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Choline induces Ca^{2+} entry in cultured sympathetic neurones isolated from rat superior cervical ganglion

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Abstract

Choline has been shown to be a specific agonist at α7 nicotinic acetylcholine receptors, which are the most Ca^{2+} permeable of the ionotropic receptor channels. Whole-cell patch recording combined with the measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$, using Indo1, in cultured rat superior cervical ganglion neurones demonstrated that application of choline induced a slowly desensitizing inward current and increased $[Ca^{2+}]_i$. The effect was dose dependent with an EC_{50} of 1.6 mM and an n_H of 1.19. The relationship between the elevation of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) and charge transfer analysed under various recording conditions showed that the $\Delta[Ca^{2+}]_i$ induced by choline resulted from an influx of Ca^{2+} through nicotinic acetylcholine receptors. The effect of choline on the membrane current and $\Delta[Ca^{2+}]_i$ was not affected by either short application or pretreatment with α-bungarotoxin (50 nM) and methyllycaconitine (1 nM), two α7 nicotinic receptors antagonists. These results indicate that activation of non-α7 nicotinic acetylcholine receptors by choline significantly increases the Ca^{2+} concentration in rat superior cervical ganglion neurones.

Keywords: Choline; Nicotinic receptor; Sympathetic neurone; Intracellular Ca^{2+}; Indo1

1. Introduction

The action of acetylcholine is believed to end primarily with its hydrolysis by acetylcholinesterase to form acetate and choline. However, it is now believed that the metabolite choline can still influence fast chemical transmission by activating nicotinic acetylcholine receptors. This leads to the interesting view that not only choline may play a role in cholinergic neurotransmission but also the action of acetylcholine may be ultimately ended by the uptake of choline rather than by the hydrolysis of acetylcholine. It is therefore important to characterise the effects of choline at nicotinic acetylcholine receptors. Currently, eleven nicotinic acetylcholine receptors subunits (α2–α7, α9–α10 and β2–β4) are known to be expressed in the rat nervous system (Elgoyhen et al., 2001; McGehee and Role, 1995) and assembled in a pentameric homomeric or heteromeric fashion to form a vast family of nicotinic acetylcholine receptors (McGehee and Role, 1995). Choline has been shown to have a different efficacy at purified nicotinic acetylcholine receptors when they are expressed in oocytes. Choline does not activate α4β2 and α3β2 nicotinic receptors and it is probably only a partial agonist at several nicotinic acetylcholine receptors such as α3β4 receptors but choline would appear to be a full agonist at homomeric α7-containing receptors (Mandelzys et al., 1995; Papke et al., 1996). Homomeric α7 nicotinic acetylcholine receptors are inhibited slowly and irreversibly by α-bungarotoxin (Couturier et al., 1990) and methyllycaconitine (Drasdo et al., 1992) and show rapid desensitization on application of an agonist. Thus the induction by choline of a fast desensitizing current irreversibly blocked by α-bungarotoxin has been interpreted as evidence for the presence of α7 nicotinic acetylcholine receptors and choline has therefore been used as a selective tool to track functional α7 receptors in native neurones (Alkondon et al., 1997). There is, however, growing evidence for the existence of atypical α7 nicotinic acetylcholine receptors in native cells since acetylcholine and/or choline can induce slowly desensitizing currents which are rapidly and reversibly blocked by α-bungarotoxin (Cuevas and Berg, 1998; Cuevas et al., 2000; Yu and Role, 1998).

So far, the effect of choline on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) has not been well documented. In rat

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sympathetic neurones, it was suggested that choline can release Ca^{2+} from internal stores (Koike et al., 1989) and thereby increase the levels of α7 mRNA expression (De Koninck and Cooper, 1995). On the other hand, non-α7 nicotinic acetylcholine receptors are permeable to Ca^{2+} to a variable extent (Fieber and Adams, 1991; Mulle et al., 1992a; Trouslard et al., 1993; Vernino et al., 1992), and homomeric α7 receptors have the highest relative permeability to Ca^{2+} among all the cationic ionotropic receptors so far described (Bertrand et al., 1993; Castro and Albuquerque, 1995; Séguéla et al., 1993). Thus by activating non-α7 and α7 nicotinic acetylcholine receptors, choline may induce Ca^{2+} entry and the subsequent rise in intracellular Ca^{2+}($\Delta[Ca^{2+}]_i$) may influence Ca^{2+}-dependent functions.

In the present study, we investigated the effect of choline on $[Ca^{2+}]_i$ in rat sympathetic neurones. We used cultured rat superior cervical ganglion neurones, which express several nicotinic acetylcholine receptors subunits α3, α4,α5, α7, β2 and β4 (Rust et al., 1994; Skok et al., 1999). Superior cervical ganglion neurones express nicotinic acetylcholine receptors containing the α3 and β4 subunits (α3β4* nicotinic acetylcholine receptors) (Covernton et al., 1994; Kristufek et al., 1999; Mandelzys et al., 1995) and both typical and atypical α7 nicotinic receptors (Cuevas et al., 2000). Using patch-clamp techniques combined with Indo1 spectrofluorimetric measurements, we determined the effect of choline on the membrane current and the associated changes in intracellular Ca^{2+} concentration. We also tried to correlate the effects of choline with the activation of a given subtype of nicotinic acetylcholine receptor. We show that choline does not mobilize internal Ca^{2+} stores but induces a significant rise in $[Ca^{2+}]_i$ by allowing Ca^{2+} entry through nicotinic receptors which are of the non-α7 type.

2. Materials and methods

2.1. Neuronal cultures

Superior cervical ganglia were dissected from young (9–15 days old) Wistar rats and dissociated according to previously published methods (Marrion et al., 1987). Rats were killed by decapitation after ether inhalation. We removed the ganglia and incubated them initially for 15 min at 37 °C in minimum essential Eagle medium α (Gibco) containing type 4 collagenase (1.5 mg/ml; Gibco) and then for 30 min at 37 °C in medium containing trypsin (1 mg/ml; Gibco). Finally, ganglia were triturated with a fire-polished Pasteur pipette and centrifuged at 900 rpm for 3 min. The supernatant was removed and replaced with culture medium, the composition of which was the following: minimum essential Eagle medium α, foetal calf serum (10%; Gibco), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco) and nerve growth factor (10 ng/ml; Allomone). Cells were directly plated into the recording chamber coated with poly-L-lysine (10 μg/ml). This chamber consisted of a glass ring (diameter 15 mm) attached to a glass cover slip with Sylgard (Rhodorsil, Rhône-Poulenc). The chamber was maintained in a 35-mm culture dish in a humid atmosphere of 95% O_2–5% CO_2 at 37 °C.

2.2. Electrophysiological recordings

Currents were recorded with whole-cell and perforated-patch clamp techniques (Horn and Marty, 1988) using a List EPC-7 amplifier and low-resistance (2–4 MΩ) electrodes. The external solution contained (in mM): NaCl 140, KCl 3 $CaCl_2$ 2.5, $MgCl_2$ 1.2, HEPES 10, glucose 11.1, pH 7.2. For conventional whole-cell experiments, the pipette was filled with a solution containing (in mM): K-acetate 110, EGTA 0.1, KCl, 20, $MgCl_2$ 1.2, HEPES 10, Indo1 0.1, pH 7.2. In perforated-patch experiments, cells were preloaded with the acetoxymethyl ester form of Indo1 (Indo1-AM) by incubation with 2 μM Indo1-AM for 45–60 min in the culture medium at 37 °C. The perforated-patch configuration was obtained using amphotericin B as the pore-forming agent (Rae et al., 1991). The pipette was filled at its tip with a solution containing (in mM): K-acetate 110, EGTA 0.1, KCl, 20, $MgCl_2$ 1.2, HEPES 10, pH 7.20 and then backfilled with the same solution containing amphotericin B (150 μg/ml). The amphotericin B (Sigma) stock solution (3 mg/100 μl) was prepared in dimethylsulfoxide just before the recording session.

2.3. Intracellular Ca^{2+} measurements

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was estimated from Indo1 fluorescence using the ratio method described by Grynkiewicz et al. (1985). The recording chamber was placed on the stage of an Olympus inverted microscope (IMT-2) fitted with UV epifluorescence illumination. UV excitation was provided by a Mercury arc lamp (Osram HBO-100, Germany) and a 360-nm excitation filter. UV light was reflected by a 380-nm dichroic mirror and transmitted to the cell by an oil immersion objective (Nikon fluorite 40×, NA 1.4). Emitted light was split by a 455 dichroic mirror to two photomultipliers (Hamamatsu type RI 104 supplied by a 900 V DC voltage source) with input filters at 405 and 485 nm. Recordings were spatially restricted to a single cell by a rectangular diaphragm. Home-built amplifiers converted the anodal photomultiplier currents to voltage and gave the ratio between the emissions at 405 and 485 nm (405/485 ratio).

Briefly, $[Ca^{2+}]_i$ was estimated by using the equation: $Ca = K_d(Fo/Fs)(R - R_{min})/(R_{max} - R)$ (Grynkiewicz et al., 1985), where K_d is the dissociation constant of Indo1 for Ca^{2+}, Fo/Fs is the maximum excursion at 485 nm, R is the recorded 405/485 emission ratio, R_{min} is the minimal value of R at zero $[Ca^{2+}]_i$ and R_{max} is the value of R at saturation $[Ca^{2+}]_i$. Signals were calibrated in vitro using mixtures, in a

variable ratio, of two solutions containing, respectively (in mM): CaEGTA 10, KCl 100 mM, HEPES 10, Indo1 0.2, pH 7.2 and $EGTAK_2$ 10, KCl 100, HEPES 10, Indo1 0.2, pH 7.20. For each mixture, the theoretical free Ca^{2+} concentration was calculated using the program ''Freeion'' designed by Fabiato (1979). The ratio 405/485 was measured for each solution and plotted against the calculated free Ca^{2+} concentration. Data points were fitted to the derived Grynkiewicz equation: $R = R_{min} + Ca(R_{max} - R_{min})/(K_d(Fo/Fs) + Ca)$. The best fit was obtained using $R_{min} = 0.05$, $R_{max} = 1.75$ and $K_d(Fo/Fs) = 310$ nM. Indo1 values were corrected for background fluorescence.

2.4. Data acquisition and treatment

Whole-cell currents and 405 and 485 signals were digitized on line using a Digitata 1200 interface (Axon Instruments) driven by pClamp 6 (Axon Instruments). Current amplitude, decay time constant of current, and 405/485 ratio were determined using Clampfit 8.2.0 (Axon Instruments). Ca^{2+} elevation was calculated by programming the Grynkiewicz equation in Excel software (Microsoft). For the dose–response curve, the data points were fitted to the Hill equation $I = I_{max}([A]^{n_H})/([A]^{n_H} + EC_{50}^{n_H})$ using Origin 5.0 (Microcal software), where EC_{50} is the half-maximal concentration of the agonist and n_H is the Hill coefficient.

We calculated the charge transfer in response to choline by integrating the current over time using Clampfit 8.2.0 software, Axon Instruments. In some cases, we calculated the peak current density by normalizing the amplitude by the cell capacitance. The capacitance was calculated from the capacitive current induced by voltage jumps from -60 to -80 mV. In most cases, the current signal was fitted by a mono-exponential function $A = A_1 \exp(-t/\tau) + \text{constant}$, where τ is the time constant. The fit was performed with Clampfit. The capacity (C) was calculated using the time constant and the equation $\tau = R_s C$, where R_s is the serial resistance. R_s was calculated by dividing the voltage jump amplitude (20 mV) by the peak amplitude of the capacitive current.

2.5. Drugs and application of substances

Acetylcholine, atropine, α-bungarotoxin, methyllycaconitine, D-tubocurarine, thapsigargin and caffeine were purchased from Sigma. Choline was from Janssen Chimica. Indo1 and Indo1-AM were from Molecular Probes. Drugs were diluted in the external solution at the concentration indicated and were applied by local perfusion using a U-tube (Fenwick et al., 1982).

2.6. Expression of results

All statistical results are given as means ± S.E.M. Statistical differences were determined using Student's t-test or one-way analysis of variance (ANOVA, Origin, Microcal software) by setting the confidence interval at 0.05.

3. Results

In all experiments, both choline and acetylcholine were applied in the presence of atropine (1 μM) to block muscarinic acetylcholine receptors. Except when stated, all recordings were made under the perforated patch configuration. No difference in the response to choline was noticed between perforated patch and conventional whole-cell configurations. The time between applications of nicotinic receptor agonist was 2 min. This interval was found necessary to allow recovery of nicotinic acetylcholine receptors from desensitization. We did not attempt to block acetylcholinesterase in the experiments when we compared the effects of choline to those produced of acetylcholine.

3.1. Choline induced an inward current and increased the free internal Ca^{2+} concentration

In the current-clamp configuration, the application of 10 mM choline for 5 s depolarized the neurones from a resting membrane potential of -61.6 ± 1.2 to -28.4 ± 1.7 mV ($n = 7$ cells). This was accompanied by a burst of two to four action potentials (Fig. 1A, left upper trace). Simultaneously, we detected a rise in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) from a basal level of 104.8 ± 15.5 to 158.5 ± 27.8 nM ($\Delta[Ca^{2+}]_i = 54.6 \pm 13.2$ nM) (Fig. 1A, left lower trace). We then tested the effect of choline in voltage-clamp mode in the same cells. At a membrane potential of -60 mV, 10 mM choline produced an inward current of -1038 ± 114 pA ($n = 7$ cells) associated with a $\Delta[Ca^{2+}]_i$ of 40.2 ± 10.6 nM (Fig. 1A, right traces).

We also expressed the response as the ratio between the increase in Ca^{2+} concentration to the charge transfer during the effect of choline and acetylcholine (M/q ratio, see Trouslard et al., 1993). At -60 mV, the mean value M/q ratio was 16.54 ± 1.73 of free Ca^{2+} per nanocoulomb (nC) of charge (nM/nC) ($n = 29$).

The decay phase of the choline current was best fitted by the sum of two exponential functions with τ fast of 289 ± 22 ms and τ slow of 2420 ± 246 ms ($n = 29$). At -60 mV, bath application of the broad-spectrum nicotinic acetylcholine receptor antagonist D-tubocurarine (60 μM) reduced the choline-evoked current (10 mM) from a control value of -1420 ± 166 to -276 ± 53 pA ($n = 5$). In these cells, the choline-associated $\Delta[Ca^{2+}]_i$ was 24.3 ± 5.4 nM in control solution and no rise could be observed in the presence of D-tubocurarine in any of the cells tested (Fig. 1B). This clearly shows the involvement of the nicotinic receptor in the effect of choline.

We compared the effects of equimolar concentrations of choline and acetylcholine (10 mM) in the same cells

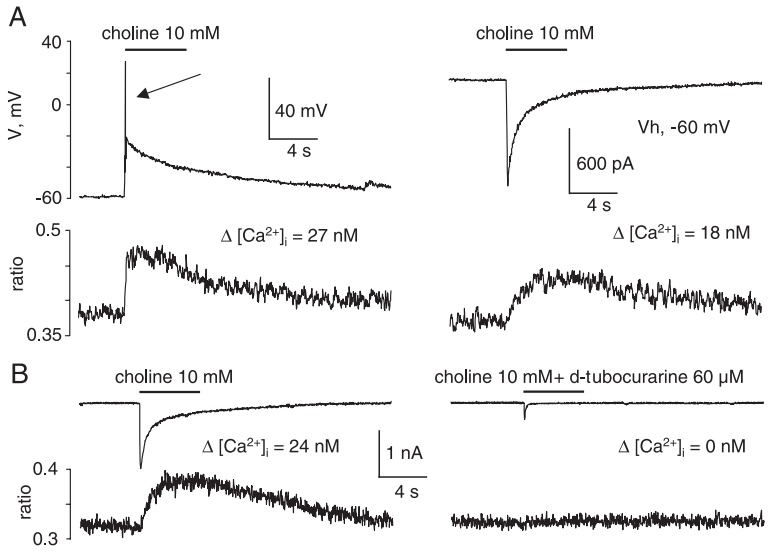

Fig. 1. Effect of choline on $[Ca^{2+}]_i$ in rat superior cervical ganglion neurones. (A) In current-clamp configuration (left traces), choline evoked a depolarization (upper trace) accompanied by a short burst of three spikes (indicated by the arrow) and increased the ratio (405/485) (lower trace), which corresponds to an intracellular Ca^{2+} increase of 27 nM from a basal $[Ca^{2+}]_i$ of 75 nM. The same cell was then voltage-clamped at −60 mV; choline applied for 5 s induced an inward current of −965 pA and increased $[Ca^{2+}]_i$ by 18 nM from its basal level. Choline was applied at 10 mM for 5 s, as indicated by the bar, using the U-tube fast application technique. (B) The choline-induced current and associated $\Delta[Ca^{2+}]_i$ were suppressed by 60 µM of D-tubocurarine in a cell clamped at −60 mV. D-tubocurarine was co-applied with choline for 5 s using the U-tube.

(Fig. 2A). The results are summarized in Table 1. The amplitude of the choline-induced membrane current was substantially less (35%) than that produced by acetylcholine whilst the total charge transfer during the responses was attenuated less (77%) by the use of choline agonist. The *M*/*q* ratio determined for choline was 55% of that induced by acetylcholine. Thus, acetylcholine seems to be more effective in increasing $[Ca^{2+}]_i$ since, for an identical charge transfer value, the $\Delta[Ca^{2+}]_i$ induced by acetylcholine was larger than that produced by choline. The decay of the acetylcholine-induced current was also best described by the sum of two exponential functions with significant faster decay time constants ($P<0.05$) than those observed with choline (Table 1). In nine cells, we also compared the effects

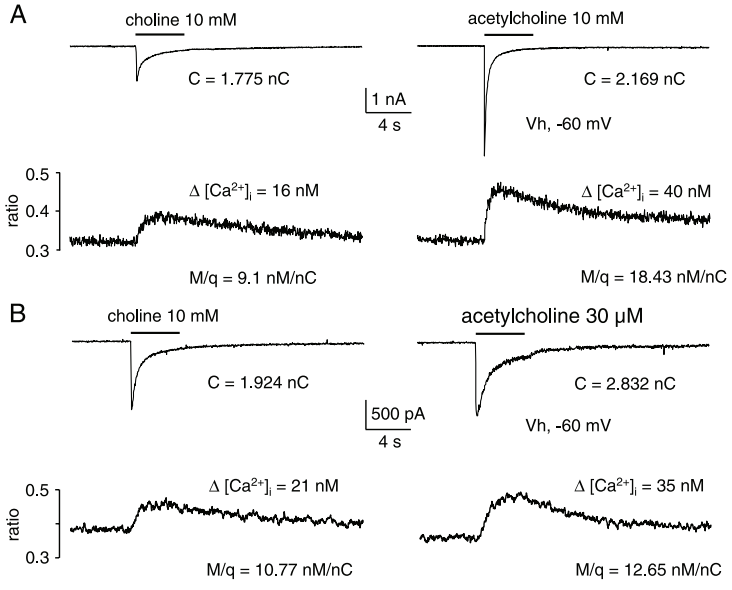

Fig. 2. Comparison of the effect of choline to that of acetylcholine. (A) Comparison of equimolar concentrations of choline and acetylcholine. Responses to 10 mM choline and acetylcholine were compared in the same neurone held at −60 mV. For each agonist we calculated the charge in nC and the $\Delta[Ca^{2+}]_i$ and expressed the response as the *M*/*q* ratio in nM of free Ca^{2+} divided by the charge in nanocoulombs. The *M*/*q* ratio of acetylcholine was significantly higher than that of choline ($P<0.05$). (B) Comparison of equipotent concentrations of choline and acetylcholine. Responses to 10 mM choline and 30 µM acetylcholine were compared in the same neurone held at −60 mV. No significant differences in the amplitude of current, charge transfer or Ca^{2+} increase were observed in response to the two agonists ($P>0.05$).

Table 1
Comparison of the basic properties of choline and acetylcholine responses

	Amplitude (pA)	Charge (nC)	$\Delta[Ca^{2+}]_i$ (nM)	*M*/*A* ratio (nM/ − nA)	*M*/*q* ratio (nM/nC)	τ fast (ms)	τ slow (ms)
Choline 10 mM	− 1320 ± 100	2.15 ± 0.30	26.9 ± 3.4	21.97 ± 3.55	14.69 ± 1.96	256 ± 25	2390 ± 333
Acetylcholine 10 mM	− 3758 ± 290[a]	2.79 ± 0.39[a]	76.2 ± 14.7[a]	20.39 ± 3.09	26.89 ± 2.44[a]	163 ± 12[a]	1772 ± 709[a]
Choline/acetylcholine	35%	77%	35%	108%	55%		
Choline 10 mM	− 794 ± 108	1.3 ± 0.38	21.6 ± 5.5	27.66 ± 4.72	20.65 ± 3.76	337 ± 44	2489 ± 305
Acetylcholine 30 μM	− 987 ± 194	1.98 ± 0.56	35.1 ± 7.5	39.89 ± 5.86	23.3 ± 3.93	487 ± 53	3338 ± 490
Choline/acetylcholine	80%	66%	62%	69%	89%		

Cells were held at − 60 mV under the perforated whole-cell configuration. Choline and acetylcholine were applied at 2-min intervals in the same cell. For each cell, an *M*/*A* ($\Delta[Ca^{2+}]_i$/ − amplitude) and an *M*/*q* ratio were determined. Values represent means ± S.E.M. for 20 cells for the comparison of equimolar concentration of agonists and for 9 cells for the comparison of equipotent concentration of agonists.

[a] Indicates significance as compared to choline response ($P < 0.05$).

of an equipotent concentration of acetylcholine and choline (Fig. 2B). This was achieved by using 30 μM for acetylcholine and 10 mM of choline. The results are summarized in Table 1. No significant differences were observed ($P > 0.05$) between the responses induced by choline and acetylcholine.

3.2. Dose dependence of the choline-induced current and associated $\Delta[Ca^{2+}]_i$

Cells were challenged with increasing concentrations of choline from 100 μM to 10 mM in cells voltage clamped at − 60 mV. As shown in Fig. 3, the current amplitude and associated $\Delta[Ca^{2+}]_i$ increased with increasing concentrations of choline. For each application, we measured the peak choline-induced current, the charge transfer during the response to choline and the associated-$\Delta[Ca^{2+}]_i$. Data were pooled from seven cells in which complete dose–response relationships of choline were obtained. The dose–response curve for the peak choline current was sigmoidal and gave an EC_{50} of 1.61 mM with a Hill coefficient of 1.19 (Fig. 4A). The dose–response curves for the charge transfer (Fig. 4B) and for the $\Delta[Ca^{2+}]_i$ (Fig. 4C) were similar, suggesting a strong correlation between the charge transfer and the associated $\Delta[Ca^{2+}]_i$. This was confirmed by the plot of the Ca^{2+} rise versus charge transfer (Fig. 4D). The relationship was linear with a slope of 10.51 ± 0.85 nM/nC, indicating that the relationship between Ca^{2+} and charge is not a function of the concentration of choline per se.

3.3. Voltage dependence of the effect of choline

The effect of 10 mM choline was measured in neurones voltage clamped at holding potentials of − 80, − 60 and

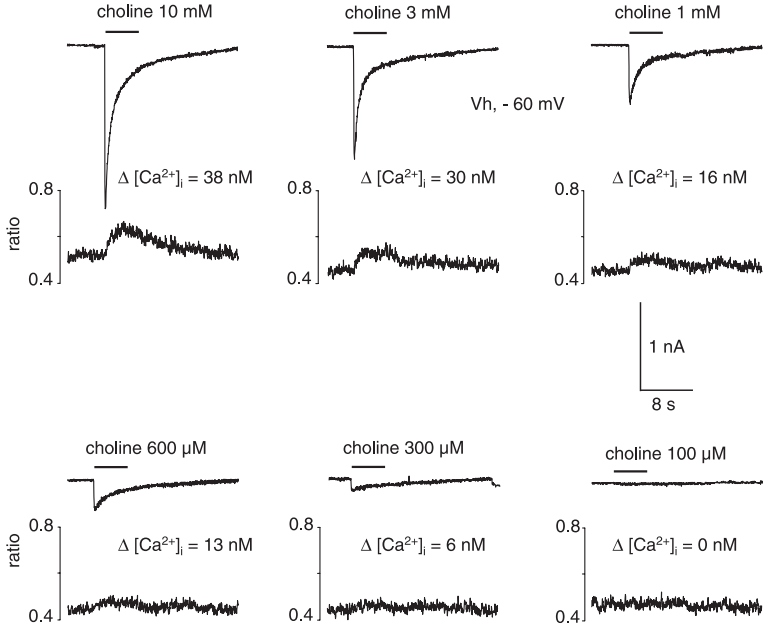

Fig. 3. Dose–response relationship of the effect of choline on cellular current and $\Delta[Ca^{2+}]_i$. Effect of successive applications of choline in decreasing concentrations from 10 to 0.1 mM on cellular current and $[Ca^{2+}]_i$ in a superior cervical ganglion neurone held at − 60 mV. Choline was applied for 5 s every 2 min. For each response, we calculated the peak current, the charge and the $\Delta[Ca^{2+}]_i$. This was done in seven cells and the plot of the dose–response curve for the mean peak choline-induced currents is shown in Fig. 4A.

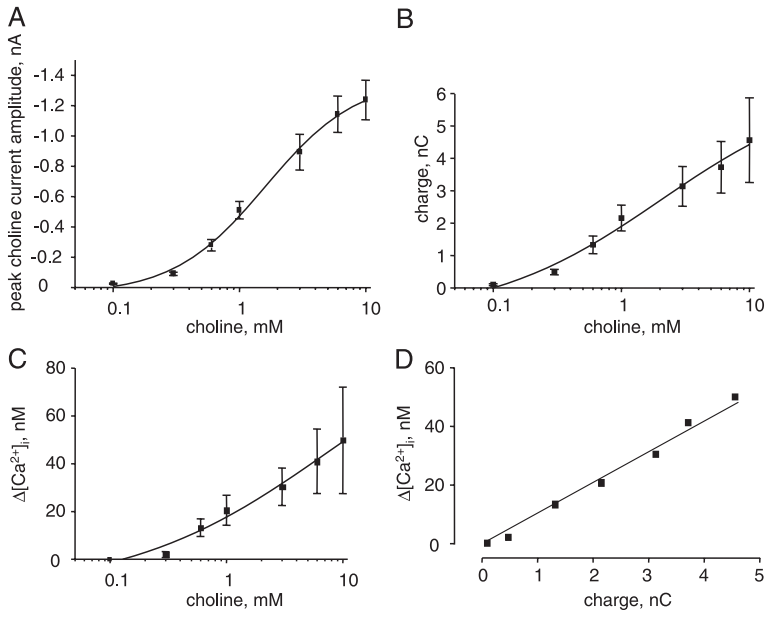

Fig. 4. Dose–response curves of the effect of choline on the peak current, charge and $\Delta[Ca^{2+}]_i$. (A) Peak current amplitude of the choline-induced current versus dose of choline. The data were best fitted with a sigmoidal function with an EC_{50} of 1.61 mM and a number of Hill of 1.19. Relationship between the charge (B) and the $\Delta[Ca^{2+}]_i$ (C) on one hand, and the concentration of choline on the other. (D) Plot of the $\Delta[Ca^{2+}]_i$ versus charge. Data were fitted with a linear function $\Delta[Ca^{2+}]_i$ = slope × charge, with a slope of 10.5 nM/nC ($r = 0.99$). This indicates that the ratio between $\Delta[Ca^{2+}]_i$ and charge is a constant and independent of the concentration of choline. Each point is the mean ± S.E.M. for seven cells (see an example in Fig. 3).

− 40 mV. As illustrated in Fig. 5A–C, the amplitude of the choline current and the associated $\Delta[Ca^{2+}]_i$ decreased with depolarization. For each response, the charge transfer and the *M*/*q* ratio at each holding potential were calculated. This procedure was performed in eight cells (using conventional whole-cell recording) and the results are illustrated in Fig. 5D. As shown, the *M*/*q* ratio remained constant at voltages between − 80 and − 40 mV, indicating that the voltage dependence of the agonist-induced Ca^{2+} rise was mainly due to the voltage dependence of the choline-induced current.

3.4. Dependence on external Ca^{2+} and internal Ca^{2+} stores

The close relationship between $\Delta[Ca^{2+}]_i$ and charge during the choline response suggested that $\Delta[Ca^{2+}]_i$ was probably due to Ca^{2+} entry through nicotinic acetylcholine receptors. This was confirmed by omitting Ca^{2+} from the

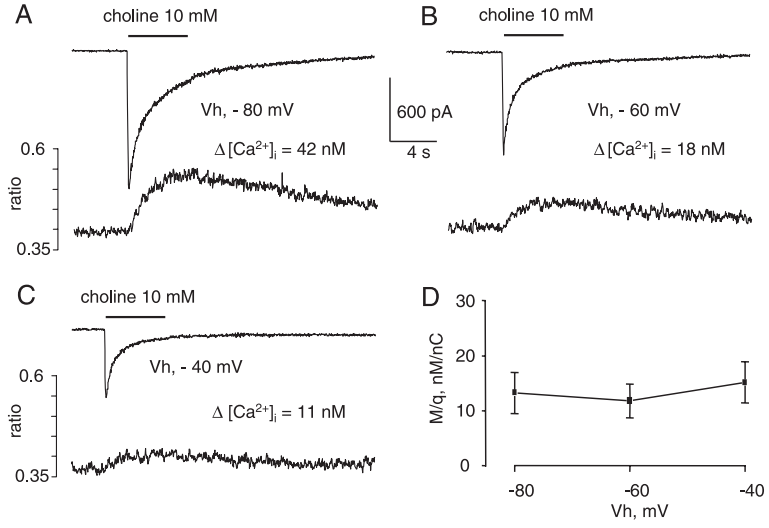

Fig. 5. Voltage dependence of the Ca^{2+} increase induced by choline. Effect of choline on cellular current and $[Ca^{2+}]_i$ were recorded at − 80 mV (A), − 60 mV (B) and − 40 mV (C). For each holding potential, we calculated the *M*/*q* ratio. (D) Plot of the mean ± S.E.M. (eight cells) versus holding potential. Note that the *M*/*q* ratio remained almost constant with a holding potential between − 80 and − 40 mV.

external medium and by comparing the choline effect in these two recording situations (Fig. 6A). Using conventional whole-cell recording, under control conditions (2.5 mM external Ca^{2+}) and at −60 mV, choline (10 mM) produced a peak inward current of −1297 ± 135 nA (n = 6 cells) associated with an increase in Ca^{2+} concentration of 36.1 ± 4.5 nM (M/q = 8.82 ± 1.94 nM/nC). In the same cells, under external Ca^{2+}-free conditions (0 Ca^{2+} + 0.5 mM EGTA) choline induced a significantly (P < 0.05) smaller current that peaked at −872 ± 120 nA (n = 6) with no increase in $[Ca^{2+}]_i$ detected. This demonstrates that external Ca^{2+} is implicated in the $\Delta[Ca^{2+}]_i$ induced by choline.

Previous results indicated that $\Delta[Ca^{2+}]_i$ induced by choline is dependent on Ca^{2+} influx in voltage-clamped neurones. We tested the involvement of internal Ca^{2+} stores in $\Delta[Ca^{2+}]_i$ induced by choline by measuring the Ca^{2+} increase and associated current induced by choline before and after Ca^{2+} depletion by co-application of caffeine (10 mM) and thapsigargin (1 μM). In five cells, a control application of choline 10 mM produced an inward current of −902 ± 117 pA and an associated Ca^{2+} increase of 30.4 ± 6.2 nM from a basal level of 100.8 ± 10.4 nM (Fig. 6B). Application of a mixture of caffeine and thapsigargin (an inhibitor of the endoplasmic Ca-ATPase) was used to both release and prevent the refilling of endoplasmic stores (Inesi and Sagara, 1994). This was repeated two to three times until the Ca^{2+} stores were depleted. The first application of caffeine and thapsigargin induced a Ca^{2+} rise of 405.9 ± 207.9 nM (Fig. 6B) whereas the third application led to a $\Delta[Ca^{2+}]_i$ of 3.0 ± 1.9 nM, indicating Ca^{2+} store depletion. The basal Ca^{2+} level was 131.1 ± 11.5 nM after the treatment with thapsigargin and caffeine. Although not significantly different from the control value, this may however indicate the involvement of the reticulum Ca^{2+} store in determining the basal Ca^{2+} level. On challenge of the cell with choline after depletion of the caffeine-sensitive store, choline induced an inward current of −813 ± 138 pA and a $\Delta[Ca^{2+}]_i$ of 30.5 ± 2.8 nM. These values were not significantly different from the control values (P>0.05).

These results demonstrate that the choline-induced Ca^{2+} increase was a result of Ca^{2+} influx and was independent of the caffeine-sensitive internal Ca^{2+} stores.

3.5. Pharmacology of the choline responses

As choline has been described as a selective agonist for α7 nicotinic acetylcholine receptors, we next tested the effect of two selective antagonists of α7 receptors, e.g. α-bungarotoxin and methyllycaconitine.

The effect of α-bungarotoxin was determined using two experimental protocols. In the first protocol, we evaluated the rapid effect of α-bungarotoxin. This was done by superfusing α-bungarotoxin 50 nM for 6 min and then monitoring the choline-induced current and the

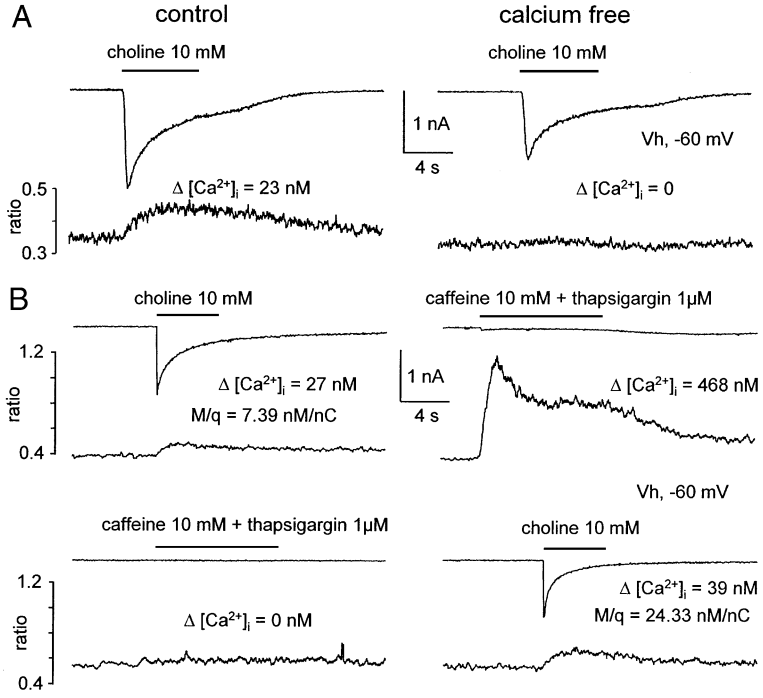

Fig. 6. Dependence of the choline response on external Ca^{2+} and caffeine-sensitive Ca^{2+} stores. (A) Comparison of choline-induced currents and associated $\Delta[Ca^{2+}]_i$ in a normal solution (2.5 mM Ca^{2+}) and Ca^{2+}-free solution (no Ca^{2+} + 0.5 mM EGTA). The holding potential was −60 mV and choline was applied at 10 mM for 5 s. (B) Depletion of caffeine-sensitive Ca^{2+} stores was achieved in this cell by two successive applications for 10 s of a mixture of 10 mM caffeine and 1 μM thapsigargin. The first application (right upper trace) induced a $\Delta[Ca^{2+}]_i$ of 467 nM whilst the second application had no effect on $[Ca^{2+}]_i$ (left lower trace). The effect of choline was not significantly different before (left upper trace) and after the depletion of caffeine-sensitive Ca^{2+} stores (right lower trace). The holding potential was −60 mV and choline was applied at 10 mM for 5 s.

associated $\Delta[Ca^{2+}]_i$. As shown in Fig. 7A, the mean peak current, the amplitude of the current measured at the half-time of application (2.5s) and the associated Ca^{2+} increase were not significantly different in the presence of 50 nM of α-bungarotoxin (ANOVA test, $P>0.05$; $n=4$ cells; Fig. 7A). The rise and the decay rates of the choline-induced currents were also unaffected by α-bungarotoxin. In a second protocol, we preincubated cultures with 50 nM α-bungarotoxin for 2 h and compared the effect of choline in treated and nontreated cells obtained from the same culture. No significant differences were noticed in the peak current density (Fig. 7B) (*t*-test; $P>0.05$). The decay kinetics of the choline-induced current and the associated $\Delta[Ca^{2+}]_i$ were also similar in these two conditions ($P>0.05$).

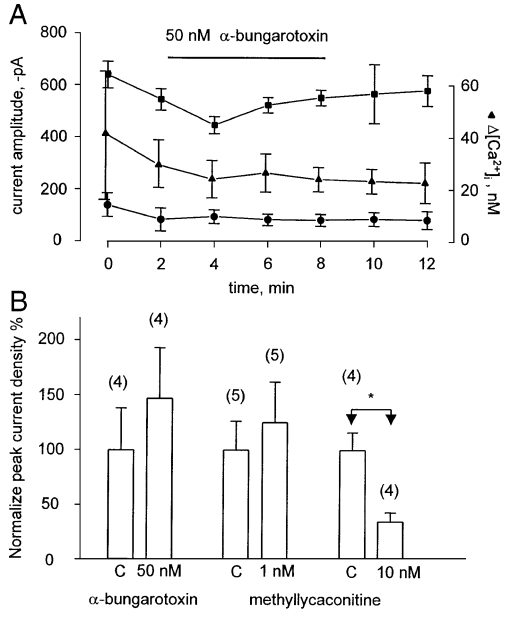

Fig. 7. Effects of α7 nicotinic acetylcholine receptor antagonists on choline responses. (A) Effect of short application of α-bungarotoxin on choline-induced current. Choline 10 mM was applied every 2 min for 5 s and α-bungarotoxin was applied for the time indicated by the bar. The graph shows the amplitude of the choline current measured at the peak (■) and at 2.5 s after the beginning of the application (●) and the choline-induced $\Delta[Ca^{2+}]_i$ (▲) versus time. α-Bungarotoxin was without effect on these three parameters. Data represent means ± S.E.M. (in four cells). Neurones were held at −60 mV under the patch-perforated configuration. (B) Effect of chronic addition of α-bungarotoxin and methyllycaconitine. For each antagonist, control (C) and treated dishes came from the same preparation. The antagonist was added for 2 h to the culture medium at a final concentration of 50 nM for α-bungarotoxin, and 1 nM and 10 nM for methyllycaconitine. To ensure no wash-out of the antagonist, the treated dish was then perfused with the external recording medium containing the same concentration of antagonist as used for the chronic addition. Choline was co-applied with the antagonist to be tested by means of the U-tube fast application. For each treatment, the mean peak density (pA/pF) of control cells and treated cells were calculated and normalized to the mean of the peak density in control cells. Bars represent the mean of the normalized peak density ± S.E.M. with the number of neurones tested indicated in parentheses. A significant effect as indicated by * was only observed with treatment with 10 nM of methyllycaconitine ($P<0.05$).

Methyllycaconitine has been shown to block the choline current mediated by native α7 nicotinic acetylcholine receptors (Alkondon et al., 1997). In the absence of methyllycaconitine, choline (3 mM) induced a mean peak current of −805 ± 104 pA and a mean associated $\Delta[Ca^{2+}]_i$ of 24.1 ± 5.2 nM ($n=10$). When choline was co-applied with 1 nM methyllycaconitine, the mean peak inward current and $\Delta[Ca^{2+}]_i$ were not significantly different from control values. We also preincubated a culture dish with methyllycaconitine for 2 h and compared the effect of 10 mM choline in treated dishes and nontreated dishes obtained from the same culture. The effect of methyllycaconitine was dose dependent as it had no effect at 1 nM whereas it significantly reduced the peak density of the choline current at the higher concentration of 10 nM (Fig. 7B).

We conclude that choline increased the internal Ca^{2+} concentration by opening permeable nicotinic acetylcholine receptors which are insensitive to 1 nM methyllycaconitine and 50 nM α-bungarotoxin and sensitive to 10 nM methyllycaconitine.

4. Discussion

The major new finding in this experiment is that choline can induce a significant increase in intracellular $[Ca^{2+}]_i$ in cultured rat sympathetic ganglion cells. We demonstrated that, under voltage-clamp conditions, at negative potentials, the $\Delta[Ca^{2+}]_i$ was due to Ca^{2+} influx through nicotinic acetylcholine receptors which were not α7 nicotinic acetylcholine receptors.

4.1. Calcium increase induced by choline is a result of Ca^{2+} influx through nicotinic acetylcholine receptors

On no occasion did we observe an increase in Ca^{2+} without recording an inward current induced by choline. Ca^{2+} stores were not depleted since, in all cells tested, caffeine was able to release Ca^{2+} from internal Ca^{2+} stores. This suggests that choline does not mobilize Ca^{2+} from internal Ca^{2+} stores as was suspected (Koike et al., 1989) but triggers Ca^{2+} entry through the membrane. This was confirmed by the observation that depletion of Ca^{2+}-sensitive stores by caffeine and thapsigargin did not significantly alter the choline-induced $\Delta[Ca^{2+}]_i$. Moreover, there was a strong correlation between the charge transfer and $\Delta[Ca^{2+}]_i$ during the choline response, shown by the analysis of the dose–response relationships or the effects of single doses at various holding potentials. The *M*/*q* values must be taken as indicative because it is difficult to properly estimate the real Ca^{2+} increase due solely to Ca^{2+} influx through nicotinic channels. Ca^{2+} concentration is subject to homeostatic regulation involving both Ca^{2+} buffering mechanisms and amplifying mechanisms such as Ca^{2+}-induced Ca^{2+} release. Thus the Ca^{2+} signal which is detected may be altered by such homeostatic mecha-

nisms. As expected, omitting Ca^{2+} from the external medium suppressed the $\Delta[Ca^{2+}]_i$ associated with the choline current. The percentage of reduction of the peak choline-induced inward current in Ca^{2+}-free external medium was 32.8% whilst the charge transfer was reduced by 24.5%. The suppression of the charge carried by Ca^{2+} has been shown to produce only a reduction in the peak amplitude of the current of $\approx$ 5% in superior cervical ganglion neurones (Rogers et al., 1997). The remaining percentage ($\approx$ 28%) probably reflects the dependence of nicotinic acetylcholine receptors on external Ca^{2+}. Indeed, external Ca^{2+} has been shown to potentiate the nicotinic-mediated current (Liu and Berg, 1999; Mulle et al., 1992b; Vernino et al., 1992) by increasing the frequency of opening of the nicotinic channel. For example, in the ciliary ganglion, the reduction of external Ca^{2+} from 2 to 0 mM reduced the α3*-nicotinic receptor-mediated current induced by 20 μM nicotine by 66% (Liu and Berg, 1999). As this percentage of reduction decreased with increasing concentration of agonists (Liu and Berg, 1999; Mulle et al., 1992b), this might explain why the amplitude of the peak current induced by 10 mM choline was only reduced by 28%.

We also compared the effect of equimolar concentrations of acetylcholine and choline, the two natural agonists of nicotinic acetylcholine receptors. As expected for a partial agonist, choline induced a current that was always smaller than that produced by acetylcholine. The ratio of the peak current choline/acetylcholine ratio was 35%. However, the charge choline/acetylcholine ratio was 77%, indicating that in terms of the charge, choline is a better agonist than expected when simply comparing the amplitude of the peak current produced by these two agonists. A reason for this is that at high concentrations, the acetylcholine current desensitizes faster than the choline current (see Table 1). There could be several explanations for this accelerated decay phase. One could be that choline and acetylcholine may have different kinetics of desensitization of nicotinic acetylcholine receptors. The kinetic properties of activation and desensitization of choline and acetylcholine have been compared at the single channel level for α7 receptors expressed by rat hippocampal neurones (Mike et al., 2000). They were shown to be identical in this preparation, but so far these properties are not known for nicotinic acetylcholine receptors expressed in superior cervical ganglion neurones and we cannot exclude this possibility. However, when comparing equipotent concentrations of acetylcholine (30 μM) and choline (10 mM), the kinetics of decay of the currents induced by the two agonists were similar. Another explanation for the different decay rate of the current observed when testing 10 mM of agonist is that acetylcholine and choline may act as blockers of opened nicotinic channels but to a different extent. Nicotinic acetylcholine receptors agonist at a high concentration behave as noncompetitive antagonists by blocking the nicotinic channel in its opened state (Arias, 1998). The extent of block is expected to depend on the size of the current. As the amplitude of the choline-mediated current is less than that mediated by acetylcholine, we expected a smaller blockade by choline. The open channel blocker behaviour of acetylcholine was sometimes seen as a typical rebound in the current trace at the end of the application of acetylcholine (not shown). This is because the dissociation of acetylcholine from its blocking site and the reopening of nicotinic receptors occurred before the dissociation of acetylcholine from its agonist binding sites. This rebound in current was rarely seen with choline. Therefore, choline could be considered a partial nicotinic agonist at nicotinic acetylcholine receptors in superior cervical ganglion neurones; however, because at high concentrations it desensitizes more slowly and/or is less potent as a nicotinic channel blocker than acetylcholine, the charge produced and therefore the increase in internal Ca^{2+} concentration are of a comparable magnitude to those induced by acetylcholine.

4.2. The Ca^{2+} rise induced by choline involves the activation of non-α7 nicotinic acetylcholine receptors

Recently, Cuevas et al. (2000) described classical α7 and atypical α7 nicotinic receptors in rat superior cervical ganglion. These currents differ in their kinetics and their sensitivity to α-bungarotoxin. Typical α7 nicotinic acetylcholine receptors mediate fast currents, which are slowly but irreversibly blocked by α-bungarotoxin whereas atypical α7 receptors mediate current, a slowly decaying current and are rapidly and reversibly blocked by α-bungarotoxin. So far, we have been unable to detect typical or atypical α7 nicotinic acetylcholine receptors. In fact, as reported by others (Mandelzys et al., 1995), we never recorded a fast desensitizing inward current induced by choline, as is observed when choline activates conventional α7 nicotinic receptors (Alkondon et al., 1997; Cuevas et al., 2000; Papke et al., 1996). In addition, in our hands the choline-induced responses were not sensitive to 50 nM α-bungarotoxin or 1 nM of methyllycaconitine. The choline-induced current was sensitive to methyllycaconitine at 10 nM, probably because at this concentration methyllycaconitine is nonselective for α7 nicotinic acetylcholine receptors. When α-bungarotoxin was applied for short periods, we did not detect a change in the kinetics of the choline-induced current, which may have been indicative of the presence of atypical α7 nicotinic acetylcholine receptors (Cuevas and Berg, 1998; Cuevas et al., 2000; Yu and Role, 1998). So far, we have no explanation for this discrepancy. We initially thought that atropine, used in our experiments to prevent muscarinic effects, might act as an α7 receptors antagonist (Zwart and Vijverberg, 1997). However, omission of atropine in some experiments did not modify the responses to choline. Another plausible explanation may be the relative low amount of α7 subunits in our preparation. Axotomy of the adult rat superior cervical ganglion decreases the amount of α7 nicotinic

acetylcholine receptor protein (Zhou et al., 2001) and the α7 mRNA level decreases by 70% within 48 h of culture of neonatal rat superior cervical ganglion neurones (De Koninck and Cooper, 1995). Such a down-regulation of α7 receptors may occur in cultures of superior cervical ganglion neurones. Several procedures have been demonstrated to up-regulate the number of α7 nicotinic receptors, such as chronic treatment with nicotine or methyllycaconitine (Molinari et al., 1998). We tested the effect of treatment with nicotine 10 μM for 1 day, but we were unable to see any change in the response to choline (data not shown). Clearly, attempts to up-regulate α7 receptor expression will require a parallel quantification of α7 transcripts under various experimental conditions.

Owing to the total absence of effect of α-bungarotoxin, we suggest that choline activates non-α7 nicotinic acetylcholine receptors which are permeable to Ca^{2+}. Superior cervical ganglion neurones express several nicotinic receptor subunits that can assemble to form various nicotinic acetylcholine receptor subtypes. For quite a long time, it has been known that rat superior cervical ganglion neurones express mainly α3β4 receptors (Britt and Brenner, 1997; Covernton et al., 1994; Mandelzys et al., 1995). It is therefore tempting to associate the effect of choline to an action at α3β4 nicotinic acetylcholine receptors. In PC12 cells, choline has indeed been shown to induce α3β4 receptor-mediated currents with 20% of the efficacy of acetylcholine (Alkondon et al., 1997), and this would fit well with the choline/acetylcholine current ratio of 35% found in this study. However, the situation in superior cervical neurones is probably more complex. Firstly, based on the comparison of pharmacological and biophysical properties of native receptors and receptors expressed in the oocytes, it has been suggested that superior cervical ganglions express nicotinic acetylcholine receptors that may contain several different α subunits and/or β subunits, among which, α3α5β2, α3β2β4 nicotinic receptors (Sivilotti et al., 1997) but more complicated combinations could be also envisaged. Secondly, there could be a large variation amongst cells and cultures in the relative amount of different receptor types (Britt and Brenner, 1997).

4.3. Physiological relevance

In superior cervical ganglion neurones, the cholinergic transmission mediated by nicotinic acetylcholine receptors is not affected by α-bungarotoxin (Brown and Fumagalli, 1977) because most of the nicotinic receptors that bind α-bungarotoxin are extrasynaptic (Kristufek et al., 1999). In contrast, synaptic nicotinic acetylcholine receptors are of the α3β4* subtype (Chiappinelli and Dreyer, 1984) and thus are potentially the target of choline. The pertinent question, and the most difficult to answer, is to know the time course of choline in the synapse and its participation in synaptic transmission. The normal extracellular concentration of choline is 5–10 μM and it is unlikely that choline at such a low concentration significantly increased $[Ca^{2+}]_i$. However, during repetitive cholinergic transmission, a high concentration of acetylcholine is probably reached in the synaptic cleft and, given the rapid turnover of acetylcholinesterase, it is conceivable that a high concentration of choline may be also reached. This has been suggested in cat superior cervical ganglion where, in the presence of eserine, an inhibitor of acetylcholinesterase, the total amount of choline released by a burst of preganglionic stimulation appears entirely in the form of acetylcholine whereas in the absence of eserine, it appears as choline (Emmelin and MacIntosh, 1956). Nevertheless, this does not prove whether choline is really present in the synaptic cleft during preganglionic stimulation. Marsh et al. (1990) have shown that tacrine, an inhibitor of acetylcholinesterase, has little effect on the fast nicotinic synaptic current but potentiates slow muscarinic synaptic transmission. This has been interpreted as reflecting the minor involvement of acetylcholinesterase in fast synaptic current, leading to the view that fast nicotinic-mediated synaptic transmission is ended by the diffusion of acetylcholine (Eccles, 1944) and by the kinetic properties of the nicotinic channel itself. However, different results have been found with otic ganglion of the parasympathetic system, where the action of acetylcholine is terminated by its enzymatic degradation by acetylcholinesterase (Callister and Sah, 1997). Here, it is conceivable that choline may prolong, to some extent, the effect of acetylcholine through an action at synaptic α3β4* nicotinic receptors. The action of choline would depend on, as has been suggested for acetylcholine itself, its clearance rate from the synaptic cleft by diffusion. But, it is worth noting that choline greatly differs from acetylcholine because choline is actively transported into cells by high-affinity choline uptake mechanisms. It would therefore be interesting to test the participation of these choline transporters in nicotinic receptor-mediated fast synaptic transmission.

In conclusion, we show that choline, a natural nicotinic receptor agonist and a metabolite of acetylcholine, acts on superior cervical ganglions to induce a significant rise in internal Ca^{2+} concentration. Under voltage-clamp conditions, this rise is due to Ca^{2+} entry through nicotinic channels, which are not sensitive to neurotoxins known to selectively block α7 nicotinic acetylcholine receptors. It is therefore possible that choline produced by the hydrolysis of acetylcholine may prolong the action of acetylcholine by activating intracellular Ca^{2+}-dependent processes.

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