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# Choline Is a Full Agonist in Inducing Activation of Neuronal Nitric Oxide Synthase via the Muscarinic $M_1$ Receptor

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**Key Words**

Choline · Muscarinic acetylcholine receptors · $M_1$ subtype · Neuronal nitric oxide synthase · Calcium signaling · Alzheimer's disease

**Abstract**

The present study was designed to characterize the interaction of choline with the $M_1$ subtype of the muscarinic acetylcholine receptor. Using Chinese hamster ovary cells transfected with neuronal nitric oxide synthase and the cloned human $M_1$ receptor (CHO $hM_1$/nNOS cells), we investigated choline's effects on production of nitric oxide and elevation of intracellular [$Ca^{2+}$]. Choline showed the properties of a full agonist in inducing the sustained plateau of increased intracellular [$Ca^{2+}$], although it was only a partial agonist in inducing the larger, transient [$Ca^{2+}$] peak. Choline was also found to act as a full agonist in stimulating the production of nitric oxide. These results have implications for efforts in the clinical treatment of dementia.



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## Introduction

The 'cholinergic hypothesis' of dementia suggests that the behavioral changes observed in Alzheimer's disease are related to a decline in cholinergic function in the brain [1]. Alzheimer patient brains show a significant reduction in the activity of choline acetyltransferase, the enzyme responsible for the synthesis of acetylcholine (ACh) [2], accompanied by extensive damage to the basal forebrain cholinergic system (especially the nucleus basalis of Meynert) [3]. Most affected are long-axon cholinergic neurons which project to areas involved in attention and memory, such as the cerebral cortex, thalamus, and hippocampus [4]. Interestingly, the neurons receiving this cholinergic innervation retain a normal density of muscarinic acetylcholine receptors (mAChR) in Alzheimer's disease, particularly of the $M_1$ subtype [5]. This receptor has been shown to be involved in learning and memory [6]. These findings have led to novel strategies for the therapy of Alzheimer's disease, attempting to alleviate the symptoms of dementia by modulating various components of the cholinergic system [6–8].

Choline is generally thought of as a relatively inactive molecule, although several studies have shown that it can have direct effects on various biological systems and signal transduction pathways. For example, choline interacts with nicotinic and mAChRs [9–12] and exhibits a higher

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potency in displacing agonist than antagonist radioligand binding to muscarinic receptors [12]. Choline also acts as a full and selective agonist at α7-containing nicotinic AChRs using the whole-cell patch-clamp method [13]. A recently developed mutant $M_1$ muscarinic receptor showed heightened sensitivity to agonists in cultured cell lines, with choline being a full agonist in stimulating phosphoinositide (PI) hydrolysis [14]. Surprisingly, choline has also been shown to be a fairly effective inhibitor of [$^3$H]ACh release in the myenteric plexus-muscle preparation, presumably by acting at presynaptic mAChRs [15].

The effect of choline on PI hydrolysis in rat brain slices has also been investigated, showing only a slight increase over controls at concentrations as high as 100 mmol/l [16]. While this effect was antagonized by atropine, the possibility that choline was metabolized to elevate ACh levels, which in turn led to the observed response, was not excluded. On the other hand, the study may have underestimated the agonistic potential of choline. PI hydrolysis is relatively proximal to the activation of muscarinic receptors. Perhaps the response to choline is more robust at later stages of signal transduction further along the pathway, such as changes in intracellular calcium and activation of nitric oxide (NO) synthase (NOS).

For these reasons, we chose to investigate the effects of choline on $Ca^{2+}$ mobilization and NOS activation in Chinese hamster ovary (CHO) cells transfected with the $M_1$ subtype muscarinic receptor and neuronal NOS. In this study, we report that choline has the ability to directly stimulate the $M_1$ subtype of mAChRs and cause substantial activation of neuronal NOS (nNOS) and elevation of intracellular $Ca^{2+}$ concentrations. These findings have implications for the clinical treatment of Alzheimer's disease and dementia.

## Materials and Methods

*Materials*

*L*-[$^3$H]arginine (64 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, Ill., USA). *L*-[$^{14}$C]citrulline (50 mCi/mmol) was purchased from DuPont-New England Nuclear (Boston, Mass., USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, N.Y., USA). Dowex AG50W-X8 ion-exchange resin was obtained from Bio-Rad (Hercules, Calif., USA). Bovine calf serum was purchased from Hyclone (Logan, Utah, USA). Geneticin, hygromycin, Pluronic F127, and fura-2/AM {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′,-tetraacetic acid pentaacetoxymethyl ester} were purchased from Calbiochem (La Jolla, Calif., USA). Choline chloride (3 × crystalline) and all other compounds were purchased from Sigma Chemical (St. Louis, Mo., USA).

*Cell Culture*

CHO cells, stably expressing both the human $M_1$ mAChR and nNOS (CHO hm1/nNOS cells), were described previously [17]. All cells were grown at 37 °C in DMEM supplemented with 10% bovine calf serum, in the presence of hygromycin and geneticin (each at 50 mg/l), in a humidified atmosphere consisting of 5% $CO_2$/95% air. For calcium experiments, the cells were grown in tissue culture flask for 4 days, harvested on the day of the experiment with phosphate-buffered saline containing 0.05 trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA), centrifuged at 300 *g* for 3 min, and resuspended twice in warmed HEPES buffer (110 mmol/l NaCl, 5.4 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 25 mmol/l glucose, 50 mmol/l HEPES, 58 mmol/l sucrose; pH 7.4 ± 0.02; 340 ± 5 mosm). For determination of nNOS activity, the cells were plated in sterile 24-well plates at a concentration of 50,000 cells/well. The cells were grown for 4 days.

*Fura-2 Calcium Measurement*

Agonist-induced changes in intracellular calcium were measured using the calcium-binding fluorescent dye fura-2, as described previously [18, 19]. Briefly, the cells were harvested, rinsed, and resuspended as described above. The cells were then loaded with the dye (ester form) by incubation in a 37 °C shaking water bath. The cells ($2–3 \times 10^7$ cells/ml) were incubated for 45 min in a volume of 2 ml, in the presence of 5 μmol/l fura-2/AM, 0.4% Pluronic F127, 0.02 g/l bovine serum albumin, and 1 mmol/l probenecid. All HEPES buffer used in $Ca^{2+}$ measurement experiments contained 1 mmol/l probenecid to inhibit fura-2 leakage out of the cells. After loading, the cells were centrifuged and resuspended at a concentration of $1 \times 10^6$ cells/ml. For the duration of the assay (4–6 h), the cells were kept in the dark on ice. Prior to fluorescence measurement, 2 ml of the cells was put in a quartz microcuvette and allowed to equilibrate at 37 °C for 5 min. Fura-2 fluorescence was measured at 37 °C with continuous stirring at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm, using a Perkin Elmer (Beaconsfield, UK) LS50B fluorescence spectrophotometer with a circulating water bath and biokinetic feedback accessory. For each trace, a maximal and minimal fluorescence range was obtained by addition of 60 mg/l digitonin and 10 mmol/l EGTA [ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic acid], respectively. In some traces, atropine or EGTA was added 5 min prior to fluorescence measurement and allowed to equilibrate before the addition of agonist. Data were collected using FL WinLab version 2.01, provided by the equipment manufacturer. Free calcium concentrations were calculated off-line according to the methods of Grynkiewicz et al. [19] using Microsoft Excel. To reduce extraneous noise (due to pipet disruption of the signal when adding agonists and other compounds), the raw data were smoothed by adjacent averaging using Microcal Origin. All calculations assumed a $K_d$ value of $Ca^{2+}$-fura-2 of 225 nmol/l.

*Measurement of nNOS Activity*

The activity of nNOS in CHO $hM_1$/nNOS cells was assayed by measuring the conversion of *L*-[$^3$H]arginine to NO and *L*-[$^3$H]citrulline as described by Wang et al. [20], with minor modifications. As described above, the cells were grown in 24-well plates for 4 days to 80–95% confluency. On the day of experiments, the plates were rinsed and blotted twice with warmed HEPES buffer (37 °C); 250 μl of HEPES was then added to each well, and the plates were placed in a 37 °C water bath, shaking at 55 rpm, and allowed to equilibrate for 20 min. 25 μl of *L*-[2,3,4,5-$^3$H]arginine monohydrochloride was add-

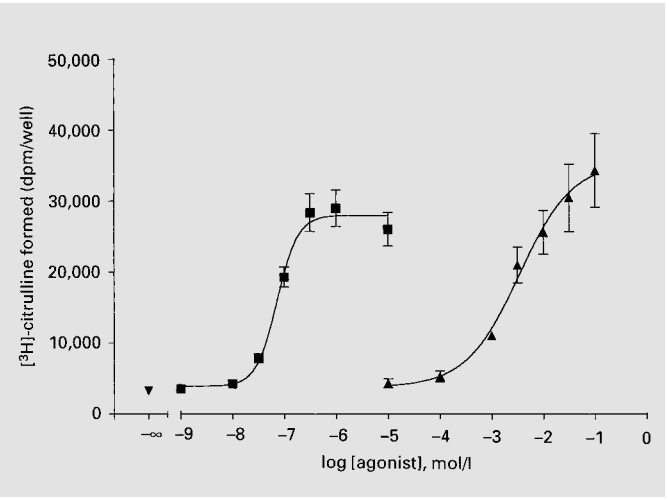


**Fig. 1.** Concentration-response curves for ACh- and choline-mediated activation of nNOS. CHO $hM_1$/nNOS cells were incubated with increasing concentrations of ACh (■) or choline (▲) for 1 h at 37 °C. Basal levels (▼) were assessed in the absence of agonist. Neuronal NOS activity was determined by quantifying the conversion of *L*-[$^3$H]arginine to *L*-[$^3$H]citrulline as described in text. Both ACh and choline showed concentration-dependent stimulation of nNOS activity. Values are mean ± SE of at least five independent experiments performed in triplicate. Where error bars are not shown, they lie within the dimensions of the symbol.

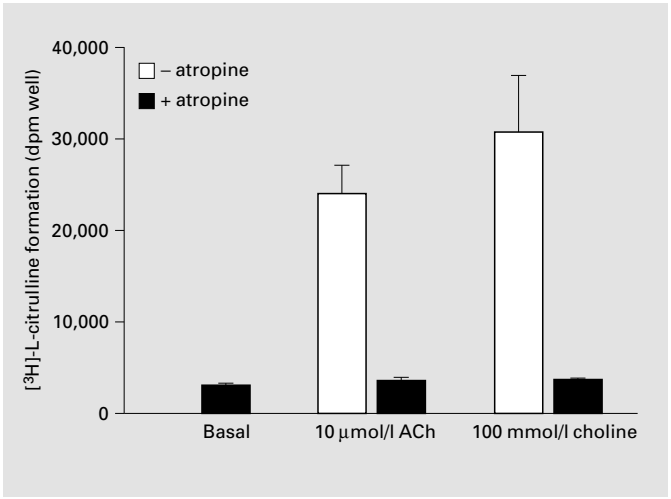


**Fig. 2.** Activation of nNOS by choline is mediated by the muscarinic receptor. CHO $hM_1$/nNOS cells were incubated with 10 mmol/l ACh or 100 mmol/l choline, alone or in the presence of 1 mmol/l atropine. Atropine was allowed to equilibrate for 5 min prior to the addition of the agonists. When compared with a two-tailed Student t test, both ACh + atropine and choline + atropine did not differ significantly from basal. Values are mean ± SE of four independent experiments performed in triplicate.

ed at a concentration of 0.6 µCi/well, followed 5 min later by varying concentrations of choline and acetylcholine. When testing the effects of 3 mmol/l EGTA or 1 µmol/l atropine, these compounds were added 5 min before the addition of [$^3$H]arginine and allowed to equilibrate. All wells had a final volume of 300 µl. After 1 h, the reaction was stopped with an excess of *L*-arginine (5 mmol/l) and EDTA (4 mmol/l). *L*-[$^3$H]citrulline was separated from *L*-[$^3$H]arginine by ion-exchange chromotography (Dowex AG50-X8 resin), and the radioactivity was determined by liquid scintillation counting and expressed as disintegrations per minute (dpm).

## Results

### *Characterization of ACh- and Choline-Induced Activation of nNOS at the Muscarinic $M_1$ Receptor*

Concentration-response relationships of choline- and ACh-mediated activation of nNOS were determined by measuring the conversion of *L*-[$^3$H]arginine to *L*-[$^3$H]citrulline under standard assay conditions. As seen in figure 1, both choline and ACh appear to have similar intrinsic activity, although ACh was more potent by over four orders of magnitude. Nonlinear regression analysis yielded the following parameters: for acetylcholine, maximum dpm/well = 28,000 ± 1,100, $pEC_{50}$ = 7.14 ± 0.07; for choline, maximum dpm/well = 36,000 ± 2,900, $pEC_{50}$ = 2.47 ± 0.24. The concentration-response relationship for ACh was typically steep, exhibiting a Hill slope coefficient of 2.14 ± 0.68. Conversely, choline's concentration-response relationship covered a wider range of concentrations, with a Hill slope coefficient of 0.84 ± 0.39. While the $pEC_{50}$ for choline is far above most published physiological concentrations of choline, the wide range of concentrations over which choline can exert its effects indicates that lower concentrations (within a reasonable physiological range) could still cause receptor stimulation. Student's paired t test (one-tailed) was performed to compare basal NO production to the lowest concentrations of choline. Choline elevated *L*-[$^3$H]citrulline formation significantly above basal at concentrations as low as 100 µmol/l and 1 mmol/l ($p = 0.017$ and $p < 0.0001$, respectively, $n = 6$), while the response to 10 µmol/l choline did not differ from basal levels ($p = 0.082$, $n = 5$).

As molecules of the choline family are important components of cell membranes, the possibility that high concentrations of choline are simply causing the disruption of the plasma membrane to allow leakage of $Ca^{2+}$ (which would subsequently activate nNOS) was tested. This pos-

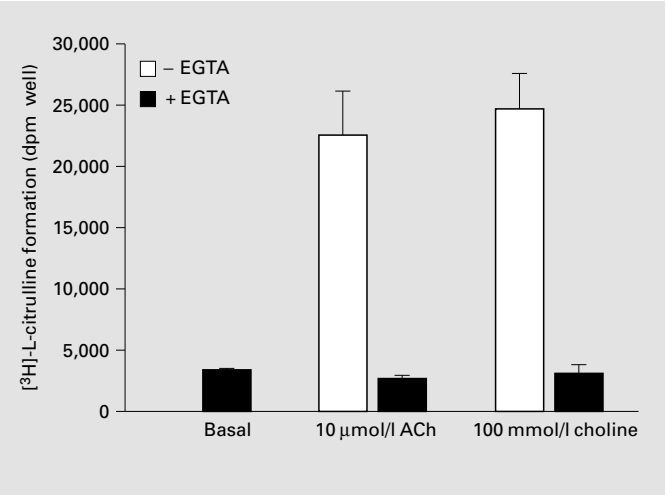


**Fig. 3.** Choline and ACh stimulation of nNOS is blocked by removal of extracellular $Ca^{2+}$ concentration gradient. Addition of 3 mmol/l EGTA 5 min prior to the addition of the agonists eliminated the nNOS response to both 10 mmol/l ACh and 100 mmol/l choline. A two-tailed Student t test showed that neither 10 mmol/l ACh + EGTA nor 100 mmol/l choline + EGTA differed significantly from basal. Values are mean ± SE of three independent experiments performed in triplicate.

sibility is unlikely, since the effects of choline were completely abolished by the muscarinic receptor antagonist, atropine (fig. 2). These data indicate a specific effect of choline via the muscarinic receptor.

Our laboratory has previously presented data indicating that prolonged $M_1$-mediated activation of nNOS requires the influx of extracellular $Ca^{2+}$ [17]. Choline's effects were, therefore, tested in the presence of 3 mmol/l EGTA which reduces extracellular $[Ca^{2+}]$ to approximately the same levels as free intracellular $[Ca^{2+}]$, effectively eliminating the $Ca^{2+}$ concentration gradient. As seen in figure 3, EGTA completely blocked the formation of *L*-[$^3$H]citrulline due to stimulation by ACh or choline.

*Measurement of ACh- and Choline-Mediated Elevations in Free Intracellular Calcium*

Changes in free $Ca^{2+}$ concentrations in response to ACh or choline stimulation were measured by fluorescence spectrometry using the radiometric dye fura-2, as described above. Our laboratory has previously shown that muscarinic receptor mediated changes in intracellular $[Ca^{2+}]$ exhibit a rapid (<1 min) and transient increase, followed by a lower plateau of elevated $[Ca^{2+}]_i$ that does not return to baseline for at least 60 min [17]. We, therefore, constructed separate ACh and choline concentration-response curves for the transient peak and sustained plateau (fig. 4). Nonlinear regression analysis of concentration-response data for the transient peak yielded the following parameters: for ACh, maximum $[Ca^{2+}]$ = 920 ± 240 nmol/l, $pEC_{50}$ = 6.54 ± 0.40, Hill slope = 0.570 ±

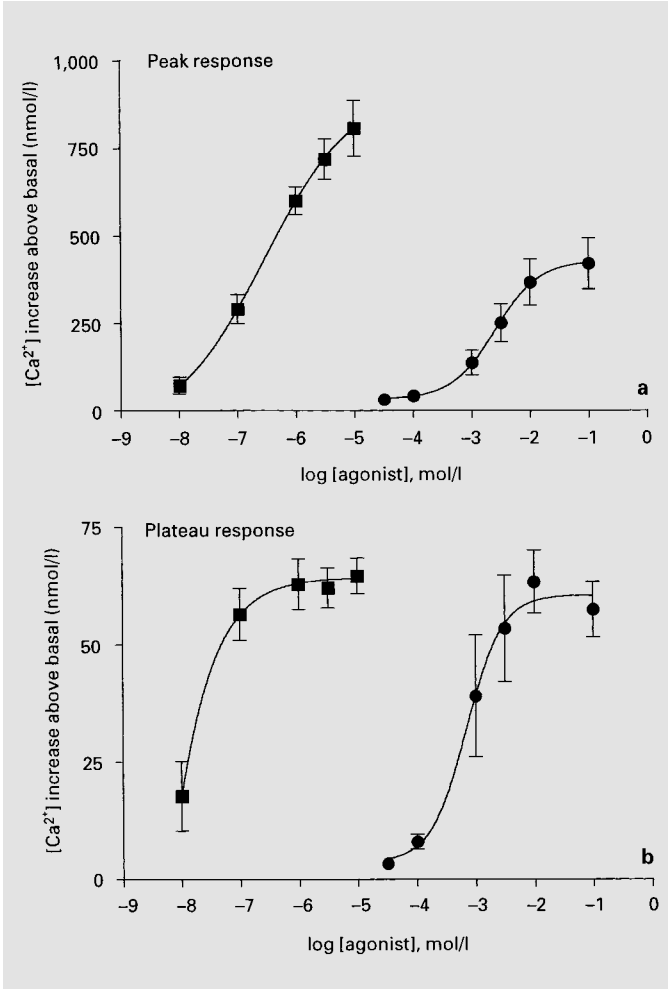


**Fig. 4.** Concentration-response curves for ACh- (■) and choline-mediated (●) elevation of intracellular $Ca^{2+}$ concentrations. Basal $Ca^{2+}$ concentrations (which were individually determined for each trace) have been subtracted out of the values presented here. Changes in intracellular $[Ca^{2+}]$ were recorded in real time using fura-2 dye loaded into CHO $hM_1$/nNOS cells. Basal $[Ca^{2+}]$ was recorded from time 0–30 s and found to have a mean of 88.63 ± 8.65 nmol/l. Varying concentrations of agonist were added at time 30 s. **a** The peak response was defined as the maximum $[Ca^{2+}]$ recorded during time 30–90 s. **b** Plateau response to muscarinic stimulation which was defined as the mean $[Ca^{2+}]$ recorded during time 120–150 s. Values are mean ± SE of at least four independent experiments performed in duplicate.

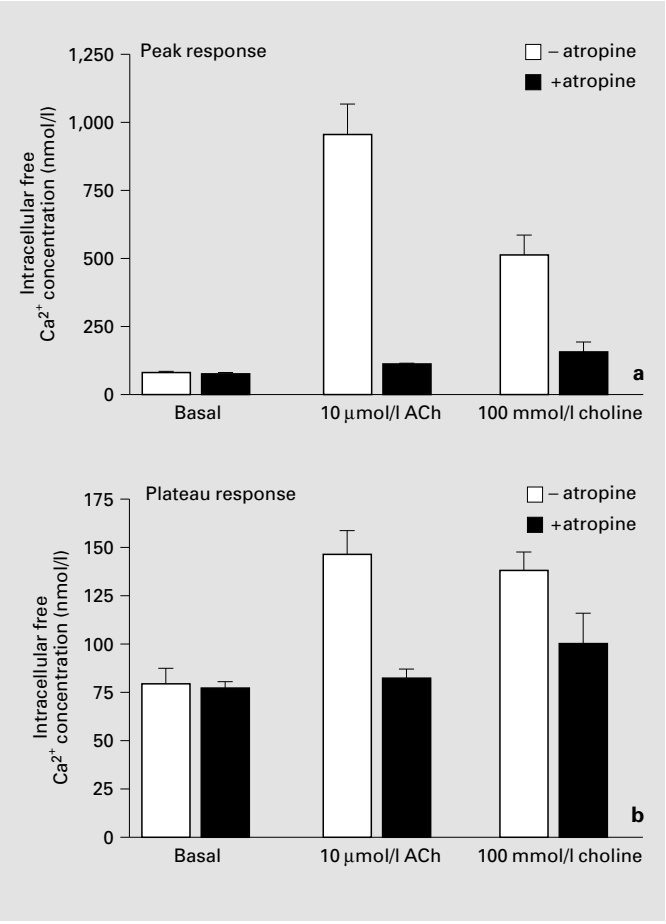


**Fig. 5.** Mobilization of intracellular $Ca^{2+}$ stores by choline is mediated by the muscarinic receptor. CHO $hM_1$/nNOS cells were stimulated with 10 mmol/l ACh or 100 mmol/l choline, alone or in the presence of 1 mmol/l atropine. Atropine was allowed to equilibrate for 5 min prior to the addition of the agonists. In the presence of atropine, neither the peak (**a**) nor plateau (**b**) measurement of $[Ca^{2+}]$ elevation in response to ACh or choline differed significantly from basal. Values are mean ± SE of four independent experiments performed in duplicate.

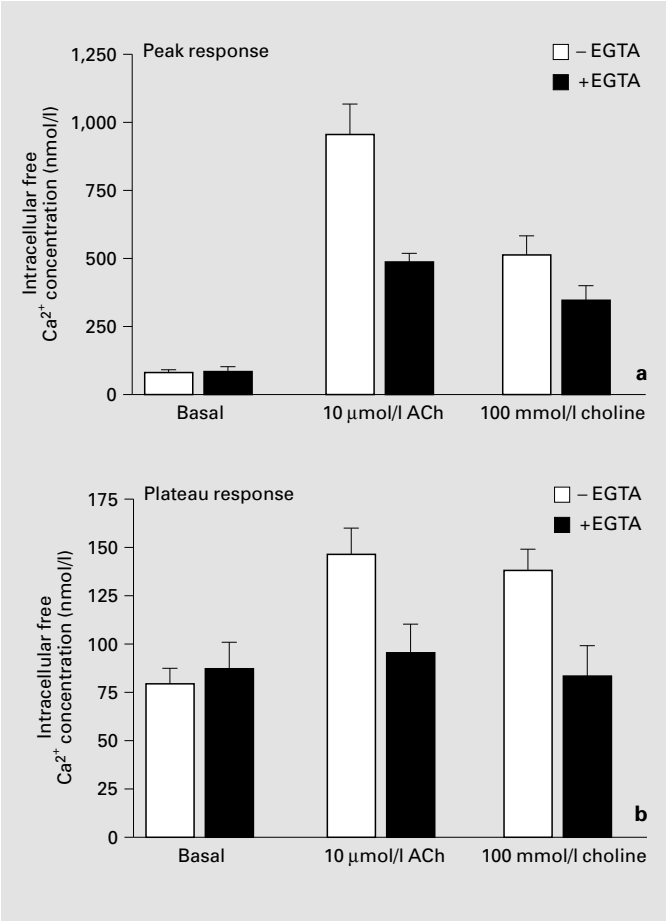


**Fig. 6.** Inhibition of agonist-mediated increases in $[Ca^{2+}]$ by pretreatment with 3 mmol/l EGTA. CHO $hM_1$/nNOS cells were stimulated with 10 mmol/l ACh or 100 mmol/l choline, alone or in the presence of 3 mmol/l EGTA. EGTA was allowed to equilibrate for 2 min prior to the addition of the agonists. EGTA showed partial inhibition of the peak response (**a**). The ACh-mediated peak response was significantly depressed in the presence of EGTA, but both the choline- and ACh-induced peaks remained significantly different from basal $Ca^{2+}$ concentrations ($p = 0.0026$ and $p < 0.0001$, respectively). Conversely, both the ACh and choline-mediated plateau responses were completely abolished in the presence of EGTA, with a Student t test showing no difference from basal (**b**). Values are mean ± SE of four independent experiments performed in duplicate.

0.435; for choline, maximum $[Ca^{2+}]$ = 430 ± 55 nmol/l, $pEC_{50}$ = 2.6 ± 0.201, Hill slope = 1.16 ± 0.60. Thus, choline showed only partial agonistic activity in the induction of the transient $[Ca^{2+}]$ peak, as a Student's t test (two-tailed) showed that the mean peak response of ACh was significantly different from the peak response induced by choline ($p = 0.0076$, d.f. = 8). Nonlinear regression analysis of concentration-response data for the sustained plateau phase of the $Ca^{2+}$ response yielded these parameters: for ACh, maximum $[Ca^{2+}]$ plateau = 63 ± 3 nmol/l, $pEC_{50}$ = 7.65 ± 6.21, Hill slope = 1.41 ± 8.29; for choline, maximum $[Ca^{2+}]$ plateau = 61 ± 7 nmol/l, $pEC_{50}$ = 3.16 ± 0.29, Hill slope = 1.409 ± 1.32. Student's t test indicated that maximum plateaus induced by choline and ACh did not significantly differ from one another ($p = 0.237$, d.f. = 8).

As with the activation of nNOS, choline's effect on intracellular $Ca^{2+}$ concentrations appears to be mediated by the muscarinic receptor. When choline was added in the presence of atropine, neither the transient peak nor

plateau measurement of intracellular [$Ca^{2+}$] differed significantly from basal levels, as shown in figure 5. In the presence of 3 mmol/l EGTA, the transient peak induced by ACh was significantly depressed ($p = 0.0031$), while the peak induced by choline was not significantly affected ($p = 0.104$). The presence of EGTA abolished the sustained intracellular [$Ca^{2+}$] plateau induced by both ACh and choline. Measurements of the plateau [$Ca^{2+}$] induced by ACh or choline in the presence of 3 mmol/l EGTA did not differ from basal $Ca^{2+}$ concentrations ($p = 0.98$ and $p = 0.85$, respectively). These results are summarized in figure 6.

## Discussion

The results of the current study are in agreement with those of earlier reports which suggested weak agonistic activity for choline, with half-maximal concentrations ranging from 1.3 to 2.5 mmol/l in the rat brain [9–12]. In contrast to earlier studies [14, 16], we report full agonist activity of choline after stimulation of nNOS and induction of a sustained $[Ca^{2+}]_i$ plateau mediated by the $M_1$ muscarinic receptor subtype. Both of these responses were found to be dependent on the presence of external $Ca^{2+}$. Choline was also shown to be a partial agonist at induction of the transient peak $[Ca^{2+}]_i$ response following muscarinic receptor stimulation. In agreement with previous binding and functional studies [9, 11, 12, 14–16], choline was at least four orders of magnitude less potent than ACh in all responses assayed in this study. To our knowledge, this is the first evidence that choline has the ability to act as a full agonist at wild-type muscarinic receptors. Although it may be argued that physiological concentrations of choline do not approach the concentrations which displayed activity in the present experiments, the highly compartmentalized nature of synapses can make the effective local concentrations of choline quite high. Hydrolysis of ACh by cholinesterase activity can also produce elevated concentrations of choline, as a single impulse can raise the synaptic concentration of ACh up to 1 mmol/l [21]. Unless it is rapidly removed from the synaptic cleft after ACh hydrolysis, this choline would be free to interact with the receptors, possibly stimulating the production of NO and elevating intracellular calcium concentrations. The use of transfected cell lines in this study should also be considered, as the agonistic effects of choline on muscarinic receptor mediated PI hydrolysis have been shown to be dependent on the level of receptor expression [14]. In this regard, it is noteworthy that the level of receptor expression in our CHO $hM_1$/nNOS cell lines ($\sim 670$ fmol/mg protein) [22] is within the range found in certain brain areas enriched with the $M_1$ receptor subtype ($\geq 1{,}000$ fmol/mg protein) [23].

Examination of data from calcium measurement experiments testing the effects of EGTA shows that while EGTA eliminates both the ACh- and choline-induced plateau response, only the ACh-induced peak response is significantly inhibited. Perhaps choline and ACh have slightly different mechanisms of $Ca^{2+}$ mobilization. ACh appears to be dependent on both external and internal calcium to achieve its peak response, while the smaller, choline-induced peak appears to come primarily from internal stores. If this is the case, then the lack of significant PI hydrolysis in other experiments points to the possibility that muscarinic receptors may be able to mobilize intracellular calcium through more than one pathway. Alternatively, it may be the case that choline is only a weak stimulator of PI hydrolysis, but the signal amplification occurring between the production of $IP_3$, mobilization of $[Ca^{2+}]_i$, and subsequent activation of nNOS is efficient enough to confer full agonist activity to choline further down the signal transduction pathway. Further experimentation is needed to resolve these discrepancies.

There are many possible implications of choline's activation of nNOS, given the varied functions of NO and its effectors, although here we are primarily concerned only with functions related to learning and memory. NO serves as an essential retrograde messenger in the induction of long-term potentiation and neuronal plasticity [24], and, more recently, NO production has been found to be essential to changes in the functional circuitry of the olfactory bulb that are thought to underlie the formation of olfactory memories [25].

As mentioned in the 'Introduction', the density of $M_1$ muscarinic receptors is relatively unaltered in Alzheimer's disease, while the long-axon neurons providing these postsynaptic receptors with ACh are most susceptible to degeneration [1, 5]. As a result of this observation, one of the most widely used treatments for Alzheimer's dementia has been the use of acetylcholinesterase inhibitors, in an effort to increase the availability of ACh that is released by the remaining presynaptic neurons. The current study may shed some light on the possible reasons underlying the disappointing results of therapeutic inhibition of acetylcholinesterase. Choline is an important metabolite for many biosynthetic pathways, and it is a central molecule in the synthesis and breakdown of phospholipids. The interactions of choline with muscarinic receptors may help regulate the rates of choline uptake and

phospholipid degradation. If this is the case, then attempts to maintain postsynaptic activity by treatment with acetylcholinesterase inhibitors could only exacerbate the degeneration of presynaptic, cholinergic neurons. Wurtman's group [26, 27] proposed a theory of Alzheimer's disease pathology that relates the selective degeneration of long-axon cholinergic neurons to abnormal choline-phospholipid metabolism. In simple terms, neurons that are required to fire repeatedly for long periods of time increase the breakdown of choline-containing phospholipids (over 50% of total cell membrane) to release additional choline for ACh synthesis. This leads to a positive feedback loop, whereby the degeneration of axon terminals and resultant neuronal death lead to an increased demand for ACh which leads to accelerated 'autocannibalism' of the membrane. One of the strong points of the theory is that it explains why marked neurodegeneration takes places before the appearance of significant memory deficits. The current results lend support to this hypothesis; since choline that is 'cannibalized' from choline-containing phospholipids is released extracellularly and must then be transported back into the cell via the high-affinity choline transporter [28], this choline would first have the chance to interact directly with muscarinic receptors and subsequently activate nNOS. This 'accidental' stimulation could help temporarily sustain learning and memory in the face of degenerating nerve terminals. There is also evidence that alterations in cell membranes (such as reduced total membrane or alteration of the ratio between phosphatidylcholine and phosphatidylethanolamine) can impair signal transduction and possibly contribute to abnormal amyloid precursor protein processing [29]. In addition, it has recently been reported that β-amyloid encourages leakage of choline across the cell membrane [30], providing another positive feedback loop encouraging the degeneration of cholinergic neurons. Neurons in Alzheimer's disease appear to be frantically attempting to compensate for the faulty metabolism of choline by increasing its uptake. High-affinity choline transporter expression and activity in rapid autopsy of Alzheimer brains was found to be up to 400% greater than in age-matched controls [31].

Precursor supplementation is another therapeutic strategy for Alzheimer's disease that has been studied fairly extensively. Researchers hope to increase the synthesis (and presumably the release) of ACh by providing an excess of choline, thereby increasing the supply of ACh to the remaining postsynaptic receptors. As with acetylcholinesterase inhibitor treatment, this strategy has met with little success. Seventeen clinical studies have been reviewed by Bartus et al. [1], and only one showed substantial improvements, with most studies showing ambiguous or negative results. However, Leathwood and Schlosser [8] have pointed out a number of faults with these studies. First, 12 of the 17 studies used free choline as the precursor which is rapidly metabolized into ethanolamine by the endogenous bacteria of the stomach. Of the studies using phosphatidylcholine, all those showing negative results used relatively low dosages. More recently, a study has been done using cytidine 5′-diphosphate choline, an intermediate in the choline-phosphatidylcholine pathway that the body efficiently absorbs as cytidine and choline [32]. This study showed promising results, improving mental performance in 60–80% of the patients. Studies with animal models of dementia and aging have also given contradictory and ambiguous results. Animal studies of choline administration's effect on ACh levels in the brain are evenly split between observation of no change in ACh levels or mild increases [8]. As in human studies, metabolites of the choline-phosphocholine pathway have been far more promising therapeutically than choline salts. Oral administration of *L*-α-glycerylphosphorylcholine to aged rats can preserve choline acetyltransferase and acetylcholinesterase reactivity and muscarinic receptor density [33]. Many of the beneficial effects seen with supplementation of choline metabolites may be more related to phosphatidylcholine's role as the major membrane phospholipid constituent (over 50%). A recent study with rats showed that oral cytidine 5′-diphosphate choline increases brain phospholipid levels [34]. These results, taken with the 'autocannibalism' hypothesis of Alzheimer's disease, suggest that acute treatment with choline supplements is not likely to be effective. By the time most people are diagnosed with Alzheimer's disease, the positive feedback loops accelerating the degradation of cholinergic membranes may be too strong to be overcome simply by adding more choline to the system. Rather, a more sustained, nutritional approach, with increased consumption of choline throughout life, seems more likely to prevent (or delay) the onset of Alzheimer's dementia.

Choline's ability to directly interact with the muscarinic receptor may also be important for the developing brain. For example, the appearance of acetylcholinesterase activity and ACh-binding sites occurs at approximately the same stage of development, while the appearance of the enzyme responsible for synthesizing ACh, choline acetyltransferase, occurs some time later [13]. These data suggest the possibility that choline might actually be the principal cholinergic neurotransmitter of the developing brain. This could have significance for the prevention of

neurodegenerative disease as well. Rat pups given high doses of choline in utero or neonatally showed lifelong memory enhancement [35].

In summary, the current study showed that choline is capable of full agonist activity in the stimulation of nNOS and sustained elevation of free intracellular calcium. Choline is about four orders of magnitude less potent that ACh, but it shows significant activity at concentrations within reasonable synaptic concentrations. This research has implications for treatment strategies of Alzheimer's disease and studies of neurodevelopment. In particular, longitudinal studies of choline phospholipid supplementation throughout various stages of development and aging are needed to establish the role of neuronal membrane integrity and composition in the pathology of Alzheimer's disease and other neurodegenerative disorders.

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