

# Ca<sup>2+</sup> permeability of nicotinic acetylcholine receptors in rat hippocampal CA1 interneurons

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Neuronal nicotinic acetylcholine receptors (nAChRs) are widely expressed in the brain where they are involved in a variety of physiological processes, including cognition and development. The nAChRs are ligand-gated cationic channels, and different subtypes are known to be differentially permeable to Ca<sup>2+</sup>; the  $\alpha$ 7-containing nAChRs are generally considered to be the most permeable. Ca<sup>2+</sup> can activate and regulate a variety of signal transduction cascades, and the influx of Ca<sup>2+</sup> through these receptors may have implications for synaptic plasticity. To determine the Ca<sup>2+</sup> permeability of the nAChRs in rat hippocampal interneurons in the slice, which contain diverse subtypes of  $\alpha$ 7- and non- $\alpha$ 7-containing nAChRs, we combined patch-clamp electrophysiology recordings with conventional fura-2 fluorescence imaging techniques. We estimated the relative Ca<sup>2+</sup> permeability of the channels by determining the ratio of the increase in [Ca<sup>2+</sup>]<sub>i</sub> level ( $\Delta$ [Ca<sup>2+</sup>]<sub>i</sub>) in the soma to the integrated transmembrane current (charge, Q) induced by the activation of the nAChRs, and compared this ratio to the highly Ca<sup>2+</sup> permeable NMDA subtype of glutamate receptor channel. In all cells tested, the  $\Delta$ [Ca<sup>2+</sup>]<sub>i</sub>/Q ratio was significantly larger (i.e. more than twice as big) for responses activated by NMDA than for  $\alpha$ 7-containing nAChRs in interneurons; the activation of the non- $\alpha$ 7 nAChRs did not produce any significant increase in [Ca<sup>2+</sup>]<sub>i</sub>. Interestingly, the Ca<sup>2+</sup> permeability of native  $\alpha$ 7 nAChRs in PC12 cells was significantly larger than in hippocampal interneurons, and not significantly different from NMDA receptors. Therefore, the  $\alpha$ 7-containing nAChRs in rat hippocampal interneurons are significantly less permeable to Ca<sup>2+</sup> than not only NMDA receptors but also  $\alpha$ 7 nAChRs in PC12 cells.

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Multiple subtypes of neuronal nicotinic acetylcholine receptor channels (nAChRs) are expressed in the brain, the functions of which have been linked to specific cognitive processes in humans and animal models, and development (Jones *et al.* 1999; Levin, 2002). Furthermore, dysfunctions in these receptors may be involved in various neurodegenerative diseases, such as Alzheimer's disease, epilepsy, and schizophrenia (Jones *et al.* 1999; Pettit *et al.* 2001; Liu *et al.* 2001). Functionally, nAChRs are ligand-gated cationic channels with the capacity to elicit local changes in cytoplasmic calcium ([Ca<sup>2+</sup>]<sub>i</sub>) levels, and different subtypes of neuronal nAChRs are known to be differentially permeable to Ca<sup>2+</sup> (Bertrand *et al.* 1993; Séguéla *et al.* 1993; Fucile, 2004). Although much evidence indicates that nAChRs can directly regulate synaptic plasticity in the hippocampus (Hunter *et al.* 1994; Fujii & Sumikawa, 2001; Ji *et al.* 2001; McGehee, 2002), the cellular mechanisms involved are not fully understood, but likely to involve Ca<sup>2+</sup>. Calcium ions can activate and regulate a

variety of signal transduction cascades, and play a key role in the short- and long-term regulation of nAChRs (Quick & Lester, 2002; Dajas-Bailador & Wonnacott, 2004; Fucile, 2004).

The influx of Ca<sup>2+</sup> through nAChRs has implications not only for synaptic plasticity, but also for the release of neurotransmitter for those presynaptic terminals that express Ca<sup>2+</sup>-permeable nAChRs (Dajas-Bailador & Wonnacott, 2004; Fucile, 2004). For example in the hippocampus, Gray *et al.* (1996) found that activation of  $\alpha$ 7-containing nAChRs increased intraterminal Ca<sup>2+</sup> levels and facilitated glutamatergic release; however, others failed to observe this effect (Vogt & Regehr, 2001). In heterologous expression systems, homomeric  $\alpha$ 7 nAChRs were shown to have a high permeability to Ca<sup>2+</sup> (Bertrand *et al.* 1993; Séguéla *et al.* 1993), as do native  $\alpha$ 7-containing nAChRs in cultured rat hippocampal neurones (Castro & Albuquerque, 1995). There is a wide range of estimates for the Ca<sup>2+</sup> permeability of both native

and heterologously expressed nAChRs, but  $\alpha 7$ -containing receptors are thought to have the highest permeability to  $\text{Ca}^{2+}$  (Fucile, 2004).

Because of the importance of nAChR function in the hippocampus in relation to cognition and synaptic plasticity, and the fact that diverse subtypes of  $\alpha 7$ - and non- $\alpha 7$ -containing nAChRs are preferentially expressed in hippocampal interneurons (Jones *et al.* 1999), it is critical to understand the  $\text{Ca}^{2+}$  permeability of nAChRs in these neurons in a more physiological setting such as the slice. To do this, we combined patch-clamp electrophysiology recordings with conventional fura-2 fluorescence imaging techniques to measure the changes in  $[\text{Ca}^{2+}]_i$  in the soma due to the activation of the various nAChRs. Recently we reported that the increase in  $[\text{Ca}^{2+}]_i$  levels due to the activation of  $\alpha 7$ -containing nAChRs in these interneurons in the slice was modest (Khiroug *et al.* 2003). Here we wanted to compare and contrast the  $\text{Ca}^{2+}$  permeability of these nAChRs (i.e. both  $\alpha 7$ - and non- $\alpha 7$ -containing) with the permeability of the NMDA subtype of the ionotropic glutamate receptor channel, which is known to be highly  $\text{Ca}^{2+}$  permeable; in fact the  $\text{Ca}^{2+}$  permeability of  $\alpha 7$  receptors expressed in *Xenopus* oocytes was greater than that for NMDA receptors expressed in the same cells (Séguéla *et al.* 1993). We also compared the  $\text{Ca}^{2+}$  permeability of native  $\alpha 7$ -containing nAChRs in cultured rat pheochromocytoma 12 (PC12) cells. Whereas we found that the relative  $\text{Ca}^{2+}$  permeability of NMDA receptors was similar to that of the  $\alpha 7$  receptors in PC12 cells, the  $\text{Ca}^{2+}$  permeability of native  $\alpha 7$ -containing nAChRs in rat hippocampal interneurons was approximately half that of both of these receptors (i.e. native  $\alpha 7$  receptors in PC12 cells and NMDA receptors in rat hippocampal interneurons). We suggest that the lower than expected  $\text{Ca}^{2+}$  permeability for hippocampal  $\alpha 7$  nAChRs may indicate a differential subunit makeup, or regulation, of these receptors.

## Methods

### Slice preparation

All experiments were carried out in accordance with guidelines approved by the NIEHS Animal Care and Use Committee, which includes minimizing the number of animals used and their suffering. Standard techniques were used to prepare 350  $\mu\text{m}$  thick acute hippocampal slices from 14- to 19-day-old rats (Fayuk & Yakel, 2004). Briefly, rats were anaesthetized with halothane (Sigma) and decapitated. Brains were quickly removed and placed into an ice-cold oxygenated, artificial cerebrospinal fluid (ACSF) containing (mM): 119 NaCl, 2.5 KCl, 1.3  $\text{MgCl}_2$ , 2.5  $\text{CaCl}_2$ , 1  $\text{NaH}_2\text{PO}_4$ , 26.2  $\text{NaHCO}_3$ , and 11 glucose. Upon dissection, brain chunks were glued to the stage of a vibrating microtome (VT1000S; Leica, Germany) for

slicing while immersed in the cooled oxygenated ACSF. Slices were then used for recordings within about 6 h, and after at least 1 h of recovery period.

### Culturing of PC12 cells

Rat adrenal medulla pheochromocytoma cells (PC12) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), and were grown in medium consisting of Kaighn's modified Ham's F12 medium (Gibco) supplemented with 15% heat-inactivated horse serum, 2.5% fetal bovine serum, 2 mM glutamine, 100 units  $\text{ml}^{-1}$  penicillin G and 100  $\mu\text{g ml}^{-1}$  streptomycin. Cells were maintained at 37°C in a humidified incubator with 5%  $\text{CO}_2$ , and plated on poly D-lysine-coated coverslips. After 2 or 3 days in culture, nerve growth factor (NGF; 50 ng  $\text{ml}^{-1}$ ) was added to differentiate the cells, and cells were used for experiments at least 3 days after NGF was added.

### Electrophysiology

Whole-cell patch-clamp recordings were performed on either CA1 interneurons (stratum radiatum or stratum oriens) or pyramidal neurons in slices, or on cultured PC12 cells. Patch pipettes (Garner 7052 or 8250 glass, with resistances of 3–4  $\text{M}\Omega$ ) were filled with a solution that contained (mM): 120 caesium gluconate, 2 NaCl, 4  $\text{Na}_2\text{ATP}$ , 0.4  $\text{Na}_2\text{GTP}$ , 4  $\text{MgCl}_2$  and 20 Hepes (pH 7.3). Slices were superfused at room temperature (18–22°C) with ACSF. Synaptic activity was blocked with TTX (1  $\mu\text{M}$ ) added to the ACSF. In some experiments, atropine (1 mM) was also added to block putative muscarinic AChR-mediated responses. Cells were clamped using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) at a holding potential of  $-70$  mV. Currents were recorded and analysed using pCLAMP software (Axon Instruments); recordings were analysed only if the holding current was less than 100 pA. Responses were induced by pressure application (Picospritzer II; General Valve Co., Fairfield, NJ, USA) of either ACh, choline, or NMDA delivered via a theta glass pipette (Harvard Apparatus, o.d. = 2 mm) pulled to a tip diameter of  $\sim 3$   $\mu\text{m}$  (P-97 puller, Sutter Instruments, Novato, CA, USA) placed 20–30  $\mu\text{m}$  from the cell body; in this way, two agonists could be rapidly applied to the same cell. Brief (20–200 ms) duration pulses at 10–20 p.s.i. were typically used to activate  $\alpha 7$ -containing nAChRs, whereas longer (5–10 s) pulses ( $< 10$  p.s.i.) were typically used to activate non- $\alpha 7$  receptors. Drugs studied were diluted at final concentrations in ACSF and were delivered to the cell through a gravity-fed multichannel perfusion system ending with a non-metallic syringe needle (250  $\mu\text{m}$  i.d., WPI) placed just above the slice surface to ensure the homogeneous coverage of the area surrounding the cell being studied.

### Imaging of [Ca<sup>2+</sup>]<sub>i</sub>

Changes in intracellular Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>) in the soma in response to pressure application of either ACh, choline, or NMDA were assessed with conventional fura-2 fluorescence imaging techniques using an Eclipse E600FN upright microscope (Nikon, Japan) with a 40× fluorescence water-immersion objective, a Lambda DG-4 light source (Sutter Instrument Company, Novato, CA, USA) equipped with a stabilized xenon arc lamp (175 W) and four-channel filter interchange system, and a Cascade cooled (−40°C) 16-bit digital camera (Roper Scientific, USA). The AQM Advance imaging software (Kinetic Imaging Ltd, UK) was utilized to acquire and store images for off-line analysis, and to synchronize with membrane current recordings. Fura-2 pentapotassium salt (200–400 μM; Molecular Probes) was dialysed into the cell via the patch pipette, its fluorescence was alternately excited with 340 and 380 nm light, and pairs of images were recorded using a 510 nm emission filter at 5 Hz with 50 ms exposure time for each wavelength used. In each cell tested, background fluorescence was monitored until it is stabilized (usually by 5 min) prior to recordings. Four by four image binning was used to increase the signal-to-noise ratio and time resolution. A region of interest was selected and the 340/380 ratio of fluorescence intensities corrected for background signal were calculated using the imaging software. The ratio values were then converted to [Ca<sup>2+</sup>]<sub>i</sub> levels according to the calibration curve obtained with the *in vitro* Ca<sup>2+</sup> calibration procedure using the Ca<sup>2+</sup> calibration kit (Molecular Probes). This procedure consisted of measurements of the 340/380 ratio in solutions containing 11 different Ca<sup>2+</sup> concentrations from 0 to 39 μM; the ratio value for 0 μM was 0.44, and for 39 μM was 8.4. We also made a calibration according to the method described by Grynkiewicz *et al.* (1985). In this case, [Ca<sup>2+</sup>]<sub>i</sub> levels were calculated using the following equation:

$$[\text{Ca}^{2+}]_i = K(R - R_{\min}) / (R_{\max} - R),$$

where  $R$  is the 340/380 ratio measured in experiments, and  $R_{\min}$  and  $R_{\max}$  are the 340/380 ratio values measured from cells filled with either a Ca<sup>2+</sup>-free intracellular solution (i.e. 10 mM EGTA and no added Ca<sup>2+</sup>), or where fura-2 was saturated with Ca<sup>2+</sup> (1 mM of added Ca<sup>2+</sup>), respectively. All the types of cells studied here (i.e. interneurons, pyramidal cells, and PC12 cells) were calibrated with this method, and we found no significant difference in  $R_{\min}$  and  $R_{\max}$  values between cells (assuming that the Ca<sup>2+</sup> signals in experiments were equally proportional to recorded 340/380 ratio changes); the  $R_{\min}$  and  $R_{\max}$  values for interneurons, pyramidal cells, and PC12 cells were, respectively ( $n = 3$  cells for each),  $0.37 \pm 0.1$  and  $6.0 \pm 0.3$ ,  $0.34 \pm 0.1$  and  $7.1 \pm 0.2$ , and  $0.30 \pm 0.1$  and  $6.1 \pm 0.1$ . The difference between these two calibration methods is

that the  $R_{\min}$  and  $R_{\max}$  values measured intracellularly (Grynkiewicz *et al.* 1985) were slightly lower than the 340/380 ratio measured *in vitro* using the Ca<sup>2+</sup> calibration kit. However, according to our calculations this slight discrepancy may affect background Ca<sup>2+</sup> measurements by no more than 20 nM, and will not have any significant effect on measured Ca<sup>2+</sup> changes. Therefore the values reported here for [Ca<sup>2+</sup>]<sub>i</sub> levels will be based on the calibrations using the *in vitro* calibration kit. Statistical analyses were performed using Origin software (OriginLab Corp., Northampton, MA, USA). Averaged data were presented as means ± s.e.m., and statistical significance was tested using ANOVA.

### Calculation of relative Ca<sup>2+</sup> permeability

We estimated the relative Ca<sup>2+</sup> permeability of the channels by the ratio of the increase in [Ca<sup>2+</sup>]<sub>i</sub> level ( $\Delta[\text{Ca}^{2+}]_i$ ) to the integrated membrane current (charge,  $Q$ ) induced by the activation of these channels in the same cell; this ratio was referred to as the Permeability Index and will be in units of moles per litre per coulomb ( $\text{m C}^{-1}$ ). The time for current integration and [Ca<sup>2+</sup>]<sub>i</sub> amplitude measurement was 1 s after the beginning of agonist application because in most cases, this was the time the maximal Ca<sup>2+</sup> response to choline occurred. Although NMDA responses typically had not peaked by 1 s, to maintain consistency we still used this time for measurement.

## Results

### [Ca<sup>2+</sup>]<sub>i</sub> level increase induced by the activation of $\alpha 7$ (but not non- $\alpha 7$ ) nAChRs in rat hippocampal interneurons in the slice

In acute slices of rat hippocampus, brief (20–200 ms) pressure applications of a maximal dose of the  $\alpha 7$  nAChR-selective agonist, choline (10 mM; Papke *et al.* 1996), to voltage-clamped CA1 stratum radiatum or stratum oriens interneurons elicited rapidly activating  $\alpha 7$ -containing nAChR-mediated responses that decayed quickly (Fig. 1A); previously we have shown that these responses are blocked by the  $\alpha 7$ -selective antagonist methyllycaconitine (MLA; Fayuk & Yakel, 2004). To measure changes in cytoplasmic Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>) in the soma in these voltage-clamped cells, we used the conventional fura-2 fluorescent imaging technique and a digital camera (see Methods). In addition to inducing an inward current response, choline also induced a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> level (Fig. 1A). The basal [Ca<sup>2+</sup>]<sub>i</sub> level was  $44 \pm 5$  nM, and choline significantly increased this by  $7 \pm 1$  nM ( $n = 41$  cells).

We also examined whether the activation of non- $\alpha 7$  nAChRs, which have previously been shown to be less permeable to Ca<sup>2+</sup> than  $\alpha 7$  nAChRs (Fucile, 2004), altered [Ca<sup>2+</sup>]<sub>i</sub> levels. To selectively activate non- $\alpha 7$  nAChRs,

longer (5 s) pressure pulses of ACh (2 mM) were typically used, along with MLA (10 nM) to block  $\alpha 7$  receptors (Fayuk & Yakel, 2004). Unlike with the activation of  $\alpha 7$  nAChRs, the activation of the non- $\alpha 7$  nAChRs did not significantly change the  $[Ca^{2+}]_i$  level (Fig. 1B).

### $[Ca^{2+}]_i$ changes induced by the activation of $\alpha 7$ nAChRs are smaller than during depolarization

The increase in  $[Ca^{2+}]_i$  level induced by choline was compared to  $[Ca^{2+}]_i$  signals induced by depolarization to activate voltage-gated  $Ca^{2+}$  channels (VGCCs). When the membrane potential was depolarized to +10 mV for either 100 ms or 1 s, the increase in  $[Ca^{2+}]_i$  level was greater than that due to the activation of  $\alpha 7$ -containing nAChRs (Fig. 2); the average increase in  $[Ca^{2+}]_i$  level was  $75 \pm 9$  nM ( $n = 8$  cells) and  $208 \pm 28$  nM ( $n = 3$  cells) for the 100 ms and 1 s depolarizations, respectively. This suggests that under our current recording conditions, we are able to detect large changes in the  $[Ca^{2+}]_i$  level, and that the modest changes induced by the activation of  $\alpha 7$ -containing nAChRs were not due to technical limitations.

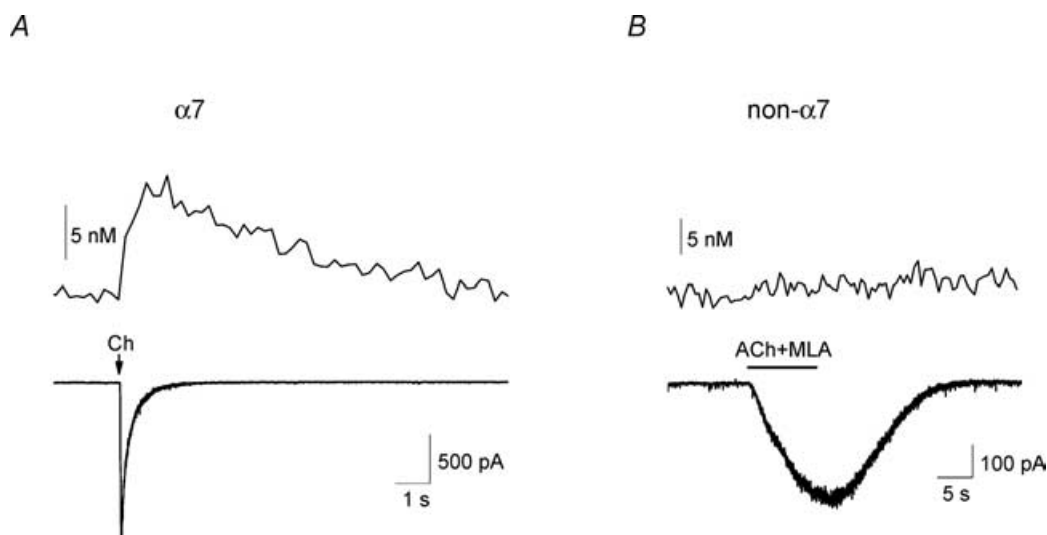
### Comparison of $[Ca^{2+}]_i$ signals induced by the activation of $\alpha 7$ nAChR and NMDA glutamate receptor subtypes

We compared the  $[Ca^{2+}]_i$  signal induced by the activation of the  $\alpha 7$  nAChR with those from the activation of

the NMDA receptor subtype of ionotropic glutamate receptor channel, the latter of which is known to be highly  $Ca^{2+}$  permeable. For the interneurone shown in Fig. 3A, the amplitude of the current response induced by NMDA (1 mM) was much smaller than that induced by choline (10 mM); however, NMDA induced a larger  $[Ca^{2+}]_i$  signal. To quantify and compare the relative  $Ca^{2+}$  permeability, we plotted the peak increase in  $[Ca^{2+}]_i$  level ( $\Delta[Ca^{2+}]_i$ ) versus the integrated membrane current (i.e. charge,  $Q$ ) (see Methods for details); the slope of this line (i.e. the  $\Delta[Ca^{2+}]_i/Q$  ratio) was termed the Permeability Index (PI; Fig. 3). For both NMDA and choline, there was a near-linear relationship between  $Q$  and  $\Delta[Ca^{2+}]_i$  (Fig. 3C). The PI was significantly larger for responses activated by NMDA ( $78 \pm 8$  mC $^{-1}$ ;  $n = 25$  cells) than for choline ( $36 \pm 3$  mC $^{-1}$ ;  $n = 41$  cells;  $P < 0.001$ ). Therefore on this basis, we estimate that the  $Ca^{2+}$  permeability of the  $\alpha 7$ -containing nAChRs in rat hippocampal interneurons is about 46% of that of NMDA receptors in the same cells.

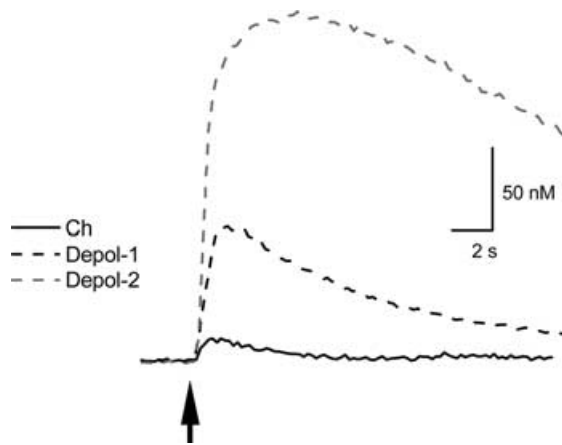
### $[Ca^{2+}]_i$ signals induced by $\alpha 7$ nAChR activation are not due to $Ca^{2+}$ intracellular store depletion, nor the activation of VGCCs

We tested whether the  $[Ca^{2+}]_i$  signals induced by activation of the  $\alpha 7$  nAChRs were due to the influx of  $Ca^{2+}$  directly through these receptors, or via other potential sources of  $Ca^{2+}$ . To test whether intracellular  $Ca^{2+}$  store depletion was involved, slices were pretreated with ryanodine and



**Figure 1.** Increased  $[Ca^{2+}]_i$  level due to activation of  $\alpha 7$ , but not non- $\alpha 7$ , nAChRs

A, brief (20–200 ms) duration pressure applications (arrows) of choline ('Ch'; 10 mM) selectively activated  $\alpha 7$ -containing nAChRs in a neurone, inducing a rapidly activating and decaying inward current response (bottom trace), and a slower activating and decaying increase in fluorescence due to an increasing  $[Ca^{2+}]_i$  level (top trace). B, in a different neurone, the activation of non- $\alpha 7$  nAChRs by longer (10 s) pressure applications of ACh (2 mM), along with the  $\alpha 7$ -selective antagonist MLA (10 nM), induced a more slowly activating inward current response (bottom trace) without any significant change in  $[Ca^{2+}]_i$  level (top trace).



**Figure 2. [Ca<sup>2+</sup>]<sub>i</sub> changes due to activation of α7 nAChRs are smaller than during depolarization**

The increase in [Ca<sup>2+</sup>]<sub>i</sub> level due to a brief application of choline (10 mM; arrow; continuous line) is smaller than the increase in [Ca<sup>2+</sup>]<sub>i</sub> level due to depolarization to +10 mV for a duration of either 100 ms ('Depol-1'; black dashed line) or 1 s ('Depol-2'; grey dashed line).

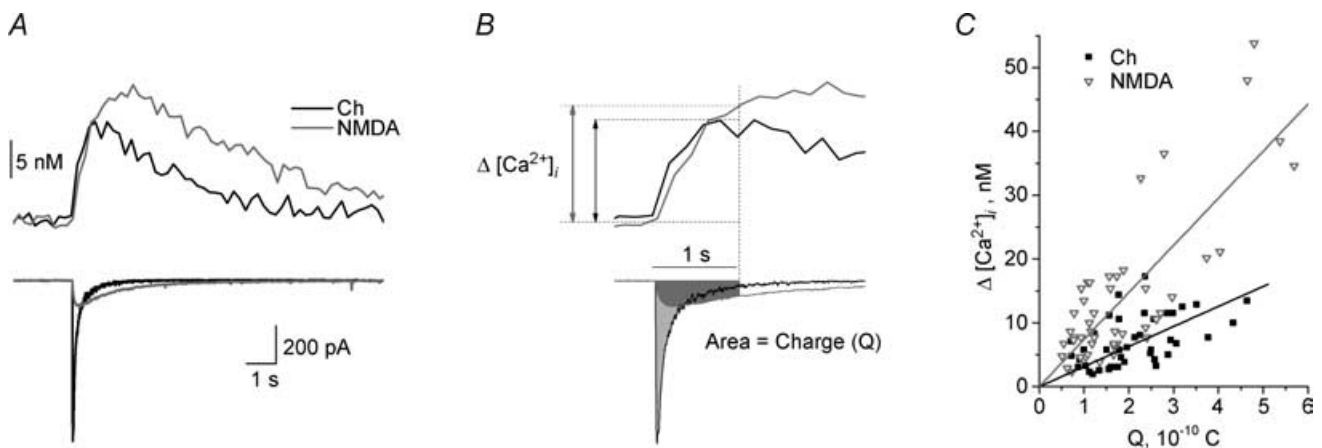
cyclopiazonic acid (CPA). Ryanodine binds to ryanodine receptors (RyRs), blocking the calcium-induced calcium release (CICR) from internal stores (McPherson *et al.* 1991). Internal stores may also release Ca<sup>2+</sup> via activation of IP<sub>3</sub> receptors (Berridge, 1993). CPA, a selective blocker of sarcoplasmic-endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA pumps; Seidler *et al.* 1989), was used to deplete the smooth endoplasmic reticulum-derived Ca<sup>2+</sup> stores.

After inducing a choline response, ryanodine and CPA (both at 20 μM) were applied for 10 min; this treatment slowly increased [Ca<sup>2+</sup>]<sub>i</sub> levels, which generally returned to baseline levels. After the addition of ryanodine and CPA, neither the peak amplitude of the [Ca<sup>2+</sup>]<sub>i</sub> signal, nor the amplitude of the choline-induced response, was significantly changed in all cells tested (*n* = 3 cells; tested both in control and with ryanodine and CPA; Fig. 4A). In addition, ryanodine and CPA had no significant effect on NMDA receptor-induced [Ca<sup>2+</sup>]<sub>i</sub> signals (*n* = 4 cells; data not shown). These data indicate that CICR does not contribute to the [Ca<sup>2+</sup>]<sub>i</sub> signals observed during either choline- or NMDA-induced responses.

We also tested whether Ca<sup>2+</sup> influx through VGCCs might have contributed to the choline- or NMDA-induced [Ca<sup>2+</sup>]<sub>i</sub> signals. To do this, VGCCs were blocked with cadmium (Cd<sup>2+</sup>; 200 μM); this did not significantly affect the Ca<sup>2+</sup> permeability index for responses to either choline (*n* = 3 cells; Fig. 4B) or NMDA (*n* = 6 cells; data not shown).

**Ca<sup>2+</sup> permeability of native α7 nAChRs in PC12 cells and NMDA receptors in pyramidal neurones**

We explored the relative Ca<sup>2+</sup> permeability of native α7 nAChRs and NMDA receptors in other cell types to determine if the lower permeability of the α7 nAChRs (as compared to NMDA receptors) was specific to



**Figure 3. Comparison of [Ca<sup>2+</sup>]<sub>i</sub> signals due to activation of α7 and NMDA receptors**

A, the application of choline (10 mM; black traces) and NMDA (1 mM; grey traces) in the same neurone induced inward current responses (bottom traces) with different peak amplitudes and kinetics, as well as increases in [Ca<sup>2+</sup>]<sub>i</sub> (top traces). Even though the amplitude of the NMDA current response was much smaller, the resulting increase in [Ca<sup>2+</sup>]<sub>i</sub> was larger than for choline. B, the total amount of Ca<sup>2+</sup> influx (as indicated by the increase in [Ca<sup>2+</sup>]<sub>i</sub> level; top) is proportional to the integrated membrane current (i.e. charge, *Q*), the latter of which is estimated as the area under the current trace (bottom). The time for current integration and [Ca<sup>2+</sup>]<sub>i</sub> amplitude measurement was 1 s after the beginning of agonist application (horizontal bar). C, the plot of the peak increase in [Ca<sup>2+</sup>]<sub>i</sub> level versus the corresponding integrated current (*Q*) for choline (filled black squares) and NMDA (open grey triangles) responses yielded a near-linear relationship (linear regressions yielded *R* and *P* values of 0.52 and < 0.003 for choline, and 0.80 and < 0.0001 for NMDA). We have termed the slope of these lines the Permeability Index (PI).

interneurons. In cultured pheochromocytoma 12 (PC12) cells, the activation of  $\alpha 7$  nAChRs with choline induced  $[Ca^{2+}]_i$  signals that were significantly larger (the PI was  $63 \pm 8 \text{ M C}^{-1}$ ;  $n = 10$  cells;  $P < 0.002$ ) than the  $[Ca^{2+}]_i$  signals induced by choline in rat hippocampal interneurons (Fig. 5). Furthermore, the PI for the  $\alpha 7$  nAChRs in PC12 cells was not significantly different from that for NMDA receptors in interneurons (Fig. 5). These data suggest that the  $Ca^{2+}$  permeability of the  $\alpha 7$ -containing nAChRs in rat hippocampal interneurons is significantly less than that for  $\alpha 7$  nAChRs in PC12 cells.

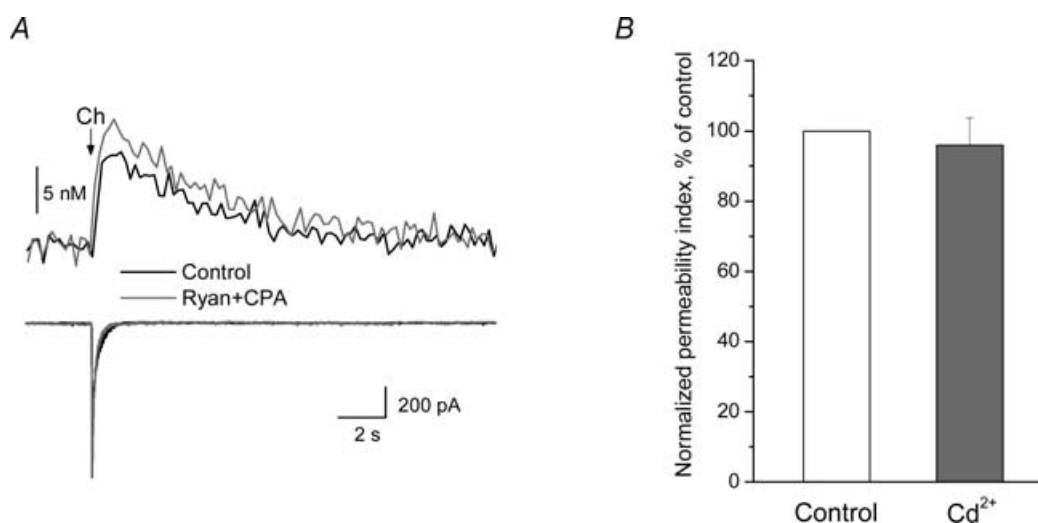
We also compared the  $Ca^{2+}$  permeability of the NMDA receptors in CA1 pyramidal neurons to that in interneurons. The PI of NMDA responses in these pyramidal neurons ( $63 \pm 7 \text{ M C}^{-1}$ ;  $n = 5$  cells) was not significantly different from either NMDA receptors in interneurons, or the  $\alpha 7$  nAChRs in PC12 cells (Fig. 5). Therefore, it appears as if the  $\alpha 7$ -containing nAChRs in rat hippocampal interneurons are significantly less permeable to  $Ca^{2+}$  than either NMDA receptors (in both interneurons and pyramidal cells) or  $\alpha 7$  nAChRs in PC12 cells.

## Discussion

In the hippocampus, nAChRs are linked to synaptic plasticity and cognitive function, which may be due in part to their ability to elicit local changes in cytoplasmic  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) levels. Different subtypes of nAChRs are known to be differentially permeable to  $Ca^{2+}$ , with the  $\alpha 7$ -containing nAChRs generally considered to be

the most highly  $Ca^{2+}$  permeable (Fucile, 2004). Rat hippocampal CA1 interneurons are known to express both  $\alpha 7$ - and non- $\alpha 7$ -containing nAChRs (Jones *et al.* 1999), and an understanding of the  $Ca^{2+}$  permeability of these receptors in the slice will help to elucidate any involvement of  $Ca^{2+}$ -dependent signal transduction cascades in nAChR-mediated synaptic plasticity. In addition, these functional data are helpful in the understanding of the molecular makeup of these receptors in these neurons.

Combining patch-clamp electrophysiological recordings with conventional fura-2 imaging techniques to measure changes in  $[Ca^{2+}]_i$  in the soma of voltage-clamped neurons, we have estimated the relative  $Ca^{2+}$  permeability of  $\alpha 7$ -containing nAChRs in rat hippocampal CA1 interneurons and compared this with the NMDA subtype of ionotropic glutamate receptor channels. We compared the ratio of the increase in somal  $[Ca^{2+}]_i$  level ( $\Delta[Ca^{2+}]_i$ ) to the integrated transmembrane current (charge, Q) induced by the activation of these channels; we refer to this ratio as the Permeability Index (PI). This method should be more sensitive to estimating the actual amount of  $Ca^{2+}$  entering cells than by using the classical Goldman-Hodgkin-Katz (GHK) constant field equations, which rely on measuring shifts in reversal potential due to changes in the concentrations of permeant ions (Fucile, 2004). In addition, the strong rectification for both  $\alpha 7$  and non- $\alpha 7$  nAChR-mediated single-channel currents in excised outside-out patches from these interneurons, in which no outward current flow through



**Figure 4.**  $[Ca^{2+}]_i$  signals due to  $\alpha 7$  nAChR activation are not due to  $Ca^{2+}$  intracellular store depletion, nor the activation of VGCCs

A, after inducing a choline response, slices were treated for 10 min with ryanodine and CPA (both at  $20 \mu\text{M}$ ) to prevent intracellular store depletion during  $\alpha 7$  nAChR activation. Neither the peak amplitude of the  $[Ca^{2+}]_i$  signal (top traces) nor the current response (bottom traces) was significantly changed by pretreatment with ryanodine and CPA. B, the application of cadmium ( $Cd^{2+}$ ;  $200 \mu\text{M}$ ) had no significant effect on the PI (3 cells; tested both in control and with  $Cd^{2+}$ ).

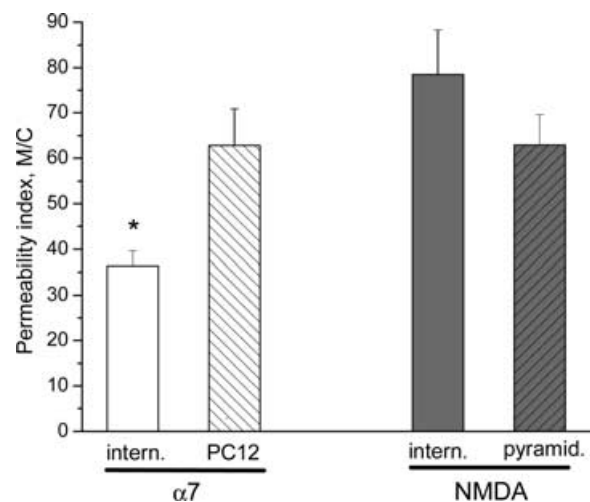
these channels was observed at positive holding potentials (Shao & Yakel, 2000), prevents the accurate determination of the reversal potential. By combining fluorescence imaging with patch-clamp recordings, we found that the relative Ca<sup>2+</sup> permeability of the  $\alpha 7$ -containing receptors in interneurons was about half that of the NMDA receptors. Furthermore, we did not detect any significant Ca<sup>2+</sup> permeability of the non- $\alpha 7$  nAChRs, although it is possible that these receptors do have significant Ca<sup>2+</sup> permeability (although significantly lower than  $\alpha 7$  receptors; Fucile, 2004), which was lower than the detection limit of our recording system. Previously we did observe non- $\alpha 7$  nAChR-mediated single-channel currents from patches excised from the soma, albeit at a low density (i.e. much lower than  $\alpha 7$  receptors; Shao & Yakel, 2000).

We considered whether the Ca<sup>2+</sup> permeability of native  $\alpha 7$  receptors in other cell types might be similar to that in rat hippocampal interneurons, or whether the Ca<sup>2+</sup> permeability of the  $\alpha 7$  receptors in interneurons might be lower than in other cell types. This is critical information since Ca<sup>2+</sup> regulates a variety of signal transduction cascades and plays a key role in the short- and long-term regulation of nAChRs (Berg & Conroy, 2002; Quick & Lester, 2002). In PC12 cells, the Ca<sup>2+</sup> permeability of native  $\alpha 7$  nAChRs, based on its PI, was significantly larger than in hippocampal interneurons, and interestingly not significantly different from that for NMDA receptors in hippocampal interneurons. Furthermore, we compared the PI of NMDA in pyramidal cells to that in interneurons, and found that there was no significant difference. These data suggest that  $\alpha 7$ -containing nAChRs in rat hippocampal interneurons are significantly less permeable to Ca<sup>2+</sup> than either NMDA receptors (in both interneurons and pyramidal cells) or  $\alpha 7$  nAChRs in PC12 cells. Furthermore, the relative Ca<sup>2+</sup> permeability of  $\alpha 7$  receptors in PC12 cells is similar to NMDA receptors, an indication that it may not be the type of cells that can explain the lower Ca<sup>2+</sup> permeability of  $\alpha 7$  nAChRs in the interneurons. However, currently we cannot rule out the possibility that  $\alpha 7$ -containing receptors selectively in interneurons are closely linked with elements that bind Ca<sup>2+</sup>, and therefore hinder its access to fura-2.

Measuring fluorescence signals in the soma to estimate the relative Ca<sup>2+</sup> influx for membrane-bound ion channels underestimates the true increases in [Ca<sup>2+</sup>]<sub>i</sub> near the mouth of the channel due to diffusion, buffering and pumps, among other factors (Berridge *et al.* 2003; Dajas-Bailador & Wonnacott, 2004). Therefore the influx of Ca<sup>2+</sup> through the  $\alpha 7$  receptors, which we have shown significantly increases somal [Ca<sup>2+</sup>]<sub>i</sub>, might be enough to regulate these channels either directly, or through Ca<sup>2+</sup>-dependent signal transduction cascades. We previously have shown that the rate of recovery from

desensitization of these  $\alpha 7$  receptors is regulated by [Ca<sup>2+</sup>]<sub>i</sub> (Khiroug *et al.* 2003). However, whether or not it is the Ca<sup>2+</sup> influx directly through the channel or through another Ca<sup>2+</sup>-dependent mechanism is at present unknown. Besides regulating ion channel function and neuronal excitability, the influx of Ca<sup>2+</sup> through the  $\alpha 7$  receptors may also regulate other processes such as neurotransmitter release, gene expression, cell proliferation, survival and death, and development (Role & Berg, 1996; Dajas-Bailador & Wonnacott, 2004).

Although we have shown here that the increase in [Ca<sup>2+</sup>]<sub>i</sub> due to the activation of the  $\alpha 7$  nAChRs was not due to calcium-induced calcium release (CICR) from internal stores, nor the activation of voltage-gated Ca<sup>2+</sup> channels (VGCCs), both of these have previously been shown to participate in the nAChR-mediated increases in [Ca<sup>2+</sup>]<sub>i</sub> in native systems. For example in unclamped cells, the activation of nAChRs will depolarize the neurones and induce the activation of VGCCs, the activation of which will dramatically increase [Ca<sup>2+</sup>]<sub>i</sub> and augment the nAChR-mediated increase in [Ca<sup>2+</sup>]<sub>i</sub> (Dajas-Bailador & Wonnacott, 2004). However local [Ca<sup>2+</sup>]<sub>i</sub> increases through  $\alpha 7$  nAChRs, rather than through VGCCs, may be more useful in regulating signal transduction cascades. For example in chick ciliary ganglion neurones, the activation of  $\alpha 7$ -containing nAChRs induces Ca<sup>2+</sup> influx, which activates the CaMK and MAPK pathways, leading to activation of the transcription factor CREB and gene expression (Chang & Berg, 2001). This effect is blocked by VGCCs, the activation of which initially leads to CREB activation; however, it also stimulates the phosphatases



**Figure 5. Ca<sup>2+</sup> permeability of native  $\alpha 7$  nAChRs in PC12 cells and NMDA receptors in pyramidal neurones**

The PI values for the choline-induced activation of  $\alpha 7$  nAChRs (left open bars) in interneurons ('intern.') was significantly smaller than in PC12 cells. For NMDA responses (right bars), there was no significant differences in the PI between interneurons and pyramidal ('pyramid.') cells.

calcineurin and PP1, which ultimately blocks CREB activation and gene expression. The nAChR-mediated  $\text{Ca}^{2+}$  influx has also previously been shown to induce CICR (Dajas-Bailador & Wonnacott, 2004). For example, glutamatergic release onto hippocampal CA3 pyramidal neurones is facilitated presynaptically by CICR induced by presynaptic nAChRs (Sharma & Vijayaraghavan, 2003). In mouse substantia nigra pars compacta neurones in the slice, the block of intracellular  $\text{Ca}^{2+}$  stores by dantrolene prevents the increase in  $[\text{Ca}^{2+}]_i$  due to the activation of  $\alpha 7$ -containing nAChRs (Tsuneki *et al.* 2000). Lastly, both CICR and VGCCs appear to contribute to the nAChR-mediated  $\text{Ca}^{2+}$  signals in SH-SY5Y cells (Dajas-Bailador *et al.* 2002).

Although the physiological significance of the  $\text{Ca}^{2+}$  influx and modulation of the  $\alpha 7$ -containing nAChRs in hippocampal interneurons remain to be determined, the lower relative  $\text{Ca}^{2+}$  permeability of these receptors than those in PC12 cells, or from a variety of heterologous expression systems (Fucile, 2004), may be a further indication of a difference in the properties, and perhaps molecular makeup, of these receptors. For example, for recombinant homomeric receptors expressed in *Xenopus* oocytes, the  $\text{Ca}^{2+}$  permeability of  $\alpha 7$  receptors was greater than that for NMDA receptors (Séguéla *et al.* 1993). Interestingly Castro & Albuquerque (1995), who estimated and compared the relative  $\text{Ca}^{2+}$  permeability of native  $\alpha 7$  nAChRs and NMDA receptors in cultured hippocampal neurones, estimated that the  $\text{Ca}^{2+}$  permeability of the  $\alpha 7$  receptors was about 59% that of the NMDA receptors, a value comparable to our estimate of 46% reported here. Perhaps this lower  $\text{Ca}^{2+}$  permeability of the hippocampal  $\alpha 7$  receptors may be due to differential modulation (Fucile, 2004). However, recently a new  $\alpha 7$  subunit isoform was discovered,  $\alpha 7-2$ , which is expressed in both central and peripheral neurones (Severance *et al.* 2004). When expressed in *Xenopus* oocytes, this  $\alpha 7-2$  subunit forms functional channels with slower desensitization kinetics, reversible block by  $\alpha$ -bungarotoxin, and higher affinity for ACh than the original  $\alpha 7-1$  subunit (Severance *et al.* 2004). However, it was not determined if the  $\text{Ca}^{2+}$  permeability of these receptors was altered.

Another possibility to explain functional diversity among  $\alpha 7$  receptors, both native and expressed, is coassembly with other nAChR subunits. Although it had been previously thought that mammalian  $\alpha 7$  subunits mostly form homomeric receptors (McGehee & Role, 1995; Chen & Patrick, 1997; Drisdell & Green, 2000), multiple functional subtypes of  $\alpha 7$ -containing nAChRs, with properties not identical to those expected of homomeric  $\alpha 7$  receptors, have been reported in rat autonomic ganglia (Cuevas & Berg, 1998; Cuevas *et al.* 2000) and hippocampal interneurons (Shao & Yakel, 2000; Sudweeks & Yakel, 2000), suggesting the possibility of heteromeric  $\alpha 7$ -containing nAChRs. In particular,

the native  $\alpha 7$ -containing receptors in the hippocampal interneurons desensitize more slowly, and have a smaller single-channel conductance in comparison to the properties of recombinant heterologously expressed receptors (Shao & Yakel, 2000; Sudweeks & Yakel, 2000). We previously found that the  $\alpha 7$  and  $\beta 2$  subunits were coexpressed in individual rat hippocampal interneurons and were both correlated with fast-activating responses (Sudweeks & Yakel, 2000), and that the  $\alpha 7$  and  $\beta 2$  subunits coassembled to form a functional heteromeric nAChR in heterologous expression systems (i.e. *Xenopus* oocytes and TSA120 cells; Khiroug *et al.* 2002). In the chick, the properties of native  $\alpha 7$  nAChRs often do not match those of heterologously expressed homomeric  $\alpha 7$  nAChRs, and  $\alpha 7$ -containing heteromeric chick nAChRs can be formed in heterologous expression systems (Anand *et al.* 1993; Yu & Role, 1998; Girod *et al.* 1999; Palma *et al.* 1999). PC12 cells express multiple diverse subtypes of  $\alpha 7$ -containing nAChRs, and single-cell RT-PCR analysis revealed that these cells express (in addition to the  $\alpha 7$  subunit)  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$  and  $\beta 4$  subunits (Virginio *et al.* 2002). Furthermore, the nAChR-mediated single-channel currents from outside-out patches in PC12 cells (Sands & Barish, 1992) do not correspond to the  $\alpha 7$ -containing nAChR single-channel currents in rat hippocampal neurones in slices (Shao & Yakel, 2000). Therefore, coassembly with other nAChR subunits, as well as other  $\alpha 7$  subunit isoforms (including potential ones yet to be discovered), may help to explain the apparent lower  $\text{Ca}^{2+}$  permeability of  $\alpha 7$  receptors in rat hippocampal interneurons.

Using the local photolysis of caged carbachol to activate nAChRs, we previously showed that the  $\alpha 7$ -containing nAChRs on these rat hippocampal interneurons were located primarily at the soma and proximal dendrites (i.e.  $< 70 \mu\text{m}$  from the soma), which was different from that of the ionotropic glutamate receptors (Khiroug *et al.* 2003). The glutamate uncaging-induced currents, including both NMDA and non-NMDA components, are expressed on the soma and along the entire dendritic field in these interneurons (Pettit & Augustine, 2000; Khiroug *et al.* 2003). Therefore, since the  $\text{Ca}^{2+}$  signal induced by the activation of  $\alpha 7$ -containing nAChRs will be through receptors located primarily on the soma, with little to no contribution from receptors on the distal dendrites, the lower relative  $\text{Ca}^{2+}$  permeability of  $\alpha 7$  receptors in rat hippocampal interneurons is not likely to be due to the differential distribution of these receptors.

In conclusion, we have found that the relative  $\text{Ca}^{2+}$  permeability of  $\alpha 7$ -containing nAChRs in rat hippocampal interneurons is approximately half that of the NMDA subtype of glutamate receptors in these neurones and native  $\alpha 7$ -containing nAChRs in PC12 cells. Although the reasons for this unexpected lower  $\text{Ca}^{2+}$



permeability are at present unknown, this may indicate a differential subunit makeup (e.g. the functional expression and coassembly with other  $\alpha 7$  subunit isoforms or non- $\alpha 7$  subunits), or regulation, of these receptors.

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