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A novel pharmacological activity of caffeine in the cholinergic system

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ABSTRACT

Cholinergic deficit is regarded as an important factor responsible for Alzheimer's disease (AD) symptoms. Acetylcholinesterase (AChE) and nicotinic receptor (AChR) are two molecular targets for the treatment of this disease. We found here that methanolic extracts of *Camellia sinensis* exhibited anticholinesterase activity and induced AChR conformational changes. From bioguided fractionation we confirmed that caffeine was the active compound exerting such effects. It is well-known that caffeine acts as an inhibitor of AChE and here we explored the effect of caffeine on the AChR by combining single channel recordings and fluorescent measurements. From single channel recordings we observed that caffeine activated both muscle and α7 AChRs at low concentrations, and behaved as an open channel blocker which was evident at high concentrations. Fluorescent measurements were performed with the conformational sensitive probe crystal violet (CrV) and AChR rich membranes from *Torpedo californica*. Caffeine induced changes in the K_D value of CrV in a concentration-dependent manner taking the AChR closer to a desentisized state. In the presence of α-bungarotoxin, an AChR competitive antagonist, high concentrations of caffeine increased the K_D value of CrV, compatible with a competition with CrV molecules for the luminal channel. Our electrophysiological and fluorescent experiments show that caffeine has a dual effect on nicotinic receptors, behaving as an agonist and an ion channel blocker, probably through distinct AChR sites with quite different affinities. Thus, caffeine or its derivatives can be considered for the design of promising multitarget-directed drugs for AD treatment by modulation of different targets in the cholinergic pathway.

1. Introduction

Tea is one of the most popular and widely consumed beverages in the world. It is produced from the leaves of *Camellia sinensis*, and, based on the processing procedures, it can be divided into different types with different constituents. Tea has been considered a healthful beverage with medicinal properties since ancient times. Some of its beneficial effects are blood lipid and weight reduction, antimicrobial, antioxidant, and anticancerogenic activities, and enhanced digestion (Hamilton-Miller, 1995; Hamilton-Miller, 2001; Chan et al., 2011).

The focus of the potential health benefits associated with tea consumption was put on its antioxidant properties (Tan et al., 2017). Pharmacological research has focused mainly on tea polyphenols, especially catechins and gallic acid have been considered the main players in these beneficial effects.

An important constituent of tea that has attracted attention in recent investigations is caffeine. It is the stimulant with psychological activity most widely consumed in the world (Christopher et al., 2005; Xu et al., 2013). Some studies have suggested a protective and even therapeutic role of caffeine on neurodegenerative disorders, like Alzheimer's and Parkinson's diseases. Until now, the only known mechanism that is affected by caffeine at concentrations close to the normal physiological level, in the μM range, is the modulation of adenosine receptors (Fredholm, 1995; Xu et al., 2010, 2016). However, other mechanisms, such as the activation of ryanodine receptors and inhibition of phosphodiesterases, which have also been demonstrated to be affected by caffeine and its derivatives, take place at high caffeine concentrations, in the range of toxic plasma levels (in the mM range) (Guerreiro et al., 2008; Daly, 2007; Kolahdouzan and Hamadeh, 2017).

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Alzheimer's Disease (AD) is the most prevalent neurodegenerative disorder in the elderly characterized by progressive cognitive decline. A disruption of the cholinergic pathways that contributes to the cognitive impairment of AD patients is described in the cerebral cortex and in the basal forebrain. The current treatment is to maintain or improve cholinergic transmission (Yanovsky et al., 2012). Two target molecules for this pharmacological treatment are the acetylcholinesterase (AChE) and the nicotinic acetylcholine receptor (AChR). AChE localizes at the synaptic cleft and is the enzyme that terminates the acetylcholine (ACh) signal at the postsynaptic membrane by hydrolysis of ACh into acetic acid and choline. The AChRs are ionotropic receptor members of the pentameric ligand gated ion channel family, that convert the binding of ACh into the opening of a cation selective channel (Albuquerque et al., 2009; Corradi and Bouzat, 2016). α7 AChRs are homomeric members of this family, localized in the central and peripheral nervous systems. In addition to AD, they have been implicated in other neurological disorders, such as Parkinson's disease, schizophrenia, bipolar disorder, autism spectrum disorder, cerebral ischemic stroke, and traumatic brain injury (Uteshev, 2014; Quik et al., 2015; Corradi and Bouzat, 2016). Muscle AChRs are heteromeric receptors ($\alpha1_2\beta1\varepsilon\delta$) located in the neuromuscular junction and are mainly implicated in myasthenic syndromes (Albuquerque et al., 2009). Nowadays, AChE inhibition is the only treatment for AD. AChE inhibitors (galantamine, huperzine A, physostigmine, etc) are considered indirect AChR agonists that locally increase endogenous neurotransmitter levels. Although much effort is put on the design of cholinergic compounds to directly improve neuronal AChR function, all potential drugs are still under research and development.

In this work we investigated the modulation of AChE and AChR by red tea obtained from *C. sinensis* leaves. Our results demonstrate that methanolic extract of red tea inhibits AChE and induces AChR conformational changes, and that caffeine is the molecule responsible for all these effects. We observed that caffeine inhibits AChE activity in a concentration-dependent manner, as previously described (Karadsheh et al., 1991; Okello et al., 2012; Pohanka and Dobes, 2013). We show for the first time that caffeine acts on AChR with a dual behavior, namely as a partial agonist and as an open channel blocker, at low and high concentrations, respectively.

2. Materials and methods

2.1. Materials

Torpedo californica specimens obtained from the Pacific coast of California (Aquatic Research Consulants, San Pedro, CA, USA) were killed by pithing, and the electric organs were dissected and stored at −70 °C until use. PNU-120596 (N-(5-Chloro-2,4-dimethoxyphenyl)-N'-(5-methyl-3-isoxazolyl)-urea) was obtained from Tocris Biosciences (Bristol, UK). Acetylcholinesterase (type VI-S lyophilized powder), crystal violet (CrV), alpha-bungarotoxin (α-BTX) and all other drugs were obtained from Sigma-Aldrich.

2.2. Methods

2.2.1. Identification of the active compound present in extracts of C. sinensis

A bioguided fractionation of a methanol:water (80:20) extract of red tea leaves was performed with solvents of increasing polarity (hexane, dichloromethane, ethyl acetate, and butanol). The active sub-extract was subject to column chromatography performed with Silica gel 60 (70–230 mesh, Merck) to obtain an active fraction, which was submitted to chromatographic separation. Analytical thin layer chromatography (TLC) was performed on Silica gel 60 F254 sheets (0.2 mm thickness, Merck) and the spots were detected with p-anisaldehyde-acetic acid spray reagent. The structural characterization of the isolated active compound was performed by NMR spectroscopy. NMR spectra were recorded with a Bruker ARX300 spectrometer operated at 300 and 150 MHz for ^1H and ^{13}C, respectively, in $CDCl_3$. Chemical shifts are given in ppm (δ) with TMS as an internal standard. All chemicals and solvents were analytical grade and solvents were purified by general methods before being used.

2.2.2. Inhibition of AChE activity

AChE water solution was prepared daily and AChE activity was measured using Ellman's method (Ellman et al., 1961). Briefly, AChE hydrolyzes acetylthiocholine, which results in the product thiocholine that reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thionitrobenzoate, a yellow product that can be detected at 405 nm. DTNB was dissolved in phosphate buffer pH 7 containing $NaHCO_3$ and was subsequently diluted with phosphate buffer pH 8 to obtain 0.25 mM DTNB. The enzymatic reaction was initiated by the addition of 20 μl of ACh 75 mM, and its hydrolysis was monitored by reading the absorbance at 405 nm every 10 s for 3 min. Galantamine (4 μg/ml) was used as positive control. The percentage of inhibition was calculated as follows:

$$\% \text{ INHIBITION} = (E-S)/E \times 100$$

Where E and S are the O.D. measured 180 s after the color reaction initiation and correspond to AChE activity in either the absence or presence of the extract analyzed, respectively.

To determine the IC_{50} (concentration that inhibits 50% of the substrate hydrolysis), the values of the percentage of hydrolysis were plotted as a function of the logarithmic increasing concentrations of plant extracts. The resulting sigmoid curve was fitted to the Boltzmann function and the IC_{50} values calculated.

2.2.3. AChR-rich membranes preparation

AChR-rich membrane fragments were prepared from *T. californica* electric tissue as described previously (Barrantes, 1982). Briefly, electric tissue was chopped into small pieces, homogenized using a Virtis homogenizer under controlled conditions, and submitted to a series of centrifugation steps ending in a high-speed sucrose gradient centrifugation. The middle fraction obtained corresponds to AChR-rich membranes with a specific activity of the order of 1.0–1.5 nmol of α-BTX sites/mg of protein (Barrantes, 1982).

2.2.4. Cell culture

HEK293 cells were transfected with mouse α, β, δ and ε subunits (for adult muscle AChR, $\alpha1_2\beta1\delta\varepsilon$), or with human α7 and Ric-3 for expression of neuronal α7 AChR, as was previously described (Bouzat et al., 1994; Corradi et al., 2007). Each receptor cDNA subunit was sub-cloned into pRBG4 plasmid for heterologous expression. For electrophysiological measurements, a plasmid encoding green fluorescent protein (GFP) was included in all transfections to allow the identification of transfected cells under fluorescence optics.

2.2.5. Fluorimetric measurements

Fluorimetric measurements were performed in a SLM model 4800 fluorimeter (SLM Instruments, Urbana, IL) using a vertically polarized light beam from Hannovia 200-W mercury/xenon arc obtained with a Glan-Thompson polarizer (4-nm excitation and emission slits).

2.2.5.1. AChR conformational state characterization. AChR-rich membranes were resuspended in buffer A (150 mM NaCl, 0.25 mM $MgCl_2$, and 20 mM HEPES buffer, pH 7.4) at a final concentration of 100 μg of protein/ml. Fluorimetric measurements were performed in 2 ml quartz cuvettes.

AChR conformational changes were evaluated using crystal violet (CrV) as described previously (Nievas et al., 2008). AChR-rich membranes resuspended in buffer A were incubated for 15 min with caffeine, QX-314, or galantamine, either alone or after pre-incubation with 1 μM of α-BTX. For the measurements conducted with AChR in the desensitized state, *T. californica* membranes were afterwards incubated with 1 mM of carbamylcholine (carb) for 15 min. The membranes were subsequently titrated with increasing concentrations of CrV (in buffer A). After each addition of CrV, the samples were incubated for 15 min before obtaining the fluorescence emission spectra. CrV was excited at 600 nm, and the fluorescence emission spectra were collected from 605 to 700 nm. Before the first addition of CrV, a background fluorescence emission spectrum was obtained for each sample. The spectrum was then substracted from the emission spectra obtained in the presence of CrV and the maximum intensity (at 623–625 nm) was measured. To determine the CrV dissociation constants (K_D), the values of CrV maximum fluorescence emission were plotted as a function of the logarithmic CrV concentrations (M). The resulting sigmoid curve was fitted to the Boltzmann function and K_D was calculated.

2.2.5.2. Binding experiments. 200 μl of AChR-rich membranes resuspended in buffer A (100 μg of protein/ml) or HEK293 cells expressing neuronal α7 AChR, two or three days after transfection, resuspended in PBS (Bouzat et al., 1994; Corradi et al., 2007) were incubated for 20 min with increasing concentrations of caffeine (0.1, 0.3, 1, 5 or 10 mM), galantamine (0.3 and 1 mM) or 10 mM carb. Each condition was afterwards incubated with Alexa 488-αBTX 10 nM for 30 min. Binding was stopped by centrifugation at 12,000 rpm for 1 min three times. The samples were resuspended with 200 μl PBS and loaded in 5 × 5 mm quartz cuvettes. Alexa 488-αBTX was excited at 488 nm, and the fluorescence emission spectra were collected from 500 to 600 nm. A background fluorescence emission spectrum was obtained. The spectrum was then subtracted from each emission spectra obtained in the presence of Alexa 488-αBTX and the maximum intensity (520 nm) was measured.

2.2.6. Electrophysiological experiments

HEK293 cells expressing muscle AChR or neuronal α7 AChR were used for single-channel measurements two or three days after transfection. Single-channel recordings were performed in the cell-attached patch-clamp configuration at room temperature (~20 °C) and at −70 mV of membrane potential. The bath and pipet solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). Agonists (ACh or caffeine) with or without PNU-120596 (PNU) were added to the pipette solution. Single-channel currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices), digitized at 200 kHz and low-pass filtered at a cutoff frequency of 10 kHz using a computer interface Instrutech ITC-18 (HEKA Instruments Inc.). Single-channel events were idealized by the half amplitude threshold criterion using the program QuB 2.0.0.28 (www.qub.buffalo.edu) with a digital low-pass filter at 9 kHz. A filter of 3 kHz was used in recordings with PNU to facilitate the analysis. Single-channel amplitudes were calculated with the Amps tool from QuB. The open and closed time histograms obtained from idealization were fitted by the maximum interval likelihood (MIL) function in QuB (Qin et al., 1997, 1996) with a dead time of 0.03 ms for muscle AChR, or 0.03 ms and 0.1 ms for α7 in the absence or the presence of PNU, respectively. This analysis was performed on the basis of a kinetic model whose probability density function curves properly fit the histograms following the maximum likelihood criteria. For muscle AChR, the best fitting was obtained with the classical kinetic model previously reported for this receptor (Bouzat et al., 1994; Corradi et al., 2007). For α7 receptor, this analysis was carried out by sequentially adding an open and/or closed state to a starting C↔O model in order to properly fit the corresponding histograms. Models contained four-five closed states and two-three open states for α7 in the presence of ACh or caffeine plus PNU, or three closed states and one-two open states for α7 in the presence of ACh or caffeine in the absence of PNU (Andersen et al., 2016; Bouzat et al., 2008; daCosta and Baenziger, 2009).

Clusters were identified as a series of closely separated openings preceded and followed by closings longer than a critical duration. Different critical closed times were calculated by MIL between each closed component. Critical time between the second and third closed components for muscle AChR (~15 ms), and between the third and fourth closed components for α7 in the presence of PNU (~60 ms) were selected in QuB to chop the idealized data and create a sub-data set that only contained clusters to define mean cluster duration and open probability within clusters (Popen).

2.2.7. Data analysis

Intergroup comparisons were carried out using one-way ANOVA test with the values representing the average ± SD of the total number of samples indicated in each figure legend. *, statistically significant differences ($p < 0.05$). ** statistically very significant differences ($p < 0.01$)

3. Results

3.1. AChE and AChR activity in the presence of methanolic extract from C. sinensis leaves

To study the effect of red tea on AChE we first investigated the enzyme activity in the presence of a methanolic extract obtained from red tea leaves. The extract caused an inhibition of AChE activity in a concentration-dependent manner, with an IC_{50} value of 0.043 mg/ml (Fig. 1A). This value is close to the IC_{50} published for pure galantamine, a recognized AChE inhibitor (Dalai et al., 2014; Tel et al., 2015). Here we used a galantamine concentration of 0.004 mg/ml which induced more than 90% AChE inhibition.

The AChR is an allosteric protein that can be in three different and interconvertible conformational states: resting state (R), in the absence of agonist; open state (O), and desensitized state (D), in the

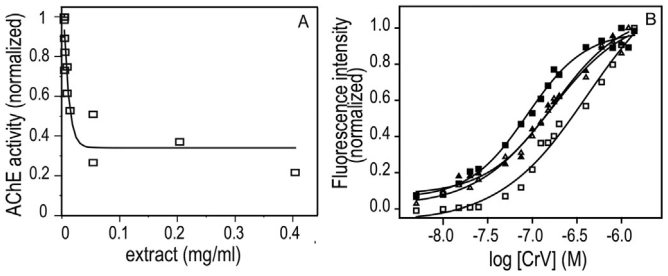

Fig. 1. A) AChE activity in the presence of methanolic extract of *C. sinensis*. AChE activity was measured 180 s after reaction initiation at an OD of 405 nm. Each point represents the mean ± S.D.; n = 3. B) Titration of AChR-rich membranes with CrV. Membranes were previously incubated for 15 min with increasing concentrations of methanolic extract of *C. sinensis*, in the absence (▲ and ■, methanolic extract and control condition, respectively) or presence (△ and □, methanolic extract and control condition, respectively) of 1 mM carb.

continuous presence of agonist. To study whether the methanolic extract obtained from *C. sinensis* can modify AChR conformational state, we used the AChR conformational-sensitive fluorescence probe crystal violet (CrV) (Nievas et al., 2008; Perillo et al., 2012), since CrV displays higher affinity for the D than for the R state (Lurtz and Pedersen, 1999), and evaluated the titration curves in the absence and presence of the methanolic extract (Fig. 1B). In the absence of carb and in the presence of the methanolic extract, a displacement of the titration curve was observed, indicating that in the presence of the extract the AChR changed its conformational state, approaching to a state close to the desensitized one, whereas in the presence of carb, the K_D of CrV for the receptor in the desensitized state did not change.

3.2. Identification of the active compound present in extracts of C. sinensis

A bioguided fractionation of a methanol:water (80:20) extract of red tea leaves was performed with solvents of increasing polarity. Those sub-extracts were evaluated in their ability to inhibit AChE activity. The dichloromethane sub-extract showed the highest inhibition power (Fig. 2A). Ten sub-fractions were obtained from the dichloromethane sub-extract by column chromatography using dicholoromethane with increasing methanol content as solvent eluent. Only sub-fraction 3 presented AChE inhibitory activity (Fig. 2B). Thin-layer chromatography suggested that there was a single molecule in this sub-fraction which was spontaneously crystallized, in pure and in excellent yield. This was confirmed by NMR spectroscopy and the active compound was identified as caffeine (Supplementary file 1). Therefore, the effects observed on AChE activity in the presence of the methanolic extract were due to its caffeine content. Although previous research has reported AChE inhibition by caffeine (Pohanka and Dobes, 2013), the effect of caffeine on AChR activity still remains unknown. We continued the study with synthetic caffeine to have total control of the identity and purity of the drug.

3.3. AChR conformational changes induced by caffeine

To explore if caffeine has a direct effect on the AChR, we first incubated *T. californica* membranes with increasing concentrations of caffeine (10-300 μM) in the absence and presence of carb, a full agonist for the AChR. After incubating the membranes with caffeine, a decrease of K_D of CrV was observed in the absence of carb, while in the presence of carb the K_D of CrV for the receptor remained constant (Fig. 3). Caffeine induced K_D changes in a saturated manner with an EC_{50} of 98.1 μM. When the same experiment was performed with increasing concentrations of carb a decrease of K_D of CrV was also observed, with an EC_{50} of 0.12 μM (Fig. 3 inset).

To deepen the caffeine-AChR interaction, we performed another series of experiments in which the membranes were preincubated with α-BTX, a specific AChR competitive antagonist that blocks ACh binding sites (Fig. 4). The presence of α-BTX induced no change in the K_D of CrV of the R state. Three different concentrations of caffeine (10, 100 and 220 μM) were tested. At low concentrations (10 and 100 μM), and in the presence of α-BTX, caffeine did not alter

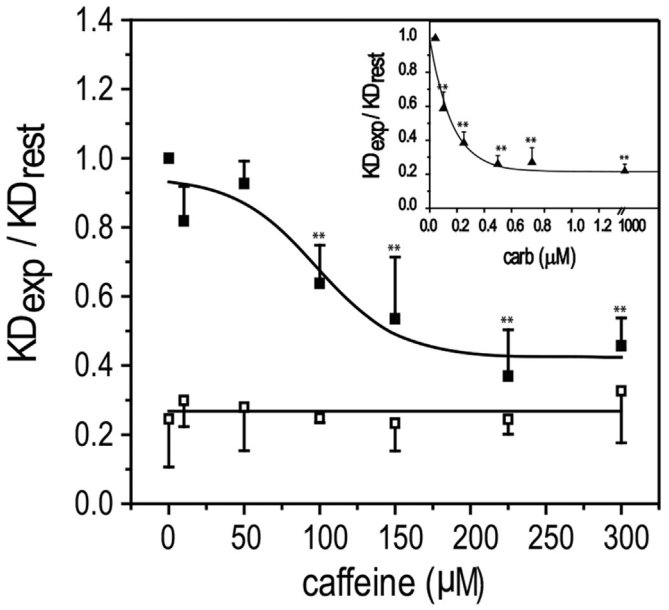

Fig. 3. Variations in the K_D-ratio of the experimentally determined K_D values to the control K_D obtained in the resting state in the presence of increasing concentrations of caffeine. The values were obtained with *T. californica* AChR-rich membrane alone (■), or in the presence of 1 mM carb (□). Each point represents the mean ± S.D.; n = 5. Inset: K_D-ratio calculated in the presence of increasing concentrations of carb.

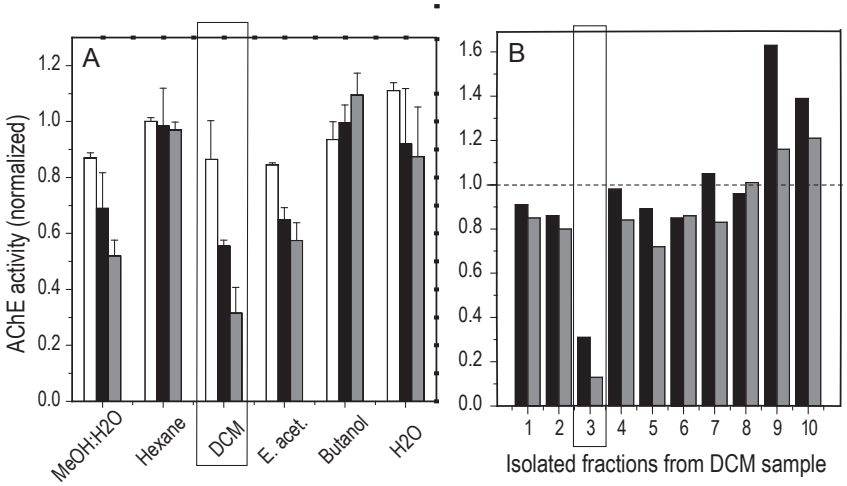

Fig. 2. AChE activity in the presence of A) different fractions obtained from the methanolic extract of *C. sinensis* (white, black and grey columns correspond to 5, 20 and 40 μl of each isolated fraction, respectively) and B) sub-fractions obtained from the DCM fraction (black and grey columns correspond to 1 and 5 μl of each isolated sub-fraction, respectively). AChE activity was measured 180 s after reaction initiation at an OD of 405 nm. Each column represents the mean ± S.D.; n = 3.

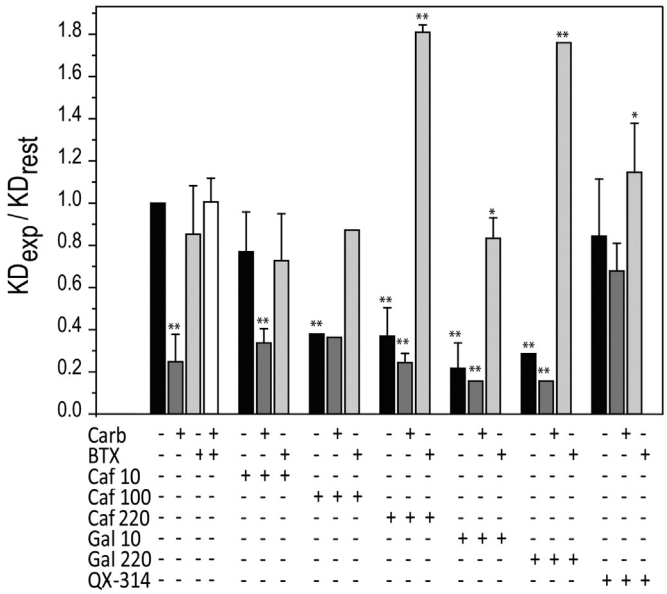

Fig. 4. Variations in the K_D-ratio of the experimentally determined K_D values to the control K_D obtained in the resting state in the presence of caffeine (10, 100 and 220 μM), galantamine (10 and 220 μM) or QX-314 (250 μM). The values were obtained with *T. californica* AChR-rich membrane alone (black columns), in the presence of 1 mM carb (dark grey columns) or preincubation with 1 μM α-BTX (light grey columns). Each column represents the mean ± S.D.; n = 4.

the K_D of CrV, whereas at high concentrations (220 μM), caffeine produced a great increase of K_D value. This increase was observed neither in the absence of α-BTX nor in the presence of carb where a constant and diminished K_D value at all caffeine concentrations was observed, nor in the absence of carb where a reduction in the K_D with caffeine was evident. (Fig. 4).

Similar experiments were performed with galantamine (10 and 220 μM), a partial agonist of AChR and commercial AChE inhibitor (Dajas-Bailador et al., 2003; Grossberg, 2003; Nordberg and Svensson, 1998). A clear decrease in K_D values was observed at both concentrations (Fig. 4). When the membranes were pre-incubated with α-BTX, the presence of 10 μM of galantamine resulted in a K_D of CrV close to the K_D of the R state. However, at 220 μM of galantamine, a similar behavior to the one reported above at high concentrations of caffeine was observed: a marked increment in K_D value. Galantamine was reported to act on AChR as a partial agonist and an open channel blocker (Akk, 2005). A further condition was tested. We performed the same study in the presence of QX-314, an AChR open channel blocker (Pascual and Karlin, 1998; Yu et al., 2003). When membranes were incubated in the presence of QX-314 and in the absence of carb, there was no change in the relative K_D value (Fig. 4); in the presence of carb K_D value was similar to the one for the R state, thus suggesting that this channel blocker did not allow the displacement of the AChR conformation equilibrium towards a D state in the presence of carb. Finally, when membranes were preincubated with α-BTX, the addition of QX-314 increased the K_D value with respect to the value for the R state, similar results were obtained with caffeine and galantamine (Fig. 4).

3.4. Muscle and neuronal nicotinic acetylcholine receptor activation by caffeine

To understand the molecular mechanism underlying the conformational changes of AChR, we studied if caffeine activates both muscle and neuronal α7 AChRs by electrophysiological measurements. To this end, mouse muscle AChR was expressed in HEK293 cells, and single channel events were recorded at 30 μM ACh to corroborate channel expression. From these recordings we observed single channel events of 5.2 ± 0.6 pA (at -70 mV of membrane potential) grouped in clusters of 140 ± 70 ms with a Popen of 0.4 ± 0.1 (Fig. 5 and Table 1). At this ACh concentration, the open time histograms showed two components whose durations and relative areas were similar to those reported previously (Table 1, Fig. 5) (Bouzat et al., 2000; Corradi et al., 2007). Closed time histograms were adjusted by three or four components, of which the two briefest corresponded to closings within clusters (Table 1) (Corradi et al., 2007). When cells were analyzed in the presence of caffeine, single-channel events were observed, which indicates that caffeine can act as an agonist for the muscle AChR (Fig. 5). At 1 μM of caffeine, no activation was observed in a total of 11 seals, in contrast to what was observed at the same concentration of ACh, where single-channel events are readily detected as isolated events (Bouzat et al., 2000). At higher caffeine concentrations (from 10 to 100 μM), single-channel events of 5.5 ± 0.5 pA were detected and the activation pattern was as isolated events without ability to activate in clusters (Fig. 5 and Table 1). Open duration histograms were adjusted by a sum of two components, whose durations were similar to those obtained at 30 μM ACh but with different relative areas (Table 1).

To gain insight into the selectivity of action of caffeine, we explored its ability to activate the human α7 AChR. To this end, we performed single channel experiments at different caffeine concentrations (100 and 300 μM) on α7 receptors expressed in HEK293 cells. In the presence of 100 μM ACh, α7 events of about 10 pA (at -70 mV of membrane potential) were detected as brief and isolated openings, or as a few openings in quick succession (bursts) similar to that reported previously (Bouzat et al., 2008) (Fig. 6A). With 100 μM of caffeine on the same set of cells, no single

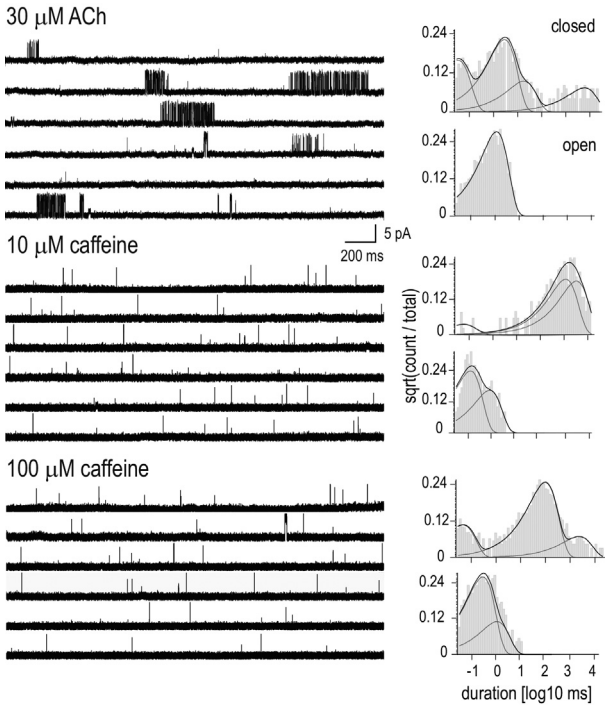

Fig. 5. Single-channel activation of muscle AChR by caffeine. *Left*) Single-channel events obtained in the presence of 30 μM of ACh or increasing concentrations of caffeine. Membrane potential -70 mV. *Right*) Open and closed time histograms obtained from the corresponding recordings.

Table 1
Single channel properties of muscle ACh receptors activated by ACh or caffeine.

Agonist	[μM]	Amp (pA)	O_1 (ms) (area)	O_2 (ms) (area)	C_1 (ms) (area)	C_2 (ms) (area)	n
ACh							
	30	5.2 ± 0.6	1.3 ± 0.3 (0.95 ± 0.09)	0.3 ± 0.2 (0.15 ± 0.10)	0.06 ± 0.04 (0.11 ± 0.05)	2.8 ± 0.9 (0.82 ± 0.07)	14
Caffeine							
	10	5.9 ± 0.1	0.97 ± 0.19 (0.43 ± 0.23)	0.13 ± 0.04 (0.63 ± 0.26)	0.04 ± 0.03 (0.02 ± 0.01)	– –	6
	50	5.4 ± 0.3	0.90 ± 0.23 (0.51 ± 0.21)	0.19 ± 0.08 (0.49 ± 0.21)	0.04 ± 0.02 (0.05 ± 0.02)	– –	5
	100	5.8 ± 0.3	1.1 ± 0.2 (0.47 ± 0.19)	0.22 ± 0.08 (0.52 ± 0.19)	0.03 ± 0.01 (0.05 ± 0.02)	– –	5

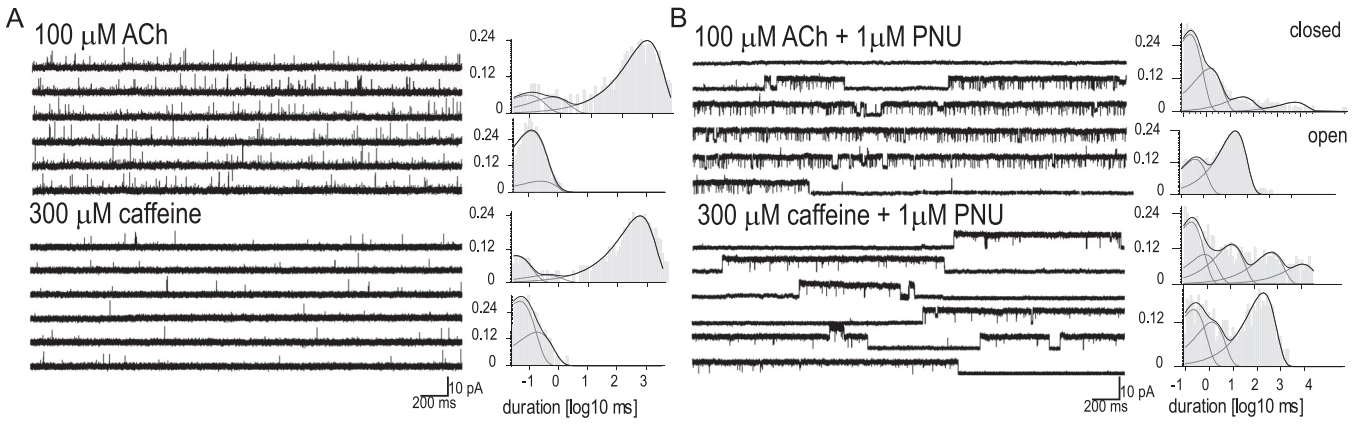

Fig. 6. Single-channel activation of human α7 receptor by caffeine. Single-channel events obtained in the presence of ACh or caffeine in the absence (A) or presence of PNU (B). Openings are shown as upward deflections and were recorded at -70 mV of membrane potential. Open and closed time histograms are shown from the corresponding recordings.

channel events were detected. Instead, some brief events were detected when caffeine concentration was increased to 300 μM (Fig. 6A), thus suggesting that caffeine probably acts as a weak agonist. Since α7 single-channel activation by ACh occurs as low-frequency brief events, and considering that caffeine could be a low efficacy agonist, we obtained single-channel recordings in the presence of PNU-120596 (PNU), a positive allosteric modulator for α7. In the presence of 100 μM of ACh and 1 μM of PNU, single-channel events were more frequent and appeared grouped in clusters of high Popen (0.92 ± 0.02) and long duration (3,100 ± 1,700 ms) (Fig. 6B). Open duration histograms were adjusted by the sum of two or three components and a clear increase in the durations with respect to those obtained without PNU was observed due to α7 potentiation. Closing duration histograms where adjusted by four or five components, of which the three briefest where considered within clusters, and the critical time to define a cluster was obtained by the intersection between the third and fourth closed component (see Methods). All the results obtained from ACh activation of human α7 AChR in the presence of PNU were similar to those previously reported (daCosta and Baenziger, 2009). To confirm that caffeine can act as an α7 agonist, we obtained single channel recordings with increasing concentration of caffeine (from 1 to 300 μM) in the presence of 1 μM of PNU. At all caffeine concentrations, single-channel events where easily detected and the activation pattern was similar to that observed from ACh plus PNU (3,800 ± 1,600 ms and 3,200 ± 80 ms for cluster duration, and 0.97 ± 0.02 and 0.96 ± 0.01 for Popen at 1 and 300 μM of caffeine, respectively) (Fig. 6B). Open and closed duration histograms were similar to those obtained from ACh activation (Fig. 6B).

All these results confirm that caffeine is an agonist for muscle and α7 neuronal AChRs.

3.5. Caffeine behaves as an allosteric agonist

Given that caffeine alone activated muscle and neuronal AChRs and also induced conformational changes on muscle AChR, we next studied whether caffeine goes to ACh binding sites through competition experiments with α-BTX. *T. californica* membranes or HEK293 cells expressing neuronal α7 AChR were first incubated with increasing concentrations of caffeine following by addition of Alexa 488-αBTX. Similar results were obtained with both AChRs. In the presence of 1 mM carb, the fluorescence intensity of Alexa 488-αBTX was statistically significant lower than the value obtained for the control condition, confirming a net displacement of α-BTX by carb (Fig. 7). However, at different caffeine concentrations, the fluorescence intensities were similar to the control value suggesting that caffeine did not affect α-BTX binding to AChR. Similar results were observed when the experiment was performed in the presence of galantamine, a known allosteric activator of AChR. All together, these results support the hypothesis that caffeine behaves as an allosteric agonist (Fig. 7).

3.6. Nicotinic acetylcholine receptors blockade by caffeine

To explore how caffeine can modulate the channel activity elicited by ACh, we obtained single channel events at 30 μM ACh in the presence of increasing concentrations of caffeine (from 0 to 20 mM). From 300 μM of caffeine, a reduction in the mean open time duration and an increase in the mean cluster duration were

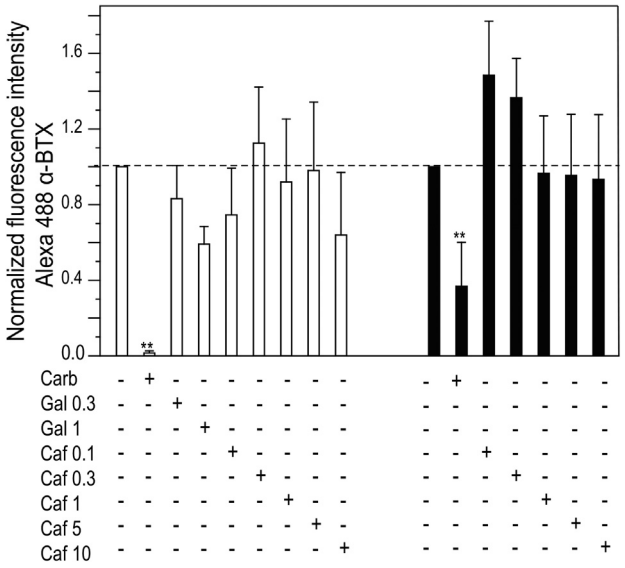

Fig. 7. Normalized fluorescence intensity of Alexa 488-αBTX binding to both muscle AChR (□) and neuronal α7 AChR (■) in the presence of 10 mM carb, galantamine (0.3 and 1 mM) or increasing concentrations of caffeine (0.1, 0.3, 1, 5 and 10 mM). Each point represents the mean ± S.D.; n = 3.

clearly observed as a function of caffeine concentration (Fig. 8). Events obtained at 30 μM ACh in the presence of 500 or 5,000 μM of caffeine showed a mean open duration of 0.61 ± 0.09 ms and 0.23 ± 0.02 ms and a mean cluster duration of 230 ± 80 ms and 450 ± 260 ms, respectively (Fig. 8). These observations are consistent with an open channel blockade, which can be represented by the classical blocking scheme (Scheme 1) (Neher and Steinbach, 1978):

$$\mathbf{C} \underset{\alpha}{\overset{\beta}{\leftrightarrow}} \mathbf{O} \underset{k_{-B}}{\overset{k_{+B}[B]}{\leftrightarrow}} \mathbf{B}$$

Scheme 1.

Where C, O and B correspond to closed, open and blocked states, respectively. Plotting the inverse of the longest open duration against caffeine concentration, a linear relation was observed and the slope of the linear regression allowed to obtain a forward blocking rate constant (k_{+B}) of $6.5 \times 10^5\ M^{-1}\ s^{-1}$ ($r^2 = 0.98$). Since the area of the briefest closed component increases with caffeine concentration (0.11 ± 0.04 and 0.77 ± 0.04 at 0 and 5,000μM of caffeine, respectively) in parallel to the decrease in the duration of the longest opening (Fig. 8), the duration of this closing can be associated with the fast blocking process. Therefore, the inverse of the briefest closed duration was used as an estimation of the unblocking rate (k_{-B}) whose value was 25,000 s^1.

Thus, our results confirm that caffeine can block the AChR pore with an estimated blocking constant ($K_B = k_{-B}/k_{+B}$) of 35 mM.

4. Discussion

In the present work we focused on finding a compound which exerts a dual biological activity: inhibitor of AChE and activator of AChR. The study was initiated with a screening of these two activities on the methanolic extract of *C. sinensis* manufactured as a

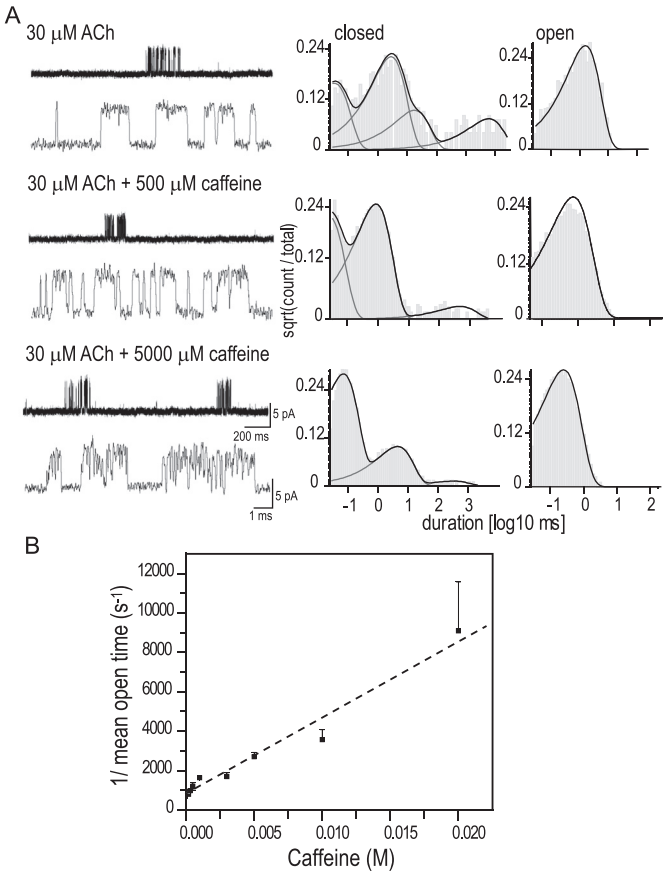

Fig. 8. Activation of muscle AChR in the presence of ACh and caffeine. A) Single channel events obtained in the presence of 30 μM of ACh plus increasing concentrations of caffeine (*left*) and their respective closed and open duration histograms (*right*). Open events are shown as upward deflection from the baseline level. Membrane potential -70 mV. B) Relationship plot for the longest open duration and caffeine concentration. Experimental data were fitted by the linear equation (1)/$\tau_{open} = \alpha + k_{+B}$ [Caf], where τ_{open} is the longest open duration, k_{+B} is the forward blocking rate constant and α is the closing rate constant. Each point represents the mean ± SD from ≥3 recordings.

red tea preparation. This extract showed an AChE inhibition with an IC_{50} value of 0.043 mg/ml (Fig. 1A). A similar result was previously reported from green and white teas (Karadsheh et al., 1991; Okello et al., 2012), thus suggesting that green, red and white teas share the agent responsible for this effect. This extract also showed *T. californica* AChR conformational perturbation (Fig. 1B), which suggested a modulation of AChR function.

Studies performed with different types of teas (green, red and black) showed that the catechin content has a direct correlation with the neuroprotective effect of tea (Schimidt et al., 2017; Singh et al., 2011). Green tea showed the greatest neuroprotective activity and it was suggested that this could be due to the content of (-)-epigallocatechin-3-gallate (EGCG), which is not detected in red and black teas. *In vitro* and *in vivo* studies have demonstrated that the decrease in oxidative stress, reactive oxygen species and apoptosis, and the increase in ACh levels by the inhibition of adenosine receptor and/or AChE are responsible for EGCG neuroprotective and neurorescue properties (Singh et al., 2011; Soung et al., 2015). Since red and black tea lack EGCG but show neuroprotective effects (Schimidt et al., 2017), another molecular agent must be responsible for the observed effects.

In order to identify the active compound, a bioguided fractionation of the *C. sinensis* methanolic extract was performed with a

group of solvents of varying polarity. The dichloromethane subextract, the most hydrophobic one, displayed the highest AChE inhibition (Fig. 2A). Bioguided subsequence fractioning of this sample by HPLC and identification of the compound by NMR analysis revealed that the active compound was caffeine (Fig. 2B and Supplementary file 1). Although previous research reported the inhibitory effect of caffeine on AChE (Pohanka and Dobes, 2013), no information is available on caffeine effect over the AChR. Therefore, caffeine could be the common agent present in all types of tea responsible, at least in part, for their beneficial effects.

In this work we demonstrated that caffeine induced conformational changes on muscle AChR (Fig. 3) which are similar to those induced by the agonist carb or the allosteric agonist galantamine. However, the potency of this effect was quite different between caffeine and carb (IC_{50} of 98.1 μM and 0.12 μM, respectively) suggesting that caffeine could be a partial agonist of AChR. Furthermore, by competitive experiments between caffeine and α-BTX, we observed that once α-BTX was bound to the AChR, low concentrations of caffeine could not perturb the AChR conformation. α-BTX binds to the orthosteric binding site and blocks the access of orthosteric ligands, maintaining the receptor in the resting state (Moore and McCarthy, 1995). Thus, two possible explanations could be given in support of this result: one according to which the binding of α-BTX to the agonist-binding site prevents caffeine binding to similar sites, and the other according to which the presence of α-BTX blocks the necessary allosteric pathway for an AChR conformational change and therefore, although caffeine goes to allosteric sites, no AChR conformational change occurs. Binding experiments with Alexa 488-αBTX and caffeine or galantamine, in both muscle and neuronal α7 AChRs, demonstrated that no competition between this fluorescence toxin and caffeine or galantamine occurred (Fig. 7), thus confirming that both caffeine and galantamine activate the AChR through an allosteric site.

Our single channel recordings verify that caffeine behaves as an agonist for adult muscle ($\alpha 1_2\beta 1\varepsilon\delta$) and neuronal (α7) AChRs (Figs. 5 and 6). We observed that caffeine activates muscle AChR as single and isolated openings without the ability to form clusters, even at high caffeine concentrations. Thus, our results confirm that caffeine behaves as a low efficacy agonist for this receptor.

Single channel recordings show a clear reduction in open time duration and an increase in cluster duration as a function of caffeine concentration, thus suggesting that caffeine also acts as an open channel blocker (Fig. 8A and B). To go deeper into this idea, we performed competitive experiments between caffeine at a high concentration and α-BTX in *T. californica* AChR-rich membranes to evaluate the CrV apparent affinity (Fig. 4). α-BTX alone induced no change in the K_D of CrV for the R state, suggesting that the blockage of both ACh binding sites by this toxin is not detectable by CrV. Using the measurement of the AChR intrinsic fluorescence as a strategy to monitor AChR conformational changes towards its desensitized state, Kawai and Raftery (2010) (Kawai and Raftery, 2010) reported that in the presence of α-BTX alone no significant AChR intrinsic fluorescence changes were observed, in agreement with our results. Furthermore, analyzing the pattern of incorporation of the hydrophobic photolabel 3-(trifluoromethyl)-3-(m-[^{125}I] iodophenyl)diazirine ([^{125}I]TID) into AChR, Moore and McCarthy (1994) (Moore and McCarthy, 1994) concluded that α-BTX increased/stabilized the amount of AChR in the R state. However, in the presence of α-BTX, high concentrations of caffeine induced values of K_D of CrV higher than the ones corresponding to the AChR in a resting state, which is already a low affinity conformation for CrV. This suggests that caffeine, at a high concentration, and without the possibility to act as an AChR activator, diminished further the affinity of CrV for AChR (Fig. 4). Taking into account that CrV binds to luminal non-competitive antagonist sites competing with phencyclidine (Lurtz and Pedersen, 1999), probably this K_D value increment could correspond to a displacement of CrV for its sites. To verify this hypothesis, similar experiments in the presence of QX-314, which is a quaternary ammonium lidocaine derivative that acts as an AChR open channel blocker (Pascual and Karlin, 1998; Yu et al., 2003), were performed. QX-314 alone induced no change in the K_D of CrV for the R state. Previous research reported that most of the drugs classified as open channel blockers bind to the AChR in the resting state with similar affinity (Moore and McCarthy, 1995), thus suggesting that the presence of QX-314 did not perturb the AChR resting conformation. However, in the presence of α-BTX, QX-314 increased K_D values of CrV (Fig. 4), reinforcing the idea that this increment can be related to a displacement of CrV from the ion channel. Considering that in the absence and presence of α-BTX, QX-314 goes to the ion channel and that only in the presence of α-BTX, the K_D of CrV increased significantly, one possible explanation for this could be that α-BTX triggers a conformational change at the channel vestibule which is not detectable by CrV or by the AChR intrinsic fluorescence, thus exposing the CrV molecules which can be easily displaced by QX-314 or caffeine. This possibility also suggests that caffeine probably behaves as an ion channel blocker at high concentrations. The potential dual effect observed with caffeine at low and high concentrations reminds galantamine behavior (a low-efficacy agonist and an open channel blocker for the AChR) (Akk, 2005). Thus, similar competition experiments were performed at high concentrations of galantamine and α-BTX (Fig. 4), confirming the increase of K_D values, and hence the displacement of CrV for the ion channel and the behavior as a channel blocker of this drug at high concentrations. The electrophysiological and fluorescent experiments carried out show that caffeine induces two opposite effects on the AChR: namely as an activator and as an ion channel blocker, both of which probably correspond to caffeine binding at different AChR sites with quite different affinities. These experiments suggest that the inhibition site would probably be the ion channel pore.

Caffeine is the most widely consumed alkaloid in the world. It is contained in many types of beverages like coffee, tea, soda and energy drinks, and in some food and medicines. The high popularity of all these sources has made people from a wide range of ages been daily exposed to caffeine effects. From oral administration, caffeine is quickly absorbed, and peak plasma concentration occurs approximately 15–120 min after consumption. Due to its hydrophobic nature, it can cross the blood-brain barrier, and cerebral spinal concentration equals caffeine plasma concentration (Fredholm et al., 1999; Kolahdouzan and Hamadeh, 2017).

Previous research has reported that caffeine acts mainly upon the central nervous system, increasing arousal and concentration, and decreasing fatigue (Kim et al., 2015). Although its long-term effects have not yet been fully understood, a number of animal studies have suggested that caffeine has neuroprotective effects (Martins et al., 2017; Schimidt et al., 2017; Singh et al., 2011; Xu et al., 2016. Schwarzschild et al., 2006). Further studies have suggested that caffeine intake from coffee or tea is inversely associated with the risk of AD, cognitive impairment, or cognitive decline (Ng et al., 2015; Okello et al., 2012). Biological effects of caffeine are now known to span a wide range of molecular targets, the most important being the following ones: i) adenosine receptors, in which xanthines act as antagonists; ii) phosphodiesterases, in which xanthines act as inhibitors; iii) calcium release ryanodine-sensitive channels in the sarcoplasmic and endoplasmic reticulum, in which xanthines act to sensitize the channels to the activation by calcium, and iv) GABAA receptors, in which xanthines act as antagonists at the benzodiazepine-positive modulatory site. Of all these mechanisms, the only one that is affected by caffeine at concentrations in the range of physiological level is the modulation

of adenosine receptors. Based on results from our work and those from previous studies, AChE is also a molecular target for caffeine at micromolar concentrations (Pohanka and Dobes, 2013). Furthermore, in this work we described for the first time the action of caffeine on AChRs in a micromolar concentration, pointing to this receptor as a new molecular target. This would not be the first time that a novel biological activity is assigned to a well-known drug.

Nowadays it is agreed that caffeine promotes neuronal survival in AD pathogenesis, which may contribute to its beneficial effects. Furthermore, clinical trials demonstrated beneficial effects of caffeine in AD progression and prevention, for example, the amount of caffeine intake during a period of 20 years is inversely associated with AD, and drinking three to five cups of coffee daily at midlife is associated with a 65% lower risk of dementia. What this demonstrates is that caffeine exerts protective effects partly by reducing caspase-3 expression, by decreasing the levels of PS1, B-secretase, and soluble amyloid-β (AB) in the hippocampus, and by reducing AB deposition in the hippocampus and entorhinal cortex. Furthermore, it can stimulate the survival pathway by increasing PKA activity and phospho-CREB levels but suppressing phospho-JNK and phospho-ERK expressions in the striatum of the Swedish mutant APP transgenic mouse model (Ng et al., 2015). Here, we propose that caffeine has a dual potentiation effect on the cholinergic pathway, behaving both as an AChE inhibitor and an AChR activator. In addition to this, caffeine itself is a rather unique compound with very high solubility in both water and nonpolar organic solvents. This chemical property is quite interesting because it facilitates partition across cell membranes, rapidly yielding high levels throughout the body, including the brain (Kolahdouzan and Hamadeh, 2017). Thus, caffeine must be considered for the design of promising multitarget-directed drugs for AD, with some well-defined target molecules and others still unknown. Here we highlight AChE as one target molecule and introduce a new one, the AChR. Further studies will define the exact mechanism by which caffeine exerts a dual effect on the AChR and will give molecular insights into the development of new drugs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.03.041.

References

Albuquerque, E.X., Pereira, E.F., Alkondon, M., Rogers, S.W., 2009. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol. Rev. 89, 73–120. https://doi:10.1152/physrev.00015.2008.

Akk, G., 2005. Galantamine activates muscle-type nicotinic acetylcholine receptors without binding to the acetylcholine-binding site. J. Neurosci. 25, 1992–2001. https://doi.org/10.1523/JNEUROSCI.4985-04.2005.

Andersen, N.D., Nielsen, B.E., Corradi, J., Tolosa, M.F., Feuerbach, D., Arias, H.R., Bouzat, C., 2016. Exploring the positive allosteric modulation of human α7 nicotinic receptors from a single-channel perspective. Neuropharmacology 107, 189–200. https://doi.org/10.1016/j.neuropharm.2016.02.032.

Barrantes, F.J., 1982. Oligomeric forms of the membrane-bound acetylcholine receptor disclosed upon extraction of the m(r) 43,000 nonreceptor peptide. J. Cell Biol. 92, 60–68.

Bouzat, C., Barrantes, F., Sine, S., 2000. Nicotinic receptor fourth transmembrane domain: hydrogen bonding by conserved threonine contributes to channel gating kinetics. J. Gen. Physiol. 115, 663–672. https://doi.org/10.1085/jgp.115.5.663.

Bouzat, C., Bartos, M., Corradi, J., Sine, S.M., 2008. The interface between extracellular and transmembrane domains of homomeric cys-loop receptors governs open-channel lifetime and rate of desensitization. J. Neurosci. 28, 7808–7819. https://doi.org/10.1523/JNEUROSCI.0448-08.2008.

Bouzat, C., Bren, N., Sine, S.M., 1994. Structural basis of the different gating kinetics of fetal and adult acetylcholine receptors. Neuron 13, 1395–1402. https://doi.org/10.1016/0896-6273(94)90424-3.

Chan, E.W., Soh, E.Y., Tie, P.P., Law, Y.P., 2011. Antioxidant and antibacterial properties of green, black, and herbal teas of Camellia sinensis. Pharmacognosy Res. 3, 266–272. https://doi.org/10.4103/0974-8490.89748.

Christopher, G., Sutherland, D., Smith, A., 2005. Effects of caffeine in non-withdrawn volunteers. Hum. Psychopharmacol. 20, 47–53. https://doi.org/10.1002/hup.658.

Corradi, J., Bouzat, C., 2016. Understanding the bases of function and modulation of α7 nicotinic receptors: implications for drug discovery. Mol. Pharmacol. 90, 288–299. https://doi: 10.1124/mol.116.104240.

Corradi, J., Spitzmaul, G., De Rosa, M.J., Costabel, M., 2007. Role of pairwise interactions between M1 and M2 domains of the nicotinic receptor in channel gating. Biophys. J. 92, 76–86. https://doi: 10.1529/biophysj.106.088757.

daCosta, C.J.B., Baenziger, J.E., 2009. A lipid-dependent uncoupled conformation of the acetylcholine receptor. J. Biol. Chem. 284, 17819–17825. https://doi.org/10.1074/jbc.M900030200.

Dajas-Bailador, F.A., Heimala, K., Wonnacott, S., 2003. The allosteric potentiation of nicotinic acetylcholine receptors by galantamine is transduced into cellular responses in neurons: Ca2+ signals and neurotransmitter release. Mol. Pharmacol. 64, 1217–1226. https://doi.org/10.1124/mol.64.5.1217.

Dalai, M.K., Bhadra, S., Chaudhary, S.K., Bandyopadhyay, A., Mukherjee, P.K., 2014. Anti-cholinesterase activity of the standardized extract of Syzygium aromaticum L. Pharmacogn Mag 10, 276–282. https://doi: 10.4103/0973-1296.133275.

Daly, J.W., 2007. Caffeine analogs: biomedical impact. Cell. Mol. Life Sci. 64, 2153–2169. https://doi.org/10.1007/s00018-007-7051-9.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Fredholm, B.B., 1995. [Should coffee be classified as a doping agent? Caffeine is definitely effective when it comes to performance capacity]. Lakart 92, 4079–4080.

Fredholm, B.B., Bättig, K., Holmén, J., Nehlig, A., Zvartau, E.E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51, 83–133.

Grossberg, G.T., 2003. Cholinesterase inhibitors for the treatment of Alzheimer's disease: getting on and staying on. Curr. Ther. Res. Clin. Exp. 64, 216–235. https://doi.org/10.1016/S0011-393X(03)00059-6.

Guerreiro, S., Toulorge, D., Hirsch, E., Marien, M., Sokoloff, P., Michel, P.P., 2008. Paraxanthine, the primary metabolite of caffeine, provides protection against dopaminergic cell death via stimulation of ryanodine receptor channels. Mol. Pharmacol. 74, 980–989. https://doi: 10.1124/mol.108.048207.

Hamilton-Miller, J.M., 1995. Antimicrobial properties of tea (Camellia sinensis L.). Antimicrob. Agents Chemother. 39, 2375–2477.

Hamilton-Miller, J.M., 2001. Anti-cariogenic properties of tea (Camellia sinensis). J. Med. Microbiol. 50, 299–302. https://doi.org/10.1099/0022-1317-50-4-299.

Karadsheh, N., Kussie, P., Linthicum, D.S., 1991. Inhibition of acetylcholinesterase by caffeine, anabasine, methyl pyrrolidine and their derivatives. Toxicol. Lett. 55, 335–342. https://doi.org/10.1016/0378-4274(91)90015-X.

Kawai, H., Raftery, M.A., 2010. Kinetics of agonist-induced intrinsic fluorescence changes in the Torpedo acetylcholine receptor. J. Biochem 147, 743–749. https://doi.org/10.1093/jb/mvq007.

Kim, Y.-S., Kwak, S.M., Myung, S.-K., 2015. Caffeine intake from coffee or tea and cognitive disorders: a meta-analysis of observational studies. Neuroepidemiology 769, 51–63. https://doi.org/10.1159/000371710.

Kolahdouzan, M., Hamadeh, M.J., 2017. The neuroprotective effects of caffeine in neurodegenerative diseases. CNS Neurosci. Ther. 23, 272–290. https://doi.org/10.1111/cns.12684.

Lurtz, M.M., Pedersen, S.E., 1999. Aminotriarylmethane dyes are high-affinity noncompetitive antagonists of the nicotinic acetylcholine receptor. Mol. Pharmacol. 55, 159–167.

Martins, A., Schimidt, H.L., Garcia, A., Colletta Altermann, C.D., Santos, F.W., Carpes, F.P., da Silva, W.C., Mello-Carpes, P.B., 2017. Supplementation with different teas from Camellia sinensis prevents memory deficits and hippocampus oxidative stress in ischemia-reperfusion. Neurochem. Int. 108, 287–295. https://doi.org/10.1016/j.neuint.2017.04.019.

Moore, M.A., McCarthy, M.P., 1995. Snake venom toxins, unlike smaller antagonists, appear to stabilize a resting state conformation of the nicotinic acetylcholine receptor. BBA - Biomembr 1235, 336–342. https://doi.org/10.1016/0005-2736(95)80022-8.

Moore, M.A., McCarthy, M.P., 1994. The effects of drugs on the incorporation of a conformationally-sensitive, hydrophobic probe into the ion channel of the nicotinic acetylcholine receptor. Biochim. Biophys. Acta 1190, 457–464.

Neher, E., Steinbach, J.H., 1978. Local anaesthetics transiently block currents through single acetylcholine receptor channels. J. Physiol 277, 153–176.

Ng, Y.P., Or, T.C.T., Ip, N.Y., 2015. Plant alkaloids as drug leads for Alzheimer's disease. Neurochem. Int. 89, 260–270. https://doi.org/10.1016/j.neuint.2015.07.018.

Nievas, G.A.F., Barrantes, F.J., Antollini, S.S., 2008. Modulation of nicotinic acetylcholine receptor conformational state by free fatty acids and steroids. J. Biol. Chem. 283, 21478–21486. https://doi.org/10.1074/jbc.M800345200.

Nordberg, A., Svensson, A.L., 1998. Cholinesterase inhibitors in the treatment of

Alzheimer's disease: a comparison of tolerability and pharmacology. Drug Saf. 19, 465–480.
Okello, E.J., Leylabi, R., McDougall, G.J., 2012. Inhibition of acetylcholinesterase by green and white tea and their simulated intestinal metabolites. Food Funct 3 (651). https://doi.org/10.1039/c2fo10174b.
Pascual, J.M., Karlin, A., 1998. Delimiting the binding site for quaternary ammonium lidocaine derivatives in the acetylcholine receptor channel. J. Gen. Physiol. 112, 611–621.
Perillo, V.L., Fernández-Nievas, G.A., Vallés, A.S., Barrantes, F.J., Antollini, S.S., 2012. The position of the double bond in monounsaturated free fatty acids is essential for the inhibition of the nicotinic acetylcholine receptor. Biochim. Biophys. Acta - Biomembr 1818, 2511–2520. https://doi.org/10.1016/j.bbamem.2012.06.001.
Pohanka, M., Dobes, P., 2013. Caffeine inhibits acetylcholinesterase, but not butyrylcholinesterase. Int. J. Mol. Sci. 14, 9873–9882. https://doi.org/10.3390/ijms14059873.
Qin, F., Auerbach, A., Sachs, F., 1997. Maximum likelihood estimation of aggregated Markov processes. Proc. Biol. Sci. 264, 375–383. https://doi.org/10.1098/rspb.1997.0054.
Qin, F., Auerbach, A., Sachs, F., 1996. Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. Biophys. J. 70, 264–280. https://doi.org/10.1016/S0006-3495(96)79568-1.
Quik, M., Zhang, D., McGregor, M., Bordia, T., 2015. Alpha7 nicotinic receptors as therapeutic targets for Parkinson's disease. Biochem. Pharmacol. 97, 399–407. https://doi: 10.1016/j.bcp.2015.06.014.
Schimidt, H.L., Garcia, A., Martins, A., Mello-Carpes, P.B., Carpes, F.P., 2017. Green tea supplementation produces better neuroprotective effects than red and black tea in Alzheimer-like rat model. Food Res. Int. 100, 442–448. https://doi.org/10.1016/j.foodres.2017.07.026.
Schwarzschild, M.A., Agnati, L., Fuxe, K., Chen, J.F., Morelli, M., 2006. Targeting adenosine A2A receptors in Parkinson's disease. Trends Neurosci. 29, 647–654. https://doi.org/1016/j.tins.2006.09.004.
Singh, B.N., Shankar, S., Srivastava, R.K., 2011. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem. Pharmacol. 82, 1807–1821. https://doi.org/10.1016/j.bcp.2011.07.093.
Soung, H.S., Wang, M.H., Tseng, H.C., Fang, H.W., Chang, K.C., 2015. (-)Epigallocatechin-3-gallate decreases the stress-induced impairment of learning and memory in rats. Neurosci. Lett. 602, 27–32. https://doi.org/10.1016/j.neulet.2015.06.035.
Tan, L.T.H., Chan, K.G., Pusparajah, P., Lee, W.L., Chuah, L.H., Khan, T.M., Lee, L.H., Goh, B.H., 2017. Targeting membrane lipid a potential cancer cure? Front. Pharmacol. 8, 1–6. https://doi.org/10.3389/fphar.2017.00012.
Tel, G., Ozturk, M., Duru, M.E., Turkoglu, A., 2015. Antioxidant and anticholinesterase activities of five wild mushroom species with total bioactive contents. Pharm. Biol. 53, 824–830. https://doi.org/10.3109/13880209.2014.943245.
Uteshev, V.V., 2014. The therapeutic promise of positive allosteric modulation of nicotinic receptors. Eur. J. Pharmacol. 727, 181–185. https://doi: 10.1016/j.ejphar.2014.01.072.
Xu, K., Di Luca, D.G., Orrú, M., Xu, Y., Chen, J.F., Schwarzschild, M.A., 2016. Neuroprotection by caffeine in the MPTP model of Parkinson's disease and its dependence on adenosine A2A receptors. Neurosci 322, 129–137. https://doi:10.1016/j.neuroscience.2016.02.035.
Xu, K., Xu, Y.H., Chen, J.F., Schwarzschild, M.A., 2010. Neuroprotection by caffeine: time course and role of its metabolites in the MPTP model of Parkinson Disease. Neurosci 167, 475–481. https://doi:10.1016/j.neuroscience.2010.02.020.
Xu, W., Kim, T.H., Zhai, D., Er, J.C., Zhang, L., Kale, A.A., Agrawalla, B.K., Cho, Y.K., Chang, Y.T., 2013. Make caffeine visible: a fluorescent caffeine “traffic light” detector. Sci. Rep. 3, 1–7. https://doi.org/10.1038/srep02255.
Yanovsky, I., Finkin-Groner, E., Zaikin, A., Lerman, L., Shalom, H., Zeeli, S., Weill, T., Ginsburg, I., Nudelman, A., Weinstock, M., 2012. Carbamate derivatives of indolines as cholinesterase inhibitors and antioxidants for the treatment of Alzheimer's disease. J. Med. Chem. 55, 10700–10715. https://doi.org/10.1021/jm301411g.
Yu, Y., Shi, L., Karlin, A., 2003. Structural effects of quinacrine binding in the open channel of the acetylcholine receptor. Proc. Natl. Acad. Sci. U. S. A 100, 3907–3912. https://doi.org/10.1073/pnas.0730718100.