L-DOPA-quinone Mediated Recovery from GIRK Channel Firing Inhibition in Dopaminergic Neurons

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Supporting Information

ABSTRACT: The oxidative degeneration of dopamine-releasing (DAergic) neurons in the substantia nigra pars compacta (SNc) has attracted much interest in preclinical research, due to its involvement in Parkinson's disease manifestations. Evidence exists on the participation of quinone derivatives in mitochondrial dysfunction, alpha synuclein protein aggregation, and protein degradation. With the aim to investigate the role of L-DOPA-quinone in DAergic neuron functions, we synthesized L-DOPA-quinone by use of 2-iodoxybenzoic acid and measured its activity in recovery from dopamine-mediated firing inhibition of SNc neurons. Noteworthy, L-DOPA-quinone counteracts firing inhibition in SNc DAergic neurons caused by GIRK opening. A possible mechanism to explain the effect of L-DOPA-quinone on GIRK channel has been proposed by computational models. Overall, the study showed the possibility that L-DOPA-quinone stabilizes GIRK in a preopen conformation through formation of a covalent adduct with cysteine-65 on the GIRK2 subunit of the protein.

KEYWORDS: L-DOPA-quinone synthesis, biological activity, dopaminergic neurons firing, Parkinson, GIRK channel

The ventral midbrain is the area where most of the dopamine (DA) released in the central nervous system originates, being synthesized and released by a local population of dopamine-releasing (DAergic) neurons arranged into two main subregions, the substantia nigra pars compacta (SNc) and the ventral tegmental area. It is known that the release of DA from these neuronal populations is not limited to the relative areas of projection, but occurs also locally, through a somato-dendritic DA release taking place within the ventral midbrain. The functional role of this locally released DA is well established, as it acts on somato-dendritic D₂ receptors (D₂Rs) located onto the DAergic neurons, also called D₂ autoreceptors, causing the opening of an inwardly rectifying potassium conductance through interposition of a G protein (GIRK channel). Thus, the overall effect of DA in the ventral midbrain is hyperpolarization of DAergic neurons' membrane potential, resulting in inhibition of their firing rate and alteration in DA transmission.^{1–6}

SNc DAergic neurons have attracted much interest in preclinical research, due to their involvement in motor control, such that their degeneration is the main hallmark of Parkinson's disease (PD) motor symptoms. Although many pathogenic factors have been suggested, oxidative stress is a widely

recognized mechanism leading to SNc DAergic neurons degeneration in PD. Indeed, it is DA itself, locally released in the SNc area, that has been indicated as a main actor of this oxidative process, particularly through its oxidation into quinones.^{7,8} Evidence exists on the participation of quinones in mitochondrial dysfunction, alpha synuclein protein aggregation, and protein degradation;⁸ however, little is known of the role of these reactive molecules in regulating SNc DAergic neurons.

L-DOPA (L-3,4-dihydroxyphenylalanine) still remains the best and most used DA pro-drug in treatment of PD.⁹ After administration, L-DOPA interacts with specific carriers and crosses the blood–brain barrier (BBB), after which it is converted to DA within the brain.¹⁰ Data are available on the relationships between the metabolic transformation of DOPA to corresponding DOPA-quinone and the emergence of side-effects during the treatment of PD.¹¹ Moreover, we demon-

Special Issue: Highlighting Medicinal Chemistry in Italy

Received: October 11, 2018

Accepted: January 9, 2019

Published: January 9, 2019

strated that unnatural DOPA-containing peptides obtained from DOPA-quinone intermediates^{12,13} were characterized by anti-PD activity on individual DAergic neurons in rat ex vivo midbrain slices.¹⁴

With the aim to investigate the role of quinone intermediates in DAergic neurons function, we focused our attention on the synthesis of L-DOPA-quinone and on its possible interactions with the GIRK channels activated in response to D₂ autoreceptor stimulation. L-DOPA-quinone was synthesized by selective oxidation of protected L-tyrosine, by using 2-iodoxybenzoic acid (IBX). Molecular dynamics (MD) simulations have been performed to rationalize the biological data, suggesting an interaction mechanism between L-DOPA-quinone and a specific cysteine residue of GIRK2 that encompasses the transmembrane protein.

RESULTS AND DISCUSSION

L-DOPA-quinone has never been isolated in the past as a consequence of its high reactivity to yield DOPACHrome, followed by polymerization to melanin.¹⁵ The use of L-DOPA-quinone that we made as intermediate for the preparation of unnatural L-DOPA-containing peptides¹⁴ suggested its increased stability when the α -amino functionality was protected. For this reason, *N*-Boc-L-Tyr *tert*-butyl ester **1** was subjected to IBX oxidation avoiding reducing conditions (Scheme 1). IBX

Scheme 1. Synthesis of L-DOPA-quinone

promotes the *ortho*-hydroxylation of phenol to catechol (*ortho*-diphenol) in the presence of a reducing medium. The reactivity of IBX is similar to that of polyphenol oxidases, passing through a relatively stable quinone intermediate. The high regioselectivity of the oxidation is a consequence of the initial intramolecular oxygen transfer from iodine(V) in a λ^5 -iodanyl intermediate to the *ortho*-position of the phenolic group.¹⁶ The oxidation of **1** was performed according to a slightly modified general procedure previously applied.^{13,17} Briefly, compound **1** (0.068 mmol) was treated with IBX (1.1 equiv) in propan-2-ol (12 mL) at 25 °C for 2 h. Hindered propan-2-ol was selected with the aim to inhibit solvolysis processes. Under these experimental conditions *N*-Boc-L-DOPA-quinone *tert*butyl ester **2** was obtained in quantitative conversion of substrate and 65% yield of product. The low mass balance was most probably due to formation of overoxidation and polymerization side-products. Compound **2** (0.01 mmol) was then deprotected by mild treatment with TFA (2.2 equiv) in CH₂Cl₂ (1 mL) at 25 °C for 2 h, to yield L-DOPA-quinone **3** as trifluoroacetate salt in 45% yield (Scheme 1).¹⁸

The spontaneous firing of Substantia nigra *pars compacta* (SNc) neurons in slices was detected as extracellular single unit spikes with a MEA device, as previously reported.¹⁹ With the aim to trigger a GIRK channel-mediated functional response, we exposed the slice to the D₂R agonist quinpirole (100 nM). We

recorded a total of 44 active neurons (5 slices) with a regular basal firing rate (1.71 ± 0.12 Hz), all of which responded with inhibition of their firing rate, as expected from typical DAergic neurons following stimulation of their somato-dendritic D₂Rs.^{1–3,5,6,19,20} The firing remained inhibited under continuous perfusion in quinpirole, such that the firing rate of the DAergic neurons was inhibited to $4.07 \pm 3.56\%$ of control after 10 to 15 min in 100 nM quinpirole. This perfusion time was selected to make sure that a clear steady-state level had been reached.²¹ However, when DOPA-quinone **3** (300 μ M) was added to the medium, still in the continuous presence of quinpirole, a partial recovery from firing inhibition was observed, attaining $81.85 \pm 19.08\%$ of control after 15 min, a value that was significantly different from that in quinpirole alone ($P < 0.01$; $F(2,12) = 15.99$, $n = 5$; Figure 1). The possible desensitization of D₂Rs by DOPA-quinone was ruled-out by looking at its effect on the GIRK-mediated response by GABA_B receptor stimulation.

Figure 1. DOPA-quinone **3** counteracts firing inhibition of SNc DAergic neurons due to GIRK opening in response to D₂R stimulation. The raster plots are single units of four sample neurons recorded from the same slice, during drugs exposure indicated on top. The figure below shows the firing frequency normalized to that measured before drug perfusion. D₂R stimulation with quinpirole (100 nM) progressively reduced the firing rate, as indicated by the partial inhibition at 2 min (white column) and attaining a maximal effect at later stage (black column). When DOPA-quinone **3** (300 μ M) was added, in the continuous presence of quinpirole, a significant firing rate recovery was observed (gray column; $P < 0.01$).

It is known that, similarly to D₂Rs, also GABA_B receptor stimulation leads to inhibition of SNc DAergic neurons firing rate through GIRK conductance opening.^{3,22} We thus took advance of this converging mechanism and tested whether DOPA-quinone could inhibit a GIRK channel-mediated firing inhibition, triggered by this different receptor pathway, independently from D₂Rs (Figure 2).

We recorded with the MEA device the firing rate of 31 neurons (in 4 slices), with a regular basal firing rate (1.89 ± 0.25 Hz), all of which were previously identified as DAergic by the transient inhibition of their firing rate in response to a brief (1–2 min) challenge with 100 μ M DA. The slices were then treated

Figure 2. DOPA-quinone 3 counteracts firing inhibition of SNc DAergic neurons due to GIRK opening in response to GABA_B receptor stimulation. The raster plots are single units of four sample neurons recorded from the same slice, during drugs exposure indicated on top. The figure below shows the firing frequency normalized to the firing rate measured before drug perfusion. The cells were first challenged with a brief (1–2 min) exposure in DA (100 μ M), in order to confirm that they were DAergic. Indeed, their firing rate was inhibited (vertically striped column). The slice was then perfused with the D₂R antagonist sulpiride (10 μ M), and a new control level of firing rate was established (horizontally striped column). In the continuous presence of sulpiride, GABA_B receptor stimulation with baclofen (300 nM) progressively reduced the firing rate, as indicated by the partial inhibition at 2 min (white column) and attaining a maximal effect at later stage (black column). However, when DOPA-quinone 3 (300 μ M) was added, in the continuous presence of baclofen and sulpiride, a significant firing rate recovery was observed (gray column; $P < 0.05$).

with the specific D₂R antagonist sulpiride (10 μ M), kept in the medium thereafter, in order to completely exclude any D₂R contribution. Following perfusion with the GABA_B receptor agonist baclofen (300 nM), DAergic neuron firing was inhibited and remained inhibited after more than 10 min of continuous baclofen perfusion to $24.94 \pm 14.46\%$ of control. However, when we added DOPA-quinone 3 (300 μ M), in the continuous presence of baclofen and sulpiride, a recovery of the firing was observed, reaching $73.64 \pm 14.42\%$ of control. The level of firing inhibition after 15 min in 300 μ M DOPA-quinone 3 and baclofen was significantly lower than that in baclofen alone ($P < 0.05$; $F(4,15) = 14.27$, $n = 4$).

These data indicated a specific role of DOPA-quinone 3 in the recovery from firing inhibition caused by GIRK channel opening in response to D₂Rs or GABA_B receptors stimulation. Recently, our research team has reported that a similar form of firing recovery may be obtained in response to DA, through an opposing depolarizing current generated by activation of the dopamine transporter (DAT).²¹ According to literature, the possibility that the same mechanism may also underlie our present results is very unlikely. Indeed, for it to occur, DOPA-quinone should activate and be uptaken by DAT, while products of DA oxidation-like quinones have been shown to inhibit rather than stimulate DAT.²³ Although future investigation may unravel presently unexpected effects on DAT, the most conceivable hypothesis is that DOPA-quinone reverts firing inhibition by acting directly onto previously opened GIRK-channels.

The mechanism by which DOPA-quinone could promote the closure, and alternatively inhibit the opening of the GIRK channel, was evaluated through a computational approach. We focused our attention on GIRK2 channel, which is the most prevalent GIRK isoform in both substantia nigra and ventral tegmental area.²⁴ DOPA-quinone is an electrophilic molecule able to react with sulfhydryl nucleophilic moieties. As an example, the formation of the 5-cysteinyldOPA (5-cDOPA) adduct is a key step in the synthesis of pheomelanin.²⁵ Covalent adducts, such as 5-cDOPA, can produce irreversible alterations of enzymes or cellular proteins activity due to the alkylation of residues in the catalytic site or in key portions of functional domains. Particularly, catecholamine-quinones were suggested to be responsible for the reduction of DA uptake by alkylating specific cysteine residues of the dopamine transporter.²³ They are also able to block the catalytic activity of brain tyrosine hydroxylase,²⁶ showing neurotoxic effects.²⁷ On the basis of this data, we developed a model for the formation of covalent DOPA-quinone adducts with cysteine residues of GIRK2, evaluating their effect on the concerted opening/closing mechanism of the GIRK2 channel.²⁸

The alkylation by sulfhydryl-modifying reagents of N-terminal cysteine 65 (C65) and C-terminal cysteine 321 (C321) residues, both placed within the cytoplasmic domain of GIRK2 channel, determine the inhibition of GIRK mediated current in GIRK1/GIRK2 heteromeric channels.²⁹ In particular, the alkylation of C65 was shown to have a high impact in current modulation, and it was found to be considerably more accessible than C321 to sulfhydryl modifying reagents, as is also demonstrated by the X-ray crystallographic structure of murine GIRK2 channel (98.3% identity with human isoform) in the preopen conformation, once complexed with β - γ G-protein subunits, dioctanoyl-l- α -phosphatidyl-D-myo-inositol-4,5-diphosphate (PIO), and Na⁺ (PDB code 4KFM). For this reason, we assumed C65 as the specific cysteine residue to which DOPA-quinone would bind. C65 is placed in the proximity of specific loops of the protein, i.e., G-loop, CD-loop, and C-linker, which have shown an essential role in the concerted opening/closing mechanism of the channel, together with the N-terminal loop to which C65 belongs (Figure 3). In fact, mutations located in these regions can significantly affect the function of GIRK channels.³⁰ In particular, the CD-loop is implied in the regulation of GIRK channel gating through conformational movements involving a network of interactions with the C-linker and the G-loop, this latter constituting the second ion gate of the channel.^{30–32} The CD-loop also constitutes part of the binding site for cellular Na⁺ (Figure 3), which demonstrated to amplify the opening of the channel in the presence of G-proteins.²⁸

The hypothesis that DOPA-quinone can disrupt the opening process of GIRK2 by alkylating C65 is also supported by an X-ray structure of GIRK2 cytoplasmic domain complexed with cadmium (Cd²⁺) ions, which suggests a mechanism for the concentration-dependent inhibitory effect of Cd²⁺ on GIRK2 activity (PDB code 3AUW).³³ In the crystal structure, the Cd²⁺ ion is located in a high-affinity site, placed at the entrance of Na⁺ binding site, where it directly interacts with C65 and with residues of both CD-loop and N-terminus in octahedral coordination geometry. Through this network of interactions, Cd²⁺ would block GIRK2 opening by trapping the channel in its closed conformation as a sort of inverse agonist, tethering between N-terminus and CD loop.³³

In order to calculate a reliable model of the 5-cDOPA adduct with C65 (PDB code 4KFM; Figure 3), we employed the

Figure 3. Crystal structure of GIRK2 channel (PDB code 4KFM). The protein loops implied in channel gating, i.e., CD-loop, G-loop, and N-terminus, are shown in green, blue, and orange, respectively, together with the sodium ion. Residues D288, H233, and E315, involved in channel gating, are displayed together with C65.

covalent docking protocol of AutoDock4.2,³⁴ using a modified set of parameters (see [Materials and Methods](#) for details).^{35,36} Since C65 is placed in front of the binding site of Na⁺ ions, we hypothesized that the catechol moiety of 5-cDOPA could even occupy such pocket, thus interfering with the agonist-like effect of Na⁺ in the modulation of GIRK2 gating. In fact, the amplification of GIRK2-mediated current is concentration-dependent since Na⁺ constitutes a mechanism of negative feedback on excessive electrical excitability, which occurs when cellular Na⁺ concentration rise enough above normal levels.³⁷ Therefore Na⁺ is not supposed to be a constitutive element of GIRK2 channel, and we removed it from the protein structure prior to docking calculations. Interestingly, the calculation suggested that 5-cDOPA was more likely to be placed outside Na⁺ binding site rather than within the pocket ([Figure 4](#)). This is probably due to the presence of the carboxylic group in the ligand, which is able to form strong interactions with the lysine residues surrounding the binding site (K64 and K90).

To assess the reliability of the docking solution and to evaluate if 5-cDOPA could effectively interfere with GIRK2 opening, molecular dynamics (MD) simulations were also performed. The MD protocol was set up using the X-ray structure of wild-type GIRK2 channel in complex with Na⁺ ions and PIO cofactors as a template. After embedding the protein in a lipid bilayer and solvating the system on the “extracellular” and “intracellular” side (see [Materials and Methods](#) for details), 50 ns of MD simulations were performed. As shown in [Figure S7](#), the total energy of the native GIRK2 rapidly (after 1 ns) reached the equilibrium. Moreover, after about 2.5 ns, the root-mean squared deviation of the protein α carbons with respect to the crystallographic structure showed an overall constant value around 2.0 Å. The same MD protocol was applied to the C65-covalently modified protein, and the interactions observed during the simulation between 5-cDOPA and the surrounding protein residues were analyzed. According to Wang and co-workers, four *Gβγ* subunits must bind GIRK2 channel to allow

Figure 4. Predicted binding mode of 5-cDOPA outside Na⁺ binding site.

its opening, revealing a concerted mechanism of gating in which all protein monomers cooperatively work to allow the necessary global conformational change.²⁸ Thus, C65 was covalently modified in all channel monomers prior to MD simulations.

The MD simulations confirmed the presence of the interactions among 5-cDOPA and the two lysine residues K64 and K90, and revealed that the amino group of 5-cDOPA was able to form a strong salt bridge with the carboxylate group of E315 ([Figure 4](#)), maintained for more than 85% of the simulation, on average. The interaction with E315, which belongs to the G-loop and is crucially involved in the channel gating mechanism, might have a key role in determining the effect of 5-cDOPA on GIRK-mediated current. In fact, mutations of this particular residue, which is extremely conserved among all GIRK channels, can substantially alter the protein functionality and/or abolish the channel-induced current.²⁹

Moreover, in the MD simulation of native GIRK2 we observed an interaction between the carboxylate group of E315 and the side chain of the residue H233 in CD-loop ([Figure 3](#)). During the movements of the CD-loop associated to channel gating, H233 should undergo a profound conformational rearrangement, inverting its orientation and climbing over the CD-loop itself ([Figure S8](#)).³⁷ Interestingly, the formation of the salt bridge between 5-cDOPA and E315 determined the disruption of the interaction between E315 and H233; this produced a modification of H233 orientation totally opposite with respect to the conformational rearrangement that should be associated to channel gating. In fact, the imidazole ring of H233 moved within Na⁺ binding site after few nanoseconds of simulation and remained trapped between CD-loop and V276, locked inside the pocket by the aromatic ring of 5-cDOPA ([Figure 4](#)). Thus, the reciprocal disposition of 5-cDOPA and H233 would prevent this latter residue to move outside Na⁺ binding site, blocking the conformational freedom of the whole CD-loop and impeding the rearrangement required for channel opening.

In addition, 5-cDOPA forms hydrogen bonds with CD-loop residues like N231 or H233 itself that reinforce the conformational restraint of the CD-loop determined by the presence of the covalent ligand. Through this network of interactions, 5-cDOPA is anchored to and interconnects with different N-

terminus and CD-loop residues. This predicted tethering, which showed to be detrimental for channel gating when induced by Cd^{2+} ions,³³ could stabilize the pre-open conformation of the channel. The tethering could thus block the flickering process, i.e., the rapid transition between open (conductive) and pre-open (nonconductive) conformations, and channel gating.³⁰ In fact, during the opening process, CD-loop and N-terminus undergo a large rearrangement, as shown in the crystal structure of GIRK2 cytoplasmic domain (CTD) in a partially open state (PDB code 3SYQ).³⁷ This double effect might constitute the mechanism through which DOPA-quinone, after the formation of the 5-cDOPA adduct, stabilizes GIRK2 preopen conformation thus inhibiting the channel-mediated current.

The same covalent docking protocol applied on C65 was also applied on C321 as a control. By just looking at the localization of the covalent adduct (Figure S6), it is possible to understand that C321 would be hard to reach by DOPA-quinone. In fact, C321 is placed in the part of the protein that stays between the outer surface (where C65 is located) and the inner surface (the channel pore). Moreover, polar and very flexible residues like K199, K200, K64, R324, Q322, and E315 narrow the access to C321 and limit the space around this residue. In agreement with these considerations, the covalent docking protocol generated only a single possible solution associated to an unfavorable ligand–protein binding affinity, probably due to both the steric hindrance and the electrostatic repulsions between 5c-DOPA and the surrounding residues. These results suggest that C321 is not likely to be alkylated by DOPA-quinone. However, MD simulation studies performed as control suggested that a concurrent alkylation of both C65 and C321 could have a strong impact on GIRK-mediated current and thus on the firing of dopaminergic neurons (see Supporting Information S9 for details).

The computational model was then extended to dopamine quinone (DA-quinone), a compound that cannot be isolated due to its very high reactivity. In particular, we evaluated whether the alkylation of C65 by DA-quinone to afford 5-S-cysteinyldopamine (5-cDA) might produce an effect on GIRK2 channel gating similar to that previously predicted for 5-cDOPA. In this latter case, covalent docking suggested that 5-cDA would preferentially occupy the Na^+ binding site, interacting with the protein loops implied in the channel gating. MD simulations highlighted that the amino group of 5-cDA is able to form interactions with the residues that can also coordinate the Na^+ when this ion is present in the binding site. Particularly, a stable interaction with the carboxylate group of D228 is observed, and it is maintained for about 80% of the simulation, on average. The amino group of 5-cDA can also arrange stable hydrogen bonds with the backbone carbonyls of D228 and other residues such as R230, thus demonstrating to well fit the pocket constituting the Na^+ binding site to which it is strongly anchored (Figure 5). The orientation of 5-cDA results in stabilization by additional H-bonds between its hydroxy groups and residues N231 and V67. Particularly, the H-bond interaction with the backbone carbonyl of V67 was observed for more than 80% of the whole MD simulation, on average.

It is worth noting that the interaction between E315 and H233 was maintained as in the reference simulation of wild-type GIRK2 (Figure 3) and that no conformational rearrangement of H233 is observed. However, 5-cDA is able to form a wide network of interactions with CD-loop residues, and thus, a deep tethering between N-terminus and CD-loop could stabilize the channel in the pre-open conformation and prevent channel

Figure 5. Predicted binding pose for 5-cDA within Na^+ binding site of GIRK2. H-bonds among 5-cDA and the surrounding protein residue are shown as dashed lines.

gating. Overall, the computational studies suggested that S-cysteinyldopamine derived from DOPA-quinone and DA-quinone could inhibit GIRK2 channel opening by promoting the tethering of G-loop, CD-loop, and N-terminus. Additionally, DOPA-quinone can also inhibit the GIRK2 gating machinery by interfering with the CD-loop movements involving H233.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00477.

Slice preparation for electrophysiology; multielectrode array recordings; drugs used for electrophysiology; covalent docking; molecular dynamics simulations; covalent docking results for DOPA-quinone bound to C321; analysis of the MD simulation; superimposed structures of GIRK2; MD simulation results of GIRK2 with C65 and C321 concurrently alkylated by DOPA-quinone; general information for reagents and synthetic procedures; general information for the HPLC system; HPLC spectrum of L-DOPA-quinone 3 (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The Filas project MIGLIORA from Regione Lazio and the project PRONAT from CNCCS SCARL are acknowledged.

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