). After exposure of the extra-patch membrane to ACh plus $ZnCl_2$ for 2–4 min, τ_{op} significantly increased in seven out of nine cells tested (Fig. 4*B*), with increments ranging from 20% (significant, one-way ANOVA, $P < 0.001$) to 100% (mean, 50 ± 10%). Open time distributions were well fitted by two exponential components before and after Zn^{2+} application (e.g. Fig. 4*C*). Neither channel conductance (38.9 ± 1.5 pS, $n = 9$) nor closed duration (not shown) was affected by Zn^{2+} application. τ_{op} did not decrease even 15 min after washout of ACh and $ZnCl_2$. No significant change of τ_{op} was observed when ACh alone was applied to the cells prior to Zn^{2+} application ($n = 3$, data not shown). We also tested whether the Zn^{2+} chelator TPEN was able to revert the Zn^{2+}-induced increase of τ_{op}. To this purpose, ACh-evoked events were recorded in cells treated for 2–4 min with TPEN (50 μM), after the effect of Zn^{2+} had been assessed. τ_{op} was reduced to control values in only one out of four cells tested (not shown), whereas in the other three cells the mean τ_{op} remained elevated. The scattering in the extent of the extra-patch Zn^{2+} effect and the lack of reversibility by TPEN suggest that the action of Zn^{2+} was mediated by some intracellular effector. To test this hypothesis, inside-out patches were exposed (1–5 min) to Zn^{2+} (200 μM, as in cell-attached experiments). Since in a different cell line (BC3H1), a 3-fold decrease of τ_{op} was observed within 20 min of patch excision (Covarrubias & Steinbach, 1990), we monitored the τ_{op} of AChR channels in transfected BOSC 23 cells. In recordings from seven inside-out patches lasting 10–20 min, no significant change of τ_{op} was observed (4.0 ± 0.5 ms within 60 s of patch excision, 4.3 ± 0.5 ms 10–20 min later, $P = 0.6$, one-way ANOVA). At the end of control recordings, three of these patches were exposed to Zn^{2+}, which was unable to modify τ_{op}. Accordingly, these data were pooled with others obtained at earlier times (3–10 min) after patch excision. In none of the eight inside-out patches examined did Zn^{2+} modify τ_{op}, which, on average, was 3.9 ± 0.4 ms ($n = 8$) under control conditions and 3.8 ± 0.4 ms in the presence of Zn^{2+} (Fig. 4*D*). The discrepancy between this study and that of Covarrubias & Steinbach (1990) might be due to differences in the cell type used (BOSC 23 *vs.* BC3H1) and/or in the recording conditions (standard external solution in the patch pipette, nominally Ca^{2+}-free solution in the bath *vs.* EGTA-containing solution in the bath and in the patch pipette). However, a detailed investigation of this point is beyond the purposes of the present study.

DISCUSSION

Zinc is a trace element crucial for embryonic development and animal growth and survival (for review see Smart *et al.* 1994). Functionally, two distinct pools of zinc exist: one is tightly bound to proteins, the other is stored in synaptic vesicles and released upon synaptic activity. In the brain, releasable Zn^{2+} mediates toxic responses (as reviewed by Choi & Koh, 1998) and modulates the function of several receptors, including excitatory and inhibitory amino acid receptors (Smart *et al.* 1994) and a neuronal nicotinic receptor (Palma *et al.* 1998). In addition, it controls nociceptive pathways in the spinal cord (Larson & Kitto, 1997). As for the neuromuscular junction, an increasing concentration of Zn^{2+} is observed in rat motoneurones in the first postnatal days (Kozma & Ferke, 1979) and exogenous Zn^{2+} influences spontaneous and evoked ACh release (Benoit & Mambrini, 1970; Nishimura, 1987; Wang & Quastel, 1990). Furthermore, the frog end-plate channel is permeable to Zn^{2+}, as demonstrated by the shift of the I_{ACh} reversal potential upon changes of the extracellular Zn^{2+} concentration (Adams *et al.* 1980). This paper provides a direct measurement of the fraction of current carried by Zn^{2+} ions through the channels of the fetal and adult types of mouse muscle nicotinic AChR expressed in transiently transfected BOSC 23 cells, as well as demonstrating that cytosolic Zn^{2+} accumulation influences AChR function. We show that γ- and ϵ-AChR channels are permeable to Zn^{2+} ions, both in the presence and in the absence of Ca^{2+} and Mg^{2+}. At 0.5 mM, i.e. a concentration possibly attained at active synapses (see Assaf & Chung, 1984), the value of $P_{f,Zn}$ for ϵ-AChRs is almost double that for γ-AChRs. A 2-fold difference has also been observed for the Ca^{2+} fractional permeability ($P_{f,Ca}$) of the two AChR types measured in BOSC 23 cells (Ragozzino *et al.* 1998) and at developing mouse synapses (Villaroel & Sakmann, 1996), suggesting that γ- and ϵ-AChRs have a differential permeability to divalent cations. At the frog end-plate, the permeability to Zn^{2+} is about 10% higher than to Ca^{2+} (Adams *et al.* 1980), in line with the observation that, for divalent cations, the smaller the size, the greater the permeability through the ϵ-AChR channels (Lewis & Stevens, 1983). Our data indicate that, for γ-AChRs, $P_{f,Zn}$ increases with the concentration of Zn^{2+}, so that at a concentration of 2 mM, the fractional permeability for Zn^{2+} (in a Ca^{2+}-, Mg^{2+}-free solution) is double that for Ca^{2+}. For ϵ-AChRs, the strong block of I_{ACh} hinders the reliable measurement of $P_{f,Zn}$ at millimolar Zn^{2+} concentrations. We further show that Ca^{2+} and Mg^{2+} at physiological concentrations do not appreciably alter the amplitude of the long-lasting increase of cytosolic Zn^{2+} concentration evoked by nicotine, and conversely Zn^{2+} up to 5 mM does not influence fractional Ca^{2+} influx. These data indicate that, at these concentrations, the permeation of each divalent cation through the AChR channels is little influenced by the presence of others.

As for other divalent cations, Zn^{2+} permeates through the receptor channel, but, at millimolar concentrations, it

reversibly reduces the amplitude of I_{ACh}. For Ca^{2+} and Mg^{2+}, the block has been ascribed to the high-affinity interaction of divalent cations with negative residues in the channel pore, which decreases channel conductance (Imoto *et al.* 1988). We show here that Zn^{2+} acts by a similar mechanism, as the addition of 1–3 mM $ZnCl_2$ to the external medium comparably reduced both the unitary conductance and the amplitude of whole-cell currents to about 70% of control values. Furthermore, the voltage independence of Zn^{2+} block and the absence of flickering behaviour in single-channel openings recorded in the presence of Zn^{2+} indicate that Zn^{2+} is not an open-channel blocker. With respect to the γ-AChR, the ϵ-AChR has one more net negative charge in both the cytoplasmic and extracellular ring close to the membrane-spanning segment M2 (Imoto *et al.* 1988), so that it might be more sensitive to divalent cations than the γ-AChR. This appears to be the case for Zn^{2+}-induced block, which is 3-fold stronger for the ϵ- than the γ-AChR.

Interestingly, both in the presence and in the absence of Ca^{2+} and Mg^{2+}, the nicotine-induced increase of the cytosolic Zn^{2+} concentration did not recover until the specific Zn^{2+} chelator TPEN was applied. This also happens in cultured cortical neurones, where Zn^{2+} is believed to mediate important physio-pathological processes (Sensi *et al.* 1997; Canzoniero *et al.* 1999), and indicates that Zn^{2+} is poorly buffered and/or extruded by cells, suggesting that Zn^{2+} may accumulate within the cells and mediate long-lasting effects. Indeed, we demonstrate that, under conditions leading to an increase of the cytosolic Zn^{2+} concentration, the open duration of the γ-AChR channel is lengthened. This effect requires the influx of a substantial amount of Zn^{2+}, as no channel modulation was observed when Zn^{2+} was included in the patch pipette. In addition, the lengthening of τ_{op} is not caused by Ca^{2+} influx through the γ-AChR channel, as there was no change of τ_{op} when cells were exposed to ACh in the absence of Zn^{2+}, while Ca^{2+} influx was the same as in the presence of Zn^{2+}. Thus, the reported increase of τ_{op} appears to be specifically related to Zn^{2+} influx. One might imagine that an increase of τ_{op} would lead to a potentiation of I_{ACh} in the presence of similar concentrations of extracellular Zn^{2+}, which was not observed. However, the effect of Zn^{2+} on single-channel open duration requires Zn^{2+} accumulation, which, under whole-cell recording conditions, is probably prevented by chelation by the EGTA contained in the patch pipette. The Zn^{2+}-induced lengthening of channel open time is quite unusual, as Zn^{2+} has been described to reduce the opening frequency of GABA-gated receptor channels without altering their open duration, or to reduce the open duration of NMDA-gated channels (as reviewed by Smart *et al.* 1994). It has been known for many years that Ca^{2+} entry favours AChR desensitization at the frog end-plate (Miledi, 1980). The data reported here suggest that the influx through the AChR channel of Zn^{2+}, another divalent cation, affects channel function (namely, its open duration) by an analogous mechanism, possibly acting through intracellular mediators (Miledi *et al.* 1989).

All these data suggest that Zn^{2+} might play a physiological role in neuromuscular transmission, as is the case for central synapses (for review see Choi & Koh, 1998). Though it has not yet been demonstrated that Zn^{2+} is released at the neuromuscular junction, Zn^{2+} is present in plasma at a concentration of about 10 μM in adult humans (Vallee & Falchuk, 1993). Thus, it might accumulate in muscle fibres during periods of intense activity, modulating AChR channel function. In conclusion, Zn^{2+} permeates through AChR channels and reduces their conductance, probably acting on extracellular regulatory sites (Lena & Changeux, 1993). Furthermore, Zn^{2+} induces the lengthening of channel open durations, possibly through cytosolic mechanisms, deserving further investigation.

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Acknowledgements

We thank Dr R. Miledi and Dr P. Bregestovski for helpful suggestions. This work was supported in part by MURST grants to F.E. and F.G.

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