Bromocriptine is a strong inhibitor of brain nitric oxide synthase: possible consequences for the origin of its therapeutic effects

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Abstract The ergot alkaloid bromocriptine (BKT) was found to act as a strong inhibitor of purified neuronal nitric oxide synthase (NOS) ($IC_{50} = 10 \pm 2$ μM) whereas it was poorly active towards inducible macrophage NOS ($IC_{50} > 100$ μM). BKT affects the activation of NOS by calmodulin, as it not only inhibits L-arginine oxidation to NO and L-citrulline but also NADPH oxidation and calmodulin-dependent cytochrome *c* reduction catalyzed by neuronal NOS. These results suggest that BKT could exert some of its therapeutic effects by interfering with the NOS-dependent formation of nitric oxide and/or superoxide ion in various tissues.

Key words: Bromocriptine; Nitric oxide synthase; Selective inhibitor; Prolactin; Migraine; Parkinson's disease

1. Introduction

The semi-synthetic ergot alkaloid bromocriptine (BKT) (Fig. 1) exhibits several therapeutic effects [1–3]: (i) it has a beneficial effect in severely disabled patients suffering from Parkinson's disease [4], (ii) it is used, like other ergot alkaloids, in the treatment of migraine, and (iii) it is a strong inhibitor of prolactin formation [5]. These effects have been related to its ability to block dopamine receptors, and to its interaction with calmodulin-induced activation of phosphodiesterases in rat and human brain [6,7].

Calmodulin (CaM) is a ubiquitous calcium binding protein which mediates a variety of biological functions [8]. One of the most important neuronal mechanisms activated by the Ca^{2+}/CaM system in the brain is the recently discovered biosynthesis of nitric oxide (NO). NO displays various functions as a cellular messenger in central and peripheral nervous systems, regulator of vascular tone and platelet aggregation, and cytotoxic agent [9,10]. Nitric oxide is produced by constitutive, Ca^{2+}/CaM-dependent, neuronal and endothelial nitric oxide synthases (nNOS and eNOS), and by inducible NOSs (iNOS) that are weakly affected by Ca^{2+} but induced by lipopolysaccharides (LPS) and cytokines [11]. NOSs are heme-thiolate proteins which catalyze the five-electron oxidation of L-arginine (L-arg) to NO and L-citrulline (L-cit). All NOSs contain two domains separated by a CaM binding site: a C-terminal reductase domain that binds NADPH, FAD and FMN and exhibits a high sequence identity to cytochrome P450 reductases, and an N-terminal oxygenase domain that binds heme, tetrahydrobiopterin (BH_4) and L-arg [12]. In fact, NOSs catalyze three kinds of reactions: (i) the reduction of electron acceptors such as cytochrome *c* (Cyt *c*) by NADPH that only requires the reductase domain and Ca^{2+}/CaM for maximal activity, (ii) the reduction of dioxygen by NADPH and (iii) the oxidation of L-arg to NO and L-cit by NADPH and O_2 that are mediated by the heme moiety and require complete NOS [13–17]. Contrary to nNOS and eNOS, the activity of iNOS is almost independent of Ca^{2+} and CaM as it binds CaM with a very high affinity [10,11].

In this paper, we report preliminary results showing that BKT is a strong inhibitor of rat brain nNOS, mainly because of its effects on the activation of nNOS by the Ca^{2+}/CaM system. Accordingly, it is a much less potent inhibitor of murine macrophage inducible iNOS. These results could explain some of the therapeutic effects of BKT.

2. Materials and methods

2.1. Chemicals

[2,3,4,5-^{3}H]L-Arginine hydrochloride (300 mCi/mmol) was purchased from Dupont-NEN. NADPH came from Boehringer and tetrahydrobiopterin from Alexis Biochemicals. Dowex 50W-X8 was from Aldrich. L-Citrulline, L-arginine hydrochloride, N^{ω}-nitro-L-arginine (NO_2Arg), dithiothreitol (DTT), calmodulin, bromocriptine, ergotamine and all other chemicals were purchased from Sigma Chemicals. RPMI 1640 and fetal calf serum (FCS) came from Gibco. *Iso*BKT was prepared from BKT according to a described procedure [18].

2.2. Enzyme preparation

Recombinant rat brain NOS (nNOS) was obtained from yeast *Saccharomyces cerevisiae* strain WR *fur1* transfected with a gene encoding the rat brain isoform following recently described procedures [19]. The A379 plasmid carrying the rat brain NOS cDNA was a generous gift from Prof. P.O. De Montellano (University of California, San Francisco, CA, USA). The culture procedures used to achieve nNOS expression in yeast are identical to those described in Sari et al. [19]. Neuronal NOS was purified through CaM-agarose affinity column chromatography (Pharmacia) as described [20] and was buffer-exchanged through a Sephadex G-50 column to 50 mM HEPES pH 7.5 just before use. It appeared 95% pure from SDS-PAGE stained with Coomassie blue and displayed average K_m and V_m values of 8.0 ± 2.0 μM and 350 ± 50 nmol L-cit min^{-1} mg $protein^{-1}$ respectively.

Murine macrophages were obtained from C3H/HeN mice injected intraperitoneally with thioglycolate broth (Institut Pasteur) 3 days before harvesting the cells. Adherent macrophages (3×10^6/ml in RPMI supplemented with 10% FCS) were incubated for 18 h at 37°C with 10 ng/ml LPS under a 5% CO_2 humidified atmosphere. Activated macrophages were washed twice and put in fresh medium containing 2 mM L-arg and various concentrations of the studied compounds. Nitrite production [21] and cell viability [22] were meas-

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Abbreviations: BKT, bromocriptine; CaM, calmodulin; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; L-arg, L-arginine; L-cit, L-citrulline; BH_4, tetrahydrobiopterin; NO_2Arg, N^{ω}-nitro-L-arginine; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; Cyt *c*, cytochrome *c*; LPS, lipopolysaccharide; FCS, fetal calf serum

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ured 24 h later following previously described methods. Inducible NOS (iNOS) was obtained from about 10^8 peritoneal murine macrophages and purified as described [23] onto a 2′,5′-ADP-agarose affinity column (Pharmacia).

Protein concentrations were determined by the Bradford protein assay kit (Bio-Rad) using bovine serum albumin as standard.

2.3. NOS activity assays

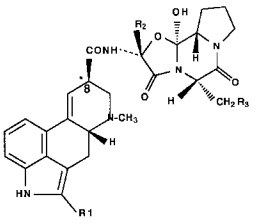
2.3.1. [^{3}H]L-Citrulline formation. NOS-dependent oxidation of L-arg to L-cit was determined according to a previously described technique [24]. Briefly, enzymatic reactions were conducted at 37°C for 5 min in 50 mM HEPES (pH 7.5) containing 5 mM DTT, 50 μM L-arg, about 500 000 cpm [2,3,4,5-^{3}H]L-arg, 1 mM NADPH, 1 mM $CaCl_2$, 10 μg/ml CaM, 20 μM BH_4, 4 μM FAD, 4 μM FMN, and the tested agents as indicated. Final incubation volumes were 100 μl. The reactions were started by the addition of protein and terminated by the addition of 500 μl of cold stop buffer (20 mM sodium acetate pH 5.5, 1 mM L-cit, 2 mM EDTA, and 0.2 mM EGTA). Samples (500 μl) were applied to columns containing 1 ml of Dowex AG 50W-X8 (Na^+ form, prepared from the H^+ form), pre-equilibrated with stop buffer and a total of 1.5 ml of stop buffer was added to eluate [^{3}H]L-cit. Aliquots were then counted on a Packard Tri-Carb 2300 liquid scintillation spectrometer. Control samples without nNOS, CaM, or NADPH were included for background determinations. Activity of iNOS was determined similarly but $CaCl_2$ and CaM were omitted.

2.3.2. NADPH consumption by nNOS. The initial rates of NADPH oxidation were quantitated spectrophotometrically at 340 nm using an extinction coefficient of 6.2 mM^{-1} cm^{-1} [14]. Cuvettes contained 1 ml of 50 mM HEPES, pH 7.4, 5 mM DTT, 1 mM $CaCl_2$, 500 μM NADPH, 4 μM FAD, 4 μM FMN, 20 μM BH_4, and, when required, L-arg (1 mM), CaM (10 μg/ml) and the tested compounds. Incubations were run for 5 min at ambient temperature on a Kontron 941 spectrophotometer and were initiated by the addition of nNOS.

2.3.3. Cytochrome c reductase activity of nNOS. The initial rates of flavin dependent reduction of Cyt *c* by nNOS were quantitated spectrophotometrically at 550 nm, using an extinction coefficient of 21 mM^{-1} cm^{-1} [14]. The reaction mixture (final volume 1 ml) contained 50 mM HEPES pH 7.5 without DTT, 2 mM $CaCl_2$, 100 μM NADPH and 40 μM Cyt *c*. Some assays contained CaM (10 μg/ml) and the studied compounds as indicated. The reactions were run for 5 min and were initiated by the addition of nNOS.

3. Results

BKT produced a concentration-dependent inhibition of the oxidation of [^{3}H]L-arg by NADPH and O_2 to [^{3}H]L-cit catalyzed by recombinant nNOS purified from yeast expressing rat brain NOS (Fig. 2). As expected, this nNOS activity was suppressed in the absence of CaM or in the presence of 25 μM NO_2Arg, a well-known NOS inhibitor [9] (Table 1). IC_{50} values of 10.0 ± 2.0 μM were obtained for BKT (Fig. 2). Interestingly, the IC_{50} values were found to be dependent upon CaM concentrations (IC_{50} = 7.0 ± 1.0 μM, 10.0 ± 2.0 μM, and 32.0 ± 2.0 μM when using 1, 10 and 50 μg/ml CaM, respectively). In addition, *iso*BKT, the C-8 epimer of BKT (Fig. 1), was a less potent inhibitor than the parent BKT, with an IC_{50} value of 35 ± 8 μM (Fig. 2). Another ergot alkaloid, ergotamine, was less active (IC_{50} = 50 ± 8 μM) (Table 1).



Bromocriptine (BKT)	R_1 = Br	R_2 = $CH(CH_3)_2$	R_3 = $CH(CH_3)_2$
iso Bromocriptine (*iso* BKT)	R_1 = Br	R_2 = $CH(CH_3)_2$	R_3 = $CH(CH_3)_2$
Ergotamine	R_1 = H	R_2 = CH_3	R_3 = C_6H_5

Fig. 1. Structures of BKT, *iso*BKT and ergotamine.

The inhibitory effects of BKT towards iNOS purified from mouse macrophages were also investigated. BKT weakly inhibited L-cit formation catalyzed by purified iNOS (about 20% inhibition at 100 μM); moreover, BKT was almost without effect on the formation of nitrite by LPS-stimulated mouse macrophages (less than 10% inhibition at 100 μM), whereas NO_2Arg strongly inhibited both iNOS activities (Table 1). These results indicate a clear selectivity of BKT for nNOS (IC_{50} more than 10 times lower for nNOS than for iNOS).

Double-reciprocal plots of the nNOS activity ([^{3}H]L-cit formation from [^{3}H]L-arg) as a function of L-arg concentration showed that increasing concentrations of BKT caused a decrease of the V_m value without changing the K_m value of L-arg for nNOS (Fig. 3, for K_m and V_m values, see Section 2). These results suggest that BKT was not a competitive inhibitor of the nNOS-dependent oxidation of L-arg. The lack of interaction of BKT at the L-arg binding site was confirmed by spectral studies showing that addition of increasing amounts (up to 50 μM) of BKT to nNOS failed to lead to any change of the visible spectrum of the heme. Furthermore, addition of BKT did not modify the K_s value of L-arg for nNOS (data not shown).

As previously reported, nNOS catalyzed the oxidation of NADPH both in the presence and in the absence of L-arg, the

Table 1
Effect of BKT, *iso*BKT and ergotamine on the formation of [^{3}H]L-cit from [^{3}H]L-arg catalyzed by purified nNOS and iNOS, and on nitrite formation from LPS-activated murine macrophages

	Activity (% C.S.)[a]		
	nNOS	iNOS	mMϕ[b]
Complete system (C.S.)	100[c]	100[d]	100[e]
−CaM	< 1	nd[f]	nd
+NO_2Arg (100 μM)	< 1	5 ± 2	17 ± 3
+BKT (25 μM)	31 ± 8	92 ± 7	97 ± 7
+BKT (100 μM)	11 ± 3	80 ± 6	93 ± 7
+*iso*BKT (25 μM)	53 ± 5	85 ± 6	nd
+Ergotamine (25 μM)	81 ± 10	nd	nd

[a][^{3}H]L-cit formation was measured as described in Section 2. Results are expressed as means ± S.E.M. from 4–6 experiments.
[b]mMϕ: LPS-activated murine macrophages. Specific activities for purified NOSs: [c]nNOS and [d]iNOS: 350 ± 50 and 80 ± 20 nmol L-cit min^{-1} mg $protein^{-1}$ respectively.
[e]Nitrite formation from LPS-stimulated murine macrophages: 35 ± 3 μmol/3 × 10^6 cells/18 h.
[f]Not determined.

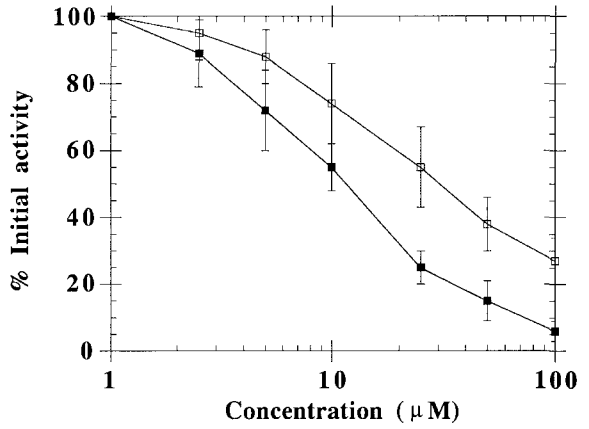


Fig. 2. Effect of increasing concentrations of BKT and *iso*BKT on [^{3}H]L-cit formation catalyzed by purified nNOS. Rates of oxidation of [^{3}H]L-arg to [^{3}H]L-cit were measured as described in Section 2 in the presence of increasing concentrations of BKT (■) or *iso*BKT (□). Activity in the absence of BKT (100%) was 350 ± 50 nmol. L-cit min^{-1} mg protein^{-1}. Results are means ± S.E.M. from 4 experiments.

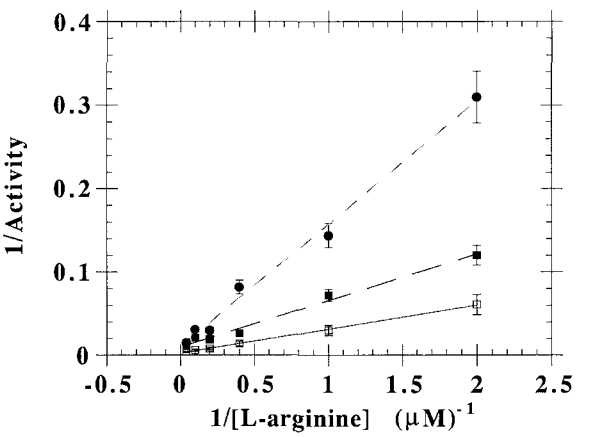


Fig. 3. Double reciprocal plot of the nNOS activity ([^{3}H]L-cit formation) as a function of L-arg concentrations, in the presence of various concentrations of BKT. Oxidation of [^{3}H]L-arg to [^{3}H]L-cit was measured as described in Section 2 in the absence (□) or in the presence of 10 (■) or 25 μM (●) BKT. Activity expressed in nmol L-cit min^{-1} mg protein^{-1}. Results are means ± S.E.M. from 3 determinations.

rate being two-fold higher in the absence of L-arg (Fig. 4) [15]. This reaction did not occur in the absence of CaM and was greatly inhibited by NO_2Arg (100 μM). BKT (10 and 50 μM) inhibited the L-arg-dependent (Fig. 4, hatched bars) and the L-arg-independent (Fig. 4, open bars) oxidation of NADPH to almost similar extents. These results further confirmed that BKT did not simply interact with nNOS at the L-arg binding site.

The hypothesis of an interaction of BKT with the CaM-dependent activation of nNOS was then tested by studying the effects of BKT on the nNOS-catalyzed reduction of Cyt *c* by NADPH. This reduction is much faster in the presence of CaM [14–17] as shown by the 15-fold stimulation of the activity (2.90 ± 0.3 μmol instead of 0.18 ± 0.03 μmol reduced Cyt *c* min^{-1} mg protein^{-1}) (Fig. 5). BKT only slightly decreased the CaM-independent activity whereas it strongly inhibited the CaM-dependent nNOS-catalyzed reduction of Cyt *c* (about 80% inhibition at 50 μM, Fig. 5). These results clearly show that BKT interfered with the CaM-dependent electron transfer from NADPH via the nNOS reductase domain.

4. Discussion

The present results show that BKT, and to a lesser extent *iso*BKT as well as some other ergot alkaloids (Boucher et al., in preparation), inhibits the various activities of nNOS. BKT inhibits the nNOS-dependent oxidation of L-arg (NO formation) with an IC_{50} value of 10 ± 2 μM, a concentration of pharmacological relevance, mainly because it interferes with the necessary activation of nNOS by CaM. This explains the much higher inhibitory effects of BKT towards nNOS compared to iNOS (either purified or in LPS-stimulated mouse macrophages), as iNOS is more tightly bound to CaM [11]. BKT could thus be compared to calmidazolium, to the antipsychotics trifluoperazine and chlorpromazine (IC_{50}: 2, 7 and 15 μM respectively) [25] and to the neuroleptic haloperidol (IC_{50}: 31 μM) [26] which are potent CaM antagonists. These results reinforce previous studies which have suggested interactions of BKT and other ergot alkaloids with CaM-dependent proteins [1,6].

The strong inhibitory effects of BKT towards nNOS could be, at least in part, at the origin of some therapeutic effects of this drug. Protective effects of BKT in patients suffering from Parkinson's disease could be related to its ability to inhibit nNOS-dependent formation of NO, $O_2^{\cdot-}$ and H_2O_2 as well as of peroxynitrite, a product arising from reaction of NO with $O_2^{\cdot-}$ which is involved in neurodegenerative diseases [27,28]. As far as the anti-migraine effects of BKT are involved, it has recently been shown that NO plays a key role in migraine [29,30]. Inhibition of nNOS-dependent formation of NO could thus participate in the anti-migraine effects observed with BKT and other ergot alkaloids. Finally, concerning the effects of BKT on prolactin secretion, it is noteworthy that several studies have established a link between NO and pro-

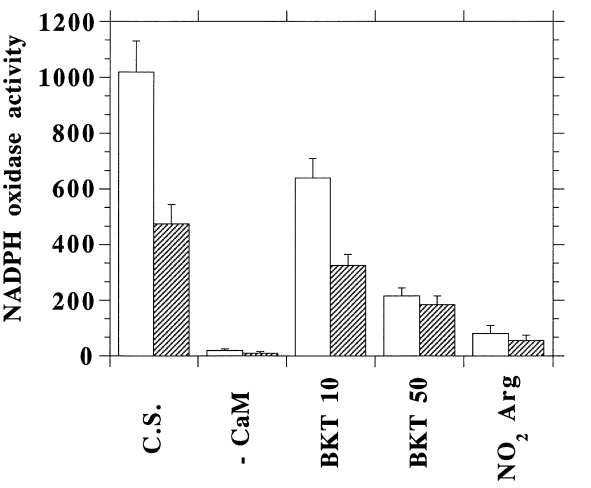


Fig. 4. Effect of CaM, BKT and NO_2Arg on NADPH oxidation catalyzed by purified nNOS. NADPH oxidation was assayed as described in Section 2. C.S.: complete system. Maximal activities were 1020 ± 110 and 475 ± 70 nmol NADPH consumed min^{-1} mg protein^{-1} in the absence (open bars) and in the presence of L-arg (1 mM, hatched bars). Experiments were performed in the absence of CaM (−CaM), in the presence of 10 or 50 μM BKT, or 100 μM NO_2Arg. Results are means ± SEM from 3 determinations.

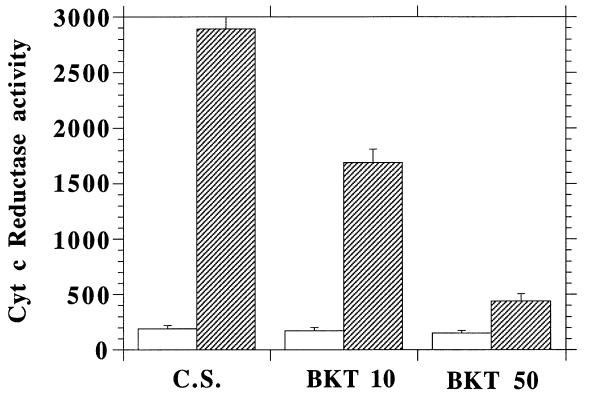


Fig. 5. Effects of BKT on the reduction of Cyt *c* by NADPH catalyzed by purified nNOS. Cyt *c* reduction was assayed as described in Section 2 without CaM (open bars) or in the presence of 10 μg/ml CaM (hatched bars). C.S.: complete system without BKT. BKT 10 or 50: C.S. contained 10 or 50 μM BKT. Activities in nmol Cyt *c* reduced min^{-1} mg $protein^{-1}$. Results are means ± S.E.M. from 3 determinations.

lactin generation. For instance, treatments of rats with NO donor such as SIN-1 have recently been found to lead to an increase in plasma prolactin levels, whereas treatment with NO_2Arg completely suppressed the steroid-induced prolactin surge in rat [31–33]. It is thus tempting to propose that the action of BKT on prolactin secretion could be mediated by NO, or a NOS-derived product. In addition, some side effects of BKT such as post-partum hypertension or digestive diseases [1] could be related to the effects of well known nNOS inhibitors, like NO_2Arg [9].

The relationships between the therapeutic effects of ergot alkaloids and NO formation require further detailed pharmacological investigations.

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