Dopaminergic pharmacology and antioxidant properties of pukateine, a natural product lead for the design of agents increasing dopamine neurotransmission

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Abstract

The dopaminergic and antioxidant properties of pukateine [(R)-11-hydroxy-1,2-methylenedioxyaporphine, PUK], a natural aporphine derivative, were analyzed in the rat central nervous system. At dopamine (DA) D₁ ([³H]-SCH 23390) and D₂ ([³H]-raclopride) binding sites, PUK showed IC₅₀ values in the submicromolar range (0.4 and 0.6 μM, respectively). When the uptake of tritiated dopamine was assayed by using a synaptosomal preparation, PUK showed an IC₅₀ = 46 μM. In 6-hydroxydopamine unilaterally denervated rats, PUK (8 mg/kg but not 4 mg/kg) elicited a significant contralateral circling, a behavior classically associated with a dopaminergic agonist action. When perfused through a microdialysis probe inserted into the striatum, PUK (340 μM) induced a significant increase in dopamine levels. In vitro experiments with a crude rat brain mitochondrial suspension showed that PUK did not affect monoamine oxidase activities, at concentrations as high as 100 μM. PUK potently (IC₅₀ = 15 μM) and dose-dependently inhibited the basal lipid peroxidation of a rat brain membrane preparation. As a whole, PUK showed a unique profile of action, comprising an increase in extracellular DA, an agonist-like interaction with DA receptors, and antioxidant activity. Thus, PUK may be taken as a lead compound for the development of novel therapeutic strategies for Parkinson disease. © 1999 Elsevier Science Inc. All rights reserved.

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Progressive degeneration of a particular subset of neurons is the pathologic basis of different neurodegenerative disorders such as Parkinson disease (PD), amyotrophic lateral sclerosis, and Alzheimer disease. PD is characterized by a slow and progressive loss of meso-striatal dopaminergic neurons, faster than that observed during normal aging (Hirsch, 1994). A pronounced deficiency in striatal and nigral dopamine (DA) is the main biochemical manifestation of PD which correlates significantly with the severity of parkinsonian disability (Bernheimer et al., 1973). Classically, the pharmacological approach for treating PD has been based on the restoration of dopaminergic neurotransmission, either by administration of DA precursors (l-dopa), DA receptor agonists, or both (Mizuno et al., 1991; Yahr, 1976). However, these therapies present disadvantages in view of their side effects and loss of efficacy observed in long-term treatments (Rinne, 1983, 1991).

A growing body of data from both experimental models and human brain studies implicates oxidative stress as a major cause in neurodegenerative diseases (Dexter et al., 1994; Ebadi et al., 1996; Jenner, 1992; Simonian and Coyle, 1996). Basically, oxidative stress refers to the cytological consequences of a mismatch be-
between the production of reactive oxygen species and the ability of cells to protect themselves against them (Simonian and Coyle, 1996). Neurons from the substantia nigra are particularly vulnerable to oxidative stress as a consequence of the normal generation of reactive oxygen species in the course of DA metabolism (Olanow and Arendash, 1994). This situation is worsened even more by the increased iron content and the relatively low levels of catalase, ferritin, and glutathione in the parkinsonian substantia nigra, as reported by different authors (Ambani et al., 1975; Dexter et al., 1989, 1990; Perry and Yang, 1986). Thus, neurons in the substantia nigra constitute a population of cells highly sensitive to imbalances in reactive oxygen species and oxidative stress.

An increasing understanding of the biochemical basis of PD and the problems related to classical therapies has given great impetus to the development of novel therapeutic alternatives. Currently, this search has been focused on drugs that are able not only to restore deficient DA transmission, but also to protect from or at least delay neuronal degeneration. Thus, compounds with DA transmission-enhancing properties associated with neuroprotective characteristics (e.g., l-deprenyl, pramipexole, bromocriptine) appear as some of the most promising antiparkinsonian therapies (Sethy et al., 1997; The Parkinson Study Group, 1993; Wu et al., 1994). Two decades ago, the semisynthetic aporphine apomorphine was used extensively in clinical pharmacology for the treatment of PD (Neumeyer et al., 1981), mainly on the basis of its dopaminergic agonism. More recently, different aporphines were shown to exhibit potent antioxidant activity in brain tissue homogenates, and boldine, a natural aporphine alkaloid, was shown to have cytoprotective effects on isolated hepatocytes and on red blood cells exposed to free-radical sources (Bannach et al., 1996; Cassels et al., 1995) [Jiménez et al., unpublished results]. Pukateine, [(R)-11-hydroxy-1, 2-methylenedioxyaporphine, PUK: Fig. 1], another aporphine alkaloid present in the bark of the pukatea tree (Laurelia novae-zelandiae), was shown to bind DA receptors in preliminary studies. Moreover, its R configuration and C-11-positioned OH group suggested that this molecule might behave as a dopaminergic agonist (Neumeyer, 1985). Aside from this putative DA transmission-enhancing property, the phenolic character of pukateine and its aporphine skeleton are strong indications that this compound might act as a potent antioxidant in brain tissue (Cassels et al., 1995).

To examine the role that aporphines, and particularly PUK, could have in the development of new alternatives for PD therapeutics, we have studied some of the dopaminergic and antioxidant properties of PUK in the central nervous system.

1. Materials and methods

1.1. Animals

Animal experiments were carried out by using male Sprague Dawley (IIBCE, Uruguay) or Charles River (France, for binding and DA uptake experiments) rats with access to food and water ad libitum, housed in groups of six in a temperature-controlled environment on a 12-h light/dark cycle.

1.2. Drugs and reagents

Chemicals for high-performance liquid chromatographic analysis and artificial cerebrospinal fluid (aCSF) were purchased from Baker (Phillipsburg, PA, USA) and were of analytical grade. All chemicals for the lipid peroxidation and monoamine oxidase (MAO) activity assays were obtained from Sigma (St. Louis, MO, USA). PUK was isolated from Laurelia novae-zelandiae bark as described elsewhere (Urzúa and Cassels, 1982), dissolved in 1% acetic acid, and taken to pH 6 by addition of 12.5 M NaOH to give the final concentrations of alkaloid required for the different experiments. The vehicle solution was also prepared with 1% acetic acid and NaOH for pH corrections, in the same way as the PUK solution. 6-Hydroxydopamine (6-OHDA) was purchased from Sigma, and dissolved in aCSF with 0.2% ascorbic acid. Boldine was isolated from Peumus boldus bark as described previously (Speisky et al., 1991), and solutions of the hydrochloride were prepared in water or phosphate buffer, depending on the assay.

1.3. Microdialysis perfusion and DA neurochemical analysis

Microdialysis probes (dialyzing length, 4 mm; diameter, 0.5 mm; CMA AB, Sweden) were stereotaxically implanted in urethane-anesthetized animals (220–270 g) and continuously perfused (flow rate, 2 μl/min.) with aCSF as previously described (Dajas-Bailador et al., 1996). Fractions (40 μl) were collected over 20-min periods in tubes containing 5 μl 0.1 M HClO₄ and, for all experiments, two fractions were considered to define basal levels. When introduced through the cannulae, PUK was perfused for 40 min. At the end of each experiment, 100 mM KCl was perfused to confirm that synap-
tic functionality was preserved. DA content was measured by using high-performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC) as previously described (Dajas-Bailador et al., 1996).

1.4. Intranigral injection of 6-OHDA and circling behavior

Animals weighing 220–270 g were injected with pentobarbital (50 mg/kg) and placed in a D. Kopf stereotaxic frame. Through a skull hole, the needle (0.022 mm o.d., 0.013 mm i.d.) of a Hamilton syringe (5 μl), attached to the stereotaxic microinjection unit (D. Kopf), was lowered into the substantia nigra. Coordinates were determined, from bregma, according to the atlas of Paxinos and Watson (1986): H: −5.2, L: −2, V: −7.2. A 16 μg/μl 6-OHDA solution was injected (0.5 μl) during 1 min, and the needle was withdrawn slowly after allowing the drug to diffuse for another minute. Controls were injected with aCSF. To select the successfully denervated animals, rats were treated with a low dose of apomorphine (0.05 mg/kg IP) 8 days after 6-OHDA injection. Circling behavior experiments were performed between 8:00 and 10:00 A.M. in a red acrylic plastic bell-shaped box (0.70 × 0.40 m), and turns (360°) were scored with a circling behavior monitor during 15 min, starting 5 min after drug injection. Rats that showed marked contralateral circling (more than 100 turns in 350 s) during the period in a volume of 25 μl. In the control experiments, volume adjustments were done with phosphate buffer. Incubations were terminated by the rapid addition of 350 μl 20% (v/v) acetic acid. Zero time samples were obtained by adding acetic acid to the incubation mixture prior to the addition of tissue homogenate.

1.6.2. Thiobarbituric acid reacting substances (TBARS) assay

TBARS formation was assayed in tissue homogenates according to a previously published procedure (Buege and Aust, 1978) with minor modifications. Briefly, after incubation was terminated, 600 μl of 0.5% thiobarbituric acid in 20% (v/v) acetic acid (pH 3.5) was added to each sample. The tubes were then incubated at 85°C for 60 min. After cooling, the samples were centrifuged at 5000 × g for 10 min, and the absorbance of the supernatant was determined at 532 nm. The absorbance reading was determined against blanks prepared in the same way as the experimental samples but without tissue. Zero time values were subtracted from experimental values for calculations. Boldine, a well-characterized aporphine antioxidant (Speisky et al., 1991), was included as a positive control.

1.7. Radioligand binding and inhibition of DA uptake experiments

Binding experiments were performed on striatal membranes. Each striatum was homogenized in 2 ml ice-cold Tris-HCl buffer (50 mM, pH 7.4 at 22°C) with a Polytron (4 s, maximal scale) and immediately diluted with Tris buffer. The homogenate was centrifuged either twice ([3H]SCH 23390-binding experiments) or four times ([3H]raclopride-binding experiments) at 20,000 × g for 10 min at 4°C with resuspension in the same volume of Tris buffer between centrifugations. For [3H]SCH 23390-binding experiments, the final pellet was resuspended in Tris buffer containing 5 mM MgSO4, 0.5 mM EDTA, and 0.02% ascorbic acid (Tris-Mg buffer), and the suspension was briefly sonicated and diluted to a protein concentration of 1 mg/ml. A 100-μl aliquot of freshly prepared membrane suspension (100 μg of striatal protein) was incubated for 1 h at 25°C with 100 μl Tris buffer containing [3H]-SCH 23390 (85.5 Ci/mmol; NEN, Paris, France; 0.25 nM final concentration) and 800 μl of Tris-Mg buffer containing the required drugs. Nonspecific binding was determined in the presence of 30 μM SK&F 38393 (RBI, Natick, USA) (Cortes et al., 1992; Protai et al., 1992) and constituted about 2 to 3% of total binding. For [3H]raclopride-binding experiments, the final pellet was resuspended in Tris buffer containing 120 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 0.1% ascorbic acid.
(Tris-ions buffer), and the suspension was treated as heretofore described. A 200-μl aliquot of freshly prepared membrane suspension (200 μg of striatal protein) was incubated for 1 h at 25°C with 200 μl of Tris-ions buffer containing [3H]raclopride (86.5 Ci/mmol; NEN, Paris, France; 0.5 nM final concentration) and 400 μl of Tris-ions buffer containing the drug being investigated. Nonspecific binding was determined in the presence of 50 μM apomorphine (Cortes et al., 1992; Protais et al., 1992) and constituted about 5 to 7% of total binding. In both cases, incubations were stopped by the addition of 3 ml of ice-cold buffer (Tris-Mg buffer or Tris-ions buffer, as appropriate) followed by rapid filtration through Whatman GF/B filters. Tubes were rinsed with 3 ml ice-cold buffer, and filters were washed with 3 × 3 ml ice-cold buffer. After the filters had been dried, radioactivity was counted in 4 ml BCS scintillation liquid (Amersham, Paris, France) at an efficiency of 45%. Filter blanks corresponded to approximately 0.5% of total binding and were not modified by drugs.

For [3H]DA uptake assays, all experimental procedures for the preparation of synaptosomes were carried out at 0–4°C. To obtain synaptosomal preparations, rats were killed by decapitation and the striatum was dissected and homogenized in 10 volumes (w/v) of 0.32 M sucrose by using 10 up and down strokes of a PTFE glass homogenizer (800 rpm). Nuclear material was removed by centrifugation at 1000 × g for 10 min. The supernatant (S1) was stored and the P1 pellet was resuspended in 10 volumes of 0.32 M sucrose and recentrifuged (1000 × g for 10 min). The two supernatants were combined, and the mixture was centrifuged at 15,000 × g for 30 min. The resulting P2 pellet was suspended in 20 volumes of ice-cold Krebs-Ringer medium previously oxygenated (95% O2, 5% CO2). The medium contained (mM): NaCl, 109; KCl, 3.6; KH2PO4, 1.1; CaCl2, 2.4; MgSO4, 0.6; NaHCO3, 25; glucose, 5.5; pH 7.6. [3H]DA uptake was evaluated on aliquots of the synaptosomal preparation. After a 5-min preincubation in Krebs-Ringer buffer containing 10 μM pargyline, [3H]DA (47 Ci/m mole; Amersham, France) was added to a final 2 nM concentration. Five-minute incubations were stopped by dilution into cold Krebs-Ringer medium followed by filtration under vacuum on Whatman GF/B filters. Filters were washed twice with 3 ml cold Krebs-Ringer medium and dried. Tissue radioactivity retained by synaptosomes was determined by liquid scintillation spectrometry. Blank values, obtained by incubating parallel samples at 0°C, were subtracted (Protais et al., 1984, 1995).

1.8. Statistical analysis

Microdialysis data were evaluated statistically by using a single factor analysis of variance (ANOVA), and Student’s t-test (two tails) for additional mean comparisons. Circling behavior experiments were analyzed by using Student’s t-test (two-tailed) for mean comparisons. Receptor-binding data were analyzed by using the hydrographic computer programs EBDA and Ligand, as described by McPherson (1985). In all cases, data are presented as mean ± SD. Statistical significance was set at p < 0.05.

2. RESULTS

2.1. Radioligand binding and DA uptake assays

At [3H]SCH 23390 (D1) and [3H]raclopride (D2) binding sites, PUK showed IC50 values in the submicromolar range (0.4 ± 0.03 and 0.6 ± 0.04 μM for SCH 23390 and raclopride sites, respectively, n = 6).

When the uptake of tritiated DA was assayed by using a synaptosomal preparation, PUK showed an IC50 = 46 ± 5 μM (n = 5).

2.2. Circling behavior experiments

Rats injected with 6-OHDA in the substantia nigra showed an almost complete depletion of DA in the striatum (>90%) 8–10 days after the injection, as measured by HPLC-EC after drug tests (data not shown), and exhibited pronounced contralateral circling with apomorphine (Fig. 2). The 4-mg/kg (n = 8) PUK dose showed no effect on circling behavior, but 8 mg/kg (n = 9) elicited significant contralateral circling (Fig. 2). This evoked behavior has been classically associated with a dopaminergic, apomorphine-like, agonist action.

2.3. Microdialysis experiments

The microdialysis technique used showed stable basal levels of 4.5 ± 2 nM DA. Perfusion of PUK (340 μM) through the microdialysis probe induced a significant increase in extracellular levels of DA in the striatum (n = 6; Fig. 3). The levels of DOPAC and HVA, the main metabolites of DA, were not modified by PUK perfusion. The percentage of PUK that crosses the mi-
ciodalysis membrane (in vitro recovery) was found to be about 24%, as measured by PUK absorbance at 300 nm (data not shown). At the end of all microdialysis experiments, KCl evoked a DA increase of more than 25-fold, ensuring that the synaptic terminals were still functional. Additional microdialysis experiments showed that PUK (8 mg/kg IP) produced no modification of DA release in the striatum (data not shown).

2.4. MAO-A and -B inhibition assays

In view of the increase in extracellular concentrations of DA caused by PUK, we tested the possible involvement of MAO inhibition in the observed effect. In vitro studies showed that PUK was completely devoid of MAO-A or MAO-B inhibitory properties, and no enzymatic inhibition was observed at concentrations as high as 100 μM (n = 4). Moreover, when PUK was preincubated with the enzyme for 30 or 60 min, no changes were observed in the enzymatic activities even after the longest incubation time (data not shown).

2.5. Lipid peroxidation assay

As shown in Fig. 4, PUK inhibited the formation of TBARS in a concentration-related fashion with an IC₅₀ = 15 μM (n = 5). The inhibition profile was very similar to that observed with boldine, the aporphine antioxidant used as a positive control (IC₅₀ = 13 μM).

3. Discussion

The search for new therapies for PD has focused on the development of compounds able to restore DA transmission and protect nigral neurons against neurodegeneration. Deprenyl, an adjuvant to levodopa in PD therapeutics (Golbe et al., 1988), was more recently addressed as a possible neuroprotective agent, retarding the progressive disability of parkinsonian patients (Tetrud and Langston, 1989; The Parkinson Study Group, 1993). Neuroprotection by deprenyl seems to be independent of its MAO-B inhibitory properties and has been associated with novel free-radical scavenging properties (Wu et al., 1994). These results, and others reporting that vitamin E consumption may be associated with reduced incidence and severity of PD, has given new emphasis to the study of antioxidant molecules that might contribute to halting neuronal degeneration and restoring DA synaptic functionality (Ebadi et al., 1996).

Early studies showed that PUK could interact with DA receptors. These results comprise the weak inhibition of DA-sensitive adenylate cyclase of the rat caudate and the displacement of [³H]apomorphine and [³H]ADTN binding to calf caudate membranes (Neu- meyer et al., 1985; Sheppard and Brughardt, 1978). In the present work, radioligand binding to striatal membranes further support this idea and confirm that PUK is capable of interacting with striatal DA receptors, showing IC₅₀ values in the submicromolar range.

The unilateral administration of 6-OHDA into the rat nigrostriatal system induces unilateral damage of the DA-containing nigral neurons (Ungerstedt, 1971; Ungerstedt and Arbuthnott, 1970). In this model, a contralateral circling behavior in response to low doses of DA agonists can be observed over time, and this turning is mediated by the development of supersensitivity of postsynaptic DA receptors on the lesioned side (Heikkila et al., 1981; Ungerstedt, 1971). PUK (8 mg/kg IP) elicited marked contralateral circling, a behavior that can be attributed to an agonist-like interaction with DA receptors. When taken together, the radioligand-binding and circling behavior experiments are in agree-
ment with the hypothesized effect of PUK on DA receptors, considering its $R$ configuration and its OH group positioned at C-11.

When perfused through the microdialysis probe, PUK produced a significant increase in the extracellular levels of striatal DA. It should be emphasized that the percentage of PUK that crosses the microdialysis membrane was determined to be about 24%. In view of that, the approximate tissue concentration of PUK surrounding the cannulae could be estimated as less than 80 $\mu$M. Given that PUK showed no inhibition of MAO activities in rat brain mitochondrial suspensions, the increase in the extracellular concentrations of DA could be attributed to the inhibition of DA uptake. This hypothesis is supported by results obtained with striatal synaptosomes, showing that PUK inhibits DA uptake with an $IC_{50} = 46$ $\mu$M. However, the possibility that PUK might be modifying extracellular DA through an increase in DA release cannot be excluded. Additional microdialysis experiments showed that PUK at 8 mg/kg IP, a dose that induced contralateral circling in denervated rats, did not evoke an increase in extracellular DA in the striatum. This finding could be explained by the fact that PUK affinity for the DA receptors is much higher than that observed for the uptake site. Consequently, the concentration of PUK that reaches striatal tissue when administered intraperitoneally would preferentially stimulate DA receptors with no effect on the DA uptake system.

A number of phenolic and nonphenolic aporphines have been shown to exhibit antioxidative activity in a variety of systems. Phenolic aporphines are expected to scavenge reactive free radicals at least by generating thermodynamically and kinetically stable phenoxy radicals, whereas nonphenolic analogues presumably act through similarly stabilized benzyl free radicals centered at C-6a (Cassels et al., 1995). On the basis of this suggested mode of action of aporphines in general, and of phenolic aporphines in particular, it was expected that PUK would exhibit antioxidative activity. Confirming this assumption, PUK showed a potent antioxidative profile in the brain tissue lipid peroxidation assay.

As a whole, PUK showed a broad profile of action, comprising an increase in extracellular DA, an agonist-like interaction with DA receptors, and antioxidative activity. The rather low $IC_{50}$ for DA uptake inhibition and DA-receptor binding does not single out PUK as a particularly attractive compound when considering the development of pharmacological alternatives for anti-parkinsonian therapies. However, that the chemical structure of PUK may be readily modified to improve its dopaminergic properties has to be taken into account. Thus, replacement of the N-methyl group by larger substituents can be expected to afford derivatives with higher affinity for at least some DA receptor subtypes, while at the same time increasing lipophilicity and possibly improving pharmacokinetics and blood-brain barrier penetration. Additionally, presumably facile electrophilic substitution on the phenolic ring C may lead to improved pharmacological properties by modulating charge distribution and overall lipophilicity and affording additional receptor-binding functionalities. These structural modifications may take advantage of PUK’s dual mode of action (increase in extracellular DA and dopaminergic agonism) and preserve the potent antioxidant, and probably cytoprotective, activity of this natural product. Molecules of this type should counterbalance the loss of dopaminergic function and protect nigral neurons from oxidative stress, which is considered to be one of the main determinants in the development of the characteristic neuronal degeneration of PD (Ebadi et al., 1996).

In conclusion, PUK shows a unique combination of dopaminergic and antioxidant properties and may be taken as a lead compound for the development of novel therapeutic strategies for PD. Future work should aim to improve the dopaminergic profile of PUK and study its putative neuroprotective role in experimental models of PD.

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