A Functional Interaction Between the SARS-CoV-2 Spike Protein and the Human α7 Nicotinic Receptor

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Research Article

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Abstract

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Infection relies on the binding of the viral spike protein (S) to angiotensin-converting enzyme 2 in host cells. The S protein has been suggested to interact with nicotinic acetylcholine receptors (nAChRs), and a potential contribution of nAChRs to COVID-19 pathophysiology has been proposed. α7 nAChR is an interesting candidate target since it is present in neuronal and non-neuronal cells, including immune cells, and has anti-inflammatory actions. We here identified a novel direct functional interaction between the α7 nAChR and the Y674-R685 S region. The S fragment exerts a dual effect, acting as a low-efficacy agonist and a non-competitive inhibitor. It activates the α7 nAChR, in line with our previous molecular dynamics simulations showing favorable binding of this accessible region of the S protein to the nAChR agonist binding pocket. However, activation requires the presence of positive allosteric modulators that enhance channel opening probability, indicating very low activation efficacy. The S fragment also induces an overlapped non-competitive inhibition, which may be the predominant effect on α7 responses. This study provides unequivocal evidence supporting a functional α7-S protein interaction, which opens doors for exploring the involvement of nAChRs in COVID-19 pathophysiology.

Introduction

The emergence of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in late 2019 changed and reshaped the world in lasting ways. Even though several COVID-19 vaccines are now available, the ongoing pandemic caused by this virus remains a global crisis with extensive negative economic, social, and health impacts. Important efforts have been made to understand the nature of this virus, its mutations, host interactions and transmission routes in order to drive vaccine and therapeutic discovery and develop effective strategies to stop the current (and future) outbreaks.

The mechanism of SARS-CoV-2 infection starts with the virus-host cell recognition, which is mediated by the viral spike (S) protein (Fig. 1A). The S protein is a homotrimeric type I fusion glycoprotein found on the surface of the virion and is composed of two subunits, S1 and S2 (Supplementary Fig. 1) [1]. S1 binds with high affinity to the human angiotensin-converting enzyme 2 (ACE2) and induces membrane fusion allowing for the delivery of the viral RNA into the host cell [2, 3]. Infectivity is enhanced by additional interactions between S1 and the neuropilin 1 receptor [4, 5]. The S protein has been suggested to contribute to COVID-19 pathophysiology also through direct or indirect interactions with other proteins, such as nicotinic acetylcholine receptors (nAChRs) [6-8], and epithelial sodium channels [9].

The potential interaction of SARS-CoV-2 with nAChRs, first postulated by Changeux and colleagues [6], was based on the fact that the sequence of the C-terminus region of the S1 subunit (region Y674-R685) has high sequence similarity with neurotoxins known to act as nAChR antagonists such as α-bungarotoxin from Bungarus multicinctus [6]. We recently examined the possible binding of the Y674-R685 region of the S protein to several nAChRs using molecular dynamics (MD) simulations [8]. These simulations predicted favourable binding of the Y674-R685 region to the agonist binding site of the
human α4β2 and α7 (Fig. 1B and C) nAChRs and the muscle-like αβγδ receptor from *Tetronarce californica* [8]. Moreover, analyses of the MD simulations of the complete and fully glycosylated S protein showed that the Y674-R685 region is accessible for binding [8]. Among nAChRs, binding of Y674-R685 to the α7 subtype is highly relevant to COVID-19 as nicotine, acting through this receptor, may regulate the expression of ACE2 [10]. Also, activation of α7 nAChR reduces inflammation and tissue damage by downregulating pro-inflammatory cytokines [11–13]. Thus, potentiation of α7 has emerged as an important strategy for modulating inflammation in different pathological contexts, including acute respiratory distress syndrome [13, 14]. Hence, ligands that bind α7 nAChR may affect the SARS-CoV-2 infectivity and the progression of COVID-19. Indeed, recently it has been shown that varenicline, a full agonist at α7 nAChR [15], reduces infectivity and disease progression in a rhesus macaque model [16].

Here, we use whole-cell and single-channel patch clamp recordings to determine whether the Y674-R685 region of the SARS-CoV-2 S protein can directly affect the human α7 nAChR. Our results reveal that this region acts as a very low-efficacy agonist of α7 since it activates the receptor but only in the presence of a potentiator. Additionally, the Y674-R685 fragment allosterically inhibits α7 responses at a wide concentration range. This functional interaction may play a role in infectivity and/or disease progression and its identification provides the foundations for new therapeutic opportunities.

**Materials And Methods**

**Chemicals.**

Acetylcholine (ACh) and 5-Hydroxyindole (5-HI) were purchased from Merck (USA). PNU-120596 (N-(5-Chloro-2,4-dimethoxyphenyl)-N'-(5-methyl-3-isoxazolyl)-urea) was obtained from Tocris Biosciences (Bristol, UK). Stock solutions were prepared in water (ACh, 5-HI) or DMSO (PNU-120596). The fragment of the S protein (S) of SARS-COV-2, called Y674-R685, contains 12 amino acids with the sequence YQTQTNSPRRAR (MW 1477.60). The peptide was synthesized (90% purity) by Designer Bioscience Ltd. (Cambridge UK) and stored as 1 mM stock solutions (in water) at -20°C.

**Expression of human α7 in mammalian cells:**

BOSC-23 cells, derived from HEK-293 cells (kindly provided by Dr. Sine, Mayo Clinic, USA), were cultured with Dulbecco’s Modified Eagle Medium (DMEM) culture medium (GIBCO, USA) supplemented with 100 µg/mL streptomycin ~ 100 IU/mL penicillin (Invitrogen, USA), 10 % Fetal Bovine Serum (FBS, Internegocios, Argentina). Human α7 cDNA subunit (GenBank accession no X70297.1) was subcloned into the pRBG4 expression vector [17, 18]. BOSC-23 cells were transfected by the calcium phosphate procedure with α7 subunit cDNA together with the α7 chaperones Ric-3 and NACHO cDNAs [19, 20]. The cDNA ratio was 1:2:1 for α7:Ric-3:NACHO, and the total amount was 4.2 µg/ 35 mm dish. Also, green fluorescence protein (GFP) cDNA plasmid was included to allow the identification of transfected cells [21, 22].
All transfections were carried out for about 8–12 h in DMEM with 10 % FBS and terminated by exchanging the medium. Cells were used for experiments two to three days after transfection, at which time maximum functional expression levels were achieved [17, 23, 24].

**Single-channel recordings in BOSC-23 cells.**

Single channels were recorded in the cell-attached patch configuration at -70 mV [24]. Each patch corresponds to a different cell (n indicates the number of independent experiments). For each condition, at least three different cell transfections from distinct days were performed (N indicates the number of cell transfections).

The bath and pipette 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). The peptide and 5-HI dissolved in water were added directly to the pipette solution. PNU-120596 in DMSO was added either to the pipette solution or to the dish. The final concentration of DMSO was lower than 0.1 % (v/v), which does not affect $\alpha_7$ activation properties [18].

Single-channel currents were digitized at 5–10 µs intervals and low-pass filtered at a cut-off frequency of 10 kHz using an Axopatch 200B patch-clamp amplifier (Molecular Devices, CA, USA). Analysis was performed with the program TAC (Bruxton Corporation, Seattle, WA, USA) with the Gaussian digital filter at 9 kHz (Final cut-off frequency 6.7 kHz) or at 3 kHz for recordings in the presence of PNU-120596. Events were detected by the half-amplitude threshold criterion [24].

Open-time histograms were fitted by the sum of exponential functions by maximum likelihood using the program TACFit (Bruxton Corporation, Seattle, WA, USA). The duration of the slowest open component was used for comparisons. Bursts of channel openings were identified as a series of closely separated openings preceded and followed by closings longer than a critical duration, which was taken as the point of intersection between closed components as previously described [23–25]. For $\alpha_7$ activated by ACh, the critical duration for defining a burst was defined by the intersection between the first and second briefest components in the closed-time histogram (≈200–400 µs). For defining bursts in the presence of 5-HI, critical times were selected between the second and third closed components (≈1–3 ms) [23, 25]. In the presence of PNU-120596 and ACh, $\alpha_7$ openings are grouped in bursts, and several bursts form long clusters. Each cluster corresponds to the activation episode of the same receptor molecule. For bursts, the critical time was set at 200–400 µs, and for clusters, the critical time was determined by the point of intersection between the third and fourth closed components (≈30–60 ms) [23]. The burst and cluster durations were taken from the slowest components of the corresponding histograms [23, 24].

**Expression of $\alpha_7$ nAChR in Xenopus laevis oocytes.**

Adult female *Xenopus laevis* were purchased from *Xenopus* One, MI, USA. *Xenopus* care and housing and care followed the UK Home Office code of practice guidelines for the species. Stage V and VI *Xenopus* oocytes were prepared as previously described [26], and then injected with 2–6 ng of human $\alpha_7$ subunit cDNA into the nucleus in a volume of 23.0 nL, using a Nanoject Automatic Oocyte Injector (Drummond,
Broomall, USA). To favour the expression of α7, its cDNA was co-injected with chaperone NACHO cDNA (GenBank accession no BC050273.1) [20, 27] at a ratio of 1 α7: 0.01 NACHO. Injected oocytes were incubated until use at 18°C in a solution (OR2) containing 82 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 5 mM HEPES; pH 7.5, supplemented with 0.1 mg/mL streptomycin, 1000 U/mL Penicillin and 100 µg/mL amikacin. Oocytes were used for electrophysiological recordings one to two days after injection [26, 27].

**Electrophysiological recordings in *Xenopus laevis* oocytes.**

Oocytes were impaled with two electrodes filled with 3 M KCl, and the voltage-clamp was maintained at -60 mV throughout the experiment. All recordings were performed at 18°C, and cells were perfused continuously with OR2 solution at pH 7.4. Currents were recorded using an automated platform equipped with standard two electrode voltage-clamp configuration (HiClamp; Multi Channel Systems, Reutlingen, Germany). This system differs from standard electrophysiology and other automated platforms because, instead of applying the compound in the perfusion, the oocyte is moved into a well from a 96-well microtiter plate containing 230 µl of the desired solution, as previously described [26]. Our limit of detection for receptor-mediated activity is 0.05–0.08% of the maximal responses elicited by 1 mM ACh, since the mechanical transfer of the oocyte to the compound-application wells can cause current artefacts. Experiments were carried out only if the resting potential of the impaled oocytes was greater than ~10 mV and the total holding current less than 0.2 µA. To assess the effects of Y674-R685 fragment compared to ACh-evoked responses of human α7 nAChR, baseline conditions were defined by applications of 100 µM ACh (approx. EC₅₀ concentration) made before and after co-applications of 1 µM Y674-R685 and 100 µM ACh. Compounds were applied for 20 s and the washout period was 5 min.

Data were filtered at 10 Hz, captured at 100 Hz using proprietary data acquisition software running under Matlab (Mathworks Inc., Natick, MA). Peak current responses were normalized to the peak ACh control responses and the data were expressed as means ± SEM from 5–6 oocytes obtained from at least three different batches of oocytes. To estimate EC₅₀ or IC₅₀ and Iₘₐₓ/Iₐₐₜₐₜ₇ control values, concentration-response data were fitted with the Hill equation using GraphPad software version 5, as previously described [26].

**Statistical analysis.**

Data are presented as mean ± SEM or mean ± SD as appropriate. Data sets that passed the Shapiro-Wilk test for normality and the Levene Median test for equal variance were analyzed using two-tailed Student’s t-test for pairwise comparisons or Mann-Whitney rank sum test with SigmaPlot 12.0 (Sysat Software, Inc.). Statistically significant differences between two groups of data were established at p values < 0.05. For each condition, n indicates the number of independent experiments, each from different cell patches, and N, the number of cell transfections, each from different days and cell batches.

**Results**

α7 nAChR activation by the Y674-R685 fragment from the SARS-CoV-2 S protein in the presence of potentiators.
Our previous MD simulations of the complex formed between the α7 nAChR and the Y674-R685 fragment from the SARS-CoV-2 S protein suggested the potential of the Y674-R685 region to interact with conserved aromatic residues within the binding pocket of the receptor (Fig. 1C) [8]. To establish unequivocally the existence of molecular functional interactions between this region of SARS-CoV-2 S protein and the human α7 nAChR, we evaluated whether the synthetic fragment could elicit macroscopic and high-resolution single-channel currents.

The macroscopic responses of the human α7 nAChR expressed in *Xenopus* oocytes to the applications of the Y674-R685 fragment at a range of concentrations (1 pM-10 mM) were examined along with control ACh-evoked responses from the same cells (Fig 2A). As shown in the figure, Y674-R685 did not elicit detectable currents in contrast to the robust responses elicited by 100 µM ACh. After 5 min wash, receptors remained responsive to subsequent control applications of ACh (Fig. 2A).

Single-channel currents from cell-attached patches from BOSC-23 cells expressing human α7 nAChR were also recorded, thus allowing for more detailed mechanistic information. Recordings were carried out in parallel with control experiments with ACh as the agonist to confirm the presence of functional α7 nAChRs in the same batch of cells. ACh (10-100 µM) evoked isolated brief openings or less often short bursts composed of a few openings in quick succession, which correspond to activation of the same receptor molecule [18,21,22,24,25] (Fig. 2B). In contrast, channel activity was not detected at a range of Y674-R685 concentrations in a total of 21 patches from different cell transfections (1 pM, n=3; 1 nM, n=3; 1 µM, n=8; 10 µM, n=3; 100 µM, n=4) (Fig. 2B).

Given that α7 nAChR activation in the presence of ACh occurs with low open probability as very brief opening events (Fig. 2B), we sought to amplify this effect using a potentiator. PNU-120596, a type II positive allosteric modulator (PAM), has been extensively used as a tool in α7 functional studies due to its ability to increase the probability of agonist-elicited channel opening and open-channel durations and to reduce desensitization [28]. We, therefore, examined whether Y674-R685 elicits α7 channel activity in the presence of PNU-120596. Note that by itself, PNU-120596 cannot induce channel activation [29].

The macroscopic currents elicited by 1 µM Y674-R685 in the presence of 10 µM PNU-120596 were recorded. Under these conditions, small currents in the low nA order were detected in 20% of the oocytes tested (n=25) whereas neither Y674-R685 nor PNU-120596 on their own elicited currents (Fig. 3A).

To gain more insights into how Y674-R685 activates the α7 nAChR in the presence of PNU-120596, we explored its effects at the single-channel level. ACh-elicited activity in the presence of 1 µM PNU-120596 is profoundly different to that in its absence (Fig. 3B). Instead of the brief isolated openings, channel activity shows long periods of high-frequency openings, named clusters, with a mean duration of about 1-3 s and an amplitude of 10 pA (-70 mV). A cluster corresponds to the activation episode of the same receptor that recovers from desensitization and oscillates between open and closed states before reaching again the more stable non-conducting desensitized state [28]. Clusters are composed of bursts with mean durations of ~300-500 ms, which comprise successive openings separated by very brief closings (Figs. 3B and 4) [23,28].
In the presence of 1 mM PNU-120596, Y674-R685 was capable of eliciting channel activity at a wide range of concentrations (1 pM to 10 mM), indicating that this region of the S protein can activate α7 nAChRs (Fig. 3B). Since the frequency of channels is variable among patches due to variations in receptor expression levels, parallel control recordings in the presence of ACh were made. When ACh and PNU-120596 were co-applied, >98% of patches showed channel activity (active patches), and the long-duration clusters described above appeared at high frequency as reported before [22] (Fig. 3B). In the presence of PNU-120596 and Y674-R685, the percentage of active patches was lower than in the presence of ACh: 65% (n=23, N=4; 1 pM Y674-R685), 40% (n=15, N=4; 1 nM Y674-R685), 67% (n=15, N=4; 1 mM Y674-R685), and 62% (n=13, N=3; 10 mM Y674-R685). Also, channel activity evoked by Y674-R685 was much more infrequent and interspaced by long silent periods when compared to that evoked by ACh (Fig. 3B). It is important to note that this type of experiments does not allow for a precise comparison of channel frequency since this parameter may be affected by the variability in the number of receptors in each patch. Nevertheless, at 1 pM Y674-R685, the frequency of channel activation episodes was very low; albeit the active patches showed long clusters resembling those elicited by ACh and PNU-120596 (Figs. 3B and 4).

The increase in Y674-R685 concentration resulted in profound changes in the channel activity pattern, as clearly illustrated in the recordings shown in Fig. 3B. The frequency of opening events appeared to increase, but the duration of the openings and the activation episodes were reduced with increasing concentrations. The typical long-duration clusters were completely absent at Y674-R685 concentrations higher than 1 µM, at which activation occurred mainly as isolated openings or in short bursts (Fig. 3B and 4).

To define the properties of the activation episodes elicited by the Y674-R685 fragment at different concentrations, the mean durations of openings, bursts, and clusters in the presence of PNU-120596 were determined (Fig. 4). At 1 pM Y674-R685, the mean durations of the longest openings, bursts and clusters were 141 ± 59 ms, 417 ± 113 ms, and 2330 ± 673 ms, respectively (n=3). These values were similar to those determined in the presence of 10 µM ACh: 148 ± 12 ms for the slowest open component (p=0.859, n=3), 550 ± 38 ms for bursts (p=0.125, n=3), and 3048 ± 516 ms for clusters (p=0.217, n=3), and also comparable to those reported before for 100 µM ACh and 1 µM PNU-120596 [23,28]. Although the mean duration of clusters between 10 µM ACh and 1 pM Y674-R685 was similar, the relative area of the component corresponding to clusters in the histogram was smaller when Y674-R685 was the agonist (relative areas were for ACh= 0.44 ± 0.09 and for Y674-R685= 0.21 ± 0.08; p=0.0264) (Fig. 4). With the increase of Y674-R685 concentration, dwell time distributions for open, bursts and clusters were shifted to briefer durations. The slowest component of each histogram became progressively briefer with increasing Y674-R685 concentrations (Fig. 4). The mean durations were 48.2 ± 13.6 ms (1 µM, n=3) and 14.5 ± 4.3 ms (10 µM, n=4) for openings; 70.2 ± 19.7 ms (1 µM, n=3) and 20.7 ± 8.0 ms (10 µM, n=4) for bursts; and 104.2 ± 41.6 ms (1 µM, n=3) and 29.9 ± 14.8 ms (10 µM, n=4) for clusters. Mean values were statistically significantly different to those determined in the presence of 10 µM ACh and 1 µM PNU-120596 (p=0.000665 and p=0.0000431 for open; p=0.0000403 and p= 0.0000106 for burst; p=0.000597 and p=0.0000681 for cluster, for 1 and 10 µM Y674-R685, respectively). At high Y674-R685
concentrations (e.g., 10 µM), the mean open duration was similar to the mean burst and cluster durations (Fig. 4), indicating that openings occurred mostly in isolation and confirming the lack of the typical long-duration clusters corresponding to potentiated α7 responses.

To further confirm that channel activation can be elicited by Y674-R685 in the presence of PNU-120596 but not in its absence, single-channel recordings in the presence of the Y674-R685 fragment alone in the pipette solution (1-10 µM) were made. Again, no channel activity was detected in all patches. However, the addition of 1 µM PNU-120596 to the dish during the course of the recording resulted in the appearance of single-channel activity in most of the silent patches (Fig. 5). Again, the frequency of opening events was systematically markedly lower, and the durations were briefer than in the conditions in which ACh was the agonist (Fig. 5).

Together, these results confirm that Y674-R685 functionally interacts with the α7 nAChR. They also show that Y674-R685 acts as a very low-efficacy agonist since channel opening requires the presence of PNU-120596 and occurs with low probability, and that the increase in concentration is accompanied by a decrease in the duration of open channel lifetime and of the activation episodes.

Because PNU-120596 is a highly efficacious type II PAM with the capability of recovering receptors from desensitization, we also tested if Y674-R685 elicited channel activity in the presence of 5-hydroxyindole (5-HI), a type I PAM which induces clear potentiation but does not recover receptors from desensitization [21,23,30]. In the presence of 2 mM 5-HI, 100 µM ACh induced prolonged openings and bursts composed of successive openings which lasted about 4 ms (Fig. 6). The histograms showed that the duration of the slowest open component was ~4-fold longer (1.28 ± 0.35 ms, n=37, versus 0.30 ± 0.06 ms, n=38) and the mean burst duration was 8-fold longer (3.60 ± 1.29 ms, n=37, versus 0.46 ± 0.12 ms, n=38) than in the absence of the PAM. Replacing ACh by Y674-R685 (1 pM-10 µM) revealed α7 channel activity. However, the frequency of opening events was lower when compared to that elicited by ACh; only few events were detected during a 15-min recording period (Fig. 6). At 10 µM Y674-R685, the mean open duration was 0.73 ± 0.07 ms (n=3) and the mean burst duration was 0.85 ± 0.13 ms (n=3); both parameters were statistically significantly briefer than the corresponding mean durations in the presence of ACh and 2 mM 5-HI (p=0.0114 and 0.005 for mean open and mean burst durations, respectively). Also, the observation that in the presence of 10 µM Y674-R685 and 2 mM 5-HI the mean duration of openings was similar to that of bursts indicates that channel opening occurred mainly as isolated events instead of in quick succession forming activation episodes, as described before for recordings with the type II PAM PNU-120596.

**Inhibition of α7 activity by Y674-R685 from S protein**

To understand the molecular mechanisms driving the inhibitory effects of Y674-R685 evidenced by the dramatic decrease in open durations, we evaluated Y674-R685 effects on α7 activated by 10 µM ACh (Fig. 7A). Given the very brief duration of α7 channels, which is close to the time resolution of our system, we included PNU-120596 to better quantify a decrease in open durations. Whereas in the presence of 1 µM PNU-120596, 10 µM ACh led to an activation pattern composed of long clusters as described above,
the inclusion of 10 µM Y674-R685 produced marked changes in this pattern: the long clusters were absent, and only markedly briefer bursts were detected (Fig. 7A). The mean duration of the slowest open component was 9.9 ± 2.6 ms (n=4), indicating a 15-fold decreased duration than in the absence of Y674-R685 (p=0.00000277). Also, the open duration was not different from the mean burst duration (11.2 ± 3.0 ms, n=4), indicating that most long openings occurred in isolation. Although clusters were not visually detected, we constructed cluster duration histograms using a critical time for cluster resolution between 10 and 20 ms, which is about 20 to 40-fold times longer than that used for burst-duration histograms. The mean duration of the slowest component of the cluster histogram was 14.1 ± 4.4 ms (n=4, p=0.0000663 with respect to clusters of 10 µM ACh and 1 µM PNU-120596), which is not different to that of bursts, thus confirming the lack of the long-duration activation episodes occurring in potentiated α7 channels.

The inhibitory effect was dependent on concentration since at 1 µM Y674-R685 the decrease in all parameters was smaller than at 10 µM, but still statistically significantly different from the control. At 1 µM Y674-R685, the mean durations were 45.7 ± 11.2 ms for open, 62.9 ±13.2 ms for bursts, and 90.5 ± 23.5 ms for clusters (n=3, p=0.000415, 0.0000296 and 0.000581 for open, bursts and clusters, respectively, with respect to ACh). The mean durations at 1 µM were also statistically significantly different to those at 10 µM Y674-R685 (p=0.00143 for mean open time, p=0.00056 for mean burst duration, and p=0.00122 for mean cluster duration), thus confirming the concentration-dependent inhibitory effect of Y674-R685.

To gain further insight into the mechanism driving the inhibitory effects of Y674-R685, we explored if channel activity elicited by ACh and PNU-120596 was affected by Y674-R685 as by methyllycaconitine (MLA), a reversible competitive α7 antagonist [15]. To better assess the impact of MLA, we followed the strategy of filling the tip of the pipette with the buffer solution containing 10 µM ACh and 1 µM PNU-120596 and the tap with the same solution but including MLA (100 nM). This strategy allowed following in real-time the effects of the antagonist during the recording of ACh-activated channels. While at the beginning of the recording, the typical pattern of high channel activity and long clusters was observed, channel activity decreased over time, ultimately disappearing after about 10-15 min in the presence of MLA (Supplementary Fig. 2, n=3). Although the frequency of channel activity was markedly reduced, the kinetic properties of the remaining activation episodes were identical to those of the control. No reduction in the duration of clusters or bursts, as described in the presence of Y674-R685, was detected in the presence of MLA (Supplementary Fig. 2).

The results together suggest that the Y674-R685 region of the S protein acts as a non-competitive antagonist of α7. To further confirm this result, we determined the effects of Y674-R685 on the peak current responses evoked by an approximate EC50 concentration of ACh (100 mM). Application of Y674-R685 with ACh inhibited peak currents, with an IC50 of 1.8 ± 0.8 mM (n = 10), but inhibition was not complete (Fig. 7B). We then investigated whether the observed antagonism was competitive or not. For these studies, 1 µM Y674-R685 was co-applied with increasing concentrations of ACh (0.1- 2000 µM) (Fig. 7B). Compared to ACh alone, Y674-R685 co-application affected ACh efficacy, reducing the maximal
currents elicited by ACh (Imax) by 30 ± 4% (n = 6) and slightly affecting its potency (EC\textsubscript{50} = 80 ± 6 μM and 131 ± 92 μM) (n = 6). These results confirm the non-competitive antagonism of ACh responses by Y674-R685.

**Discussion**

The sequence homology between the S protein of SARS-CoV-2 and snake toxins that bind to nAChRs suggested that these receptors may play a role in the pathophysiology of COVID-19 [6, 7]. Subsequent MD simulations support a possible interaction between the Y674-R685 region of the SARS-CoV-2 S protein and nAChRs [8].

Here, we provide the first molecular evidence of a direct functional interaction between human α\textsubscript{7} nAChR and the Y674-R685 region of the SARS-CoV-2 S protein. This interaction, which takes place within the picomolar to micromolar concentration range, results in a dual effect involving low efficacious agonism and non-competitive antagonism.

α\textsubscript{7} is a homeric nAChR highly expressed in both neuronal and non-neuronal cells, which is emerging as a potential drug target for neurological, neurodegenerative and inflammatory disorders [31–33]. It responds to ACh by opening an intrinsic ion channel permeable to cations, triggering rapid membrane depolarization and calcium influx [34]. α\textsubscript{7} activation is unique as it shows the most rapid desensitization and the highest calcium permeability of the family [35]. Although indirect crosstalk may occur [10], determining a direct functional interaction between SARS-CoV-2 S protein and the α\textsubscript{7} nAChR is the cornerstone for supporting the hypothesis related to the involvement of this receptor in the COVID-19 inflammatory disease. Here, by taking advantage of the power of single-channel recordings, we provide novel information regarding the molecular functional effects and the underlying mechanisms of the interaction between the α\textsubscript{7} nAChR and the Y674-R685 region of the SARS-CoV-2 S protein.

We found that Y674-R685 activates the α\textsubscript{7} receptor at picomolar concentrations but with exceptionally low efficacy. The efficacy for activation is so low that only in the presence of PAMs channel activity can be detected, and, even in their presence, the frequency of channel activity induced by the fragment is markedly lower than the one elicited by ACh. In addition, ACh-elicited responses are reduced by the S protein fragment, indicating an overlapped function as agonist and inhibitor. Given that endogenous α\textsubscript{7} PAMs, with the exception of calcium, have not been reported so far, the S protein may physiologically play an inhibitory role at α\textsubscript{7}. α\textsubscript{7} nAChR also mediates metabotropic responses, which include release of calcium from intracellular stores induced by the calcium influx and the triggering of signal transduction pathways [13, 34, 36, 37]. This raises important questions regarding how these metabotropic responses are modified by the S protein and their impact on COVID-19 pathophysiology.

PAMs, which reduce the energy barrier for activation, are emerging as novel therapeutic tools for neurological, neurodegenerative, and inflammatory disorders as they potentiate α\textsubscript{7} responses in the presence of an agonist [31, 32, 38, 39]. They have been classified as type I PAMs, which enhance agonist-
induced macroscopic currents, and type II PAMs, that also delay desensitization and recover receptors from desensitized states [23, 40, 41]. At the single channel level, PAMs enhance open channel durations and induce activation in episodes in which the channel oscillates between open and closed conformations, thus generating an activity pattern markedly different from the isolated, infrequent submillisecond-openings in the absence of PAMs [23]. Depending on the type and efficacy of the PAM, the potentiated activation episodes can range from milliseconds (as shown here for 5-HI) to seconds (as demonstrated here for PNU-120596). Despite the marked differences between 5-HI and PNU-120596 regarding their efficacy for potentiation and influence on receptor desensitization, Y674-R685 activates the α7 nAChR in the presence of both PAMs but not in their absence, indicating its action as a low-efficacy agonist. Also, in the presence of both PAMs, reduction in the duration of the opening events with increasing Y674-R685 concentration is observed, thus supporting the additional role of the fragment as an α7 inhibitor.

At 1 pM Y674-R685, channel activity in the presence of PNU-120596 is markedly infrequent but kinetically indistinguishable from the ACh-elicited one. The open channel lifetime, and architecture and duration of the activation episodes (clusters) are similar in the presence of ACh or 1 pM Y674-R685. As the Y674-R685 concentration increases, the frequency of openings increases, but their durations become progressively briefer, with long-duration clusters no longer present. In ACh-elicited channels, the Y674-R685 fragment also produces a concentration-dependent reduction of open and cluster durations, an effect different to that produced by the competitive antagonist MLA. Thus, the type of single-channel inhibition together with the results from macroscopic current recordings support a mechanism of non-competitive inhibition.

The ability to act as a low-efficacy agonist is consistent with data from our MD simulations predicting that Y674-R685 can bind directly to the agonist binding site located in the extracellular domain of the α7 nAChR, leading in some conformations to a semi-capped loop C [8]. Loop C is one of the three loops that form the principal face of the orthosteric binding site [42]. Upon agonist binding, loop C closes to cap the agonist, an event associated with priming and channel opening [43]. Loop C capping aids the anchoring of the bound agonist to the orthosteric binding site. It has also been suggested that agonists induce more compact loop conformations, while partial agonists and antagonists produce incomplete closure or prevent loop C capping [44, 46].

Our previous MD simulations of Y674-R685 bound to the α7 nAChR identified the guanidinium group of R682 in the SARS-COV-2 S protein as the key anchoring point to the α7 nAChR (Fig. 1C), forming strong interactions with several residues lining the receptor's ligand binding pocket [8]. These simulations also show that, when bound to α7, the Y674-R685 region of the SARS-CoV-2 S protein adopts many different conformations within the binding pocket, ranging from highly compact to fully extended configurations (Supplementary Fig. 3). Some of these binding modes allow for the formation of the key interactions necessary to activate the receptor (Fig. 1C), while others do not (Supplementary Fig. 4) [8]. Although the α7 nAChR has five identical orthosteric binding sites, agonist occupancy of only one is required to elicit channel activation [25]. Thus, it could be possible that the Y674-R685 fragment occupancy of the binding
sites in multiple orientations, some of which cannot trigger activation, may contribute to its low efficacy, as described before for partial agonists [45]. Additionally, the partial closure of loop C in the presence of the Y674-R685 [8] observed in some of our previous simulations may also reduce the efficacy for channel opening (Supplementary Fig. 5).

As shown here for Y674-R685, several compounds of different structures have dual opposite effects on nAChRs, including α7, acting as agonists and non-competitive antagonists (also called negative allosteric modulators, NAMs) [22, 47, 48]. NAMs comprise a wide range of structurally different compounds that inhibit receptor function by binding to sites distinct to the orthosteric sites (allosteric) [39]. These allosteric sites can be located in different receptor domains, including the extracellular [49] and transmembrane domains [28, 50]. The site for the allosteric inhibition of Y674-R685 remains to be determined. The negative modulation may occur by different molecular mechanisms, comprising channel block, enhanced desensitization, or decrease probability of opening. Given the complex kinetics of the α7 receptor, distinguishing how the fragment exerts allosteric inhibition is not possible from our experiments. However, the type of concentration-dependent reduction of bursts and clusters suggests slow channel blockade since fast flickering, typical of fast open-channel blockers, was not observed [51]. Due to the presence of three arginine residues, the Y674-R685 fragment (sequence YQTQTNSPRRAR) is highly charged, with an overall charge of +3, which may be compatible with channel block.

Overall, our results reveal a functional interaction between the Y674-R685 region of the SARS-CoV-2 S protein and the α7 nAChR, with the predominant effect probably being inhibitory since activation cannot occur in the absence of PAMs. Although too speculative at this stage, this finding raises the possibility that the hyper-inflammatory response observed in some COVID-19 patients may be partly due to inhibition of α7 nAChRs. It has been shown that activation of α7 nAChR in the immune system protects from excessive production of pro-inflammatory cytokines (e.g., TNF-α) [12]. Impairment of this receptor subtype results in an overproduction of cytokines and enhanced tissue damage [52]. In this context, therapeutic interventions focused on α7 nAChR may be valuable to be explored.

**Abbreviations**

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

nAChR: Nicotinic Acetylcholine Receptor.

ACh: Acetylcholine.

MLA: methyllycaconitine

PAM: Positive Allosteric Modulator.

5-HI: 5-Hydroxyindole.

PNU-120596: (N-(5-Chloro-2,4-dimethoxyphenyl)- N’-(5-methyl-3-isoxazolyl)-urea)
S protein: SARS-CoV-2 Spike protein

MD: Molecular dynamics

Declarations

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Figures
Figure 1

Three-dimensional structures of the SARS-CoV-2 S protein and the human α7 nAChR. A. The model represents the complete, fully glycosylated S protein in the closed state after furin cleavage [1]. The protein is rendered as a blue cartoon with the glycans depicted in green. The receptor binding motifs (residues S438-Q506) and the Y674-R685 region are highlighted in yellow and red, respectively. The Y674-R685 region was shown to be accessible for binding in previous MD simulations of the fully glycosylated S protein [8]. B. Cryo-EM structure of the human α7 nAChR (PDB code: 7K0X) [42]. This receptor is a
homopentamer formed of five α7 subunits. Each subunit is composed of an extracellular (ECD), transmembrane (TMD) and intracellular (ICD) domain. The agonist binding site is located in the ECD at the interface between two neighbouring subunits. In this structure, epibatidine (red spheres) is bound to the agonist binding site. The green spheres represent the calcium ions. C. MD simulations of Y674-R685 bound to the human α7 nAChR show favourable binding to the binding pocket [8]. Example of conformations from simulations in which the most important interactions with conserved key aromatic residues are present. Left: Overall view of the Y674-R685: α7(ECD) complex. Right: Close-up view of interactions formed by R682 and Q675 within agonist binding site. The α7 receptor and Y674-R685 are coloured in dark blue and orange, respectively. Interactions between side chains of R682 and Q675 and the aromatic rings of TrpB (α7W171), TyrC1 (α7Y210), TyrC2 (α7Y217) and TyrA (α7Y115) are shown with dashed lines.

Figure 2

The Y674-R685 fragment cannot elicit detectable α7 responses. A. Macroscopic responses of the human α7 nAChR. Representative traces from single oocytes expressing human α7 nAChR to applications of ACh (100 µM) or 1 pM, 1 µM or 10 µM of Y674-R685. Drug applications were for 20 s followed by a 300 s washout. B. Single-channel currents of the human α7 nAChR recorded from cell-attached patches in the presence of 100 µM ACh or 10 µM Y674-R685. No channel activity was detected at a range 1 pM-100 µM concentration. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter: 9 kHz.
Figure 3

Activation of the human α7 nAChR by Y674-R685 in the presence of the PAM PNU-120596. A. Macroscopic currents were recorded from oocytes expressing the human α7 nAChR after a pulse of 100 µM ACh (control) or 1 µM Y674-R685 in the absence or presence of PNU-120596. Note that PNU-120596 does not elicit currents by itself. B. Single-channel currents of the human α7 nAChR in the presence of the type II PAM PNU-120596 (1 µM) activated by 100 µM ACh (left), or Y674-R685 at 1 pM or 10 µM (right). For each condition typical channel traces are shown. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter: 3 kHz.
Figure 4

Single-channel recordings of the human α7 nAChR in the presence of Y674-R685. Single-channel currents of human α7 nAChR in the presence of the type II PAM PNU-120596 (1μM) activated by 100 μM ACh, or Y674-R685 at 1 pM or 10 μM. For each condition, channel traces at two different temporal scales are shown. Channel openings are shown as upward deflections. Representative open, burst and cluster duration histograms are shown for each condition. The open, burst and cluster durations correspond to the durations of the slowest components of each histogram. The dashed lines show how these mean durations change among different conditions. Membrane potential: -70 mV. Filter: 3 kHz.
Figure 5

Channel activity elicited by Y674-R685 in the absence or presence of the PAM. Top: Single-channel currents of human α7 activated by 100 µM ACh appear mainly as brief isolated openings. Addition of 1 µM PNU-120596 to the extracellular solution surrounding a cell-attached patch results in a marked increase in current in the continued presence of 100 µM ACh in the patch pipette. Bottom: No channel activity is elicited by Y674-R685 at a range of concentrations in the absence of PAMs. Addition of 1 µM PNU-120596 to the extracellular solution surrounding the silent patch reveals channel activity, indicating that only in the presence of the PAM Y674-R685 can activate α7. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter 3 kHz.
Figure 6

Activation of the human α7 nAChR by Y674-R685 in the presence of the type I PAM, 5-HI. Single-channel currents were recorded from cells expressing the human α7 nAChR in the presence of 2 mM 5-HI as the PAM and 100 µM ACh or 10 µM Y674-R685 as agonists. Traces at two different scales are shown for each condition. Membrane potential: -70 mV, Filter: 9 kHz. Representative open and burst duration histograms are shown for each agonist. The bar chart shows the mean durations of open and bursts for each agonist. *p<0.05, **p<0.01.
Figure 7

Channel blockade of the human α7 nAChR by Y674-R685. A. Single-channel currents elicited by 10 μM ACh and potentiated by 1 μM PNU-120596 were recorded in the absence (control) or presence of 1 or 10 μM Y674-R685. Left: Typical channel traces are shown at two different temporal scales. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter: 3 KHz. Right: Bar chart showing the mean durations of openings, bursts and clusters in the absence of the peptide and in the presence of 1 or 10 μM Y674-R685. ** p<0.01; *** p<0.001. B. Concentration response curve (CRC) for the inhibition of the α7 nAChR by Y674-R685. Left: CRC for the inhibition of the peak ACh current responses of α7 by increasing concentrations of Y674-R685. Increasing concentrations of Y674-R685 (10 nM to 30
µM) were co-applied with control ACh (100 µM). Each data point represents the average normalized response of six cells (± SEM). Right: Competition CRC data (red) for 1 µM Y674-R685 co-applied with different ACh concentrations (0.1-2000 µM). For comparison, ACh CRC data alone (black) are shown at the same concentrations. Data were fitted to the Hill equation.

**Supplementary Files**

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