Biochemical Pharmacology, Vol. 31, No. 22, pp. 3693–3694, 1982.
Printed in Great Britain.

0006-2952/82/223693-02 $03.00/0

The effects of (−)*N*-(chloroethyl)-norapomorphine on [^{3}H]-apomorphine binding to striatal dopamine receptors

(*Received* 1 *April* 1982; *accepted* 12 *May* 1982)

Recently a novel aporphine derivative, (−)*N*-(chloroethyl)-norapomorphine [(−)NCA], has been shown irreversibly to inhibit striatal dopamine function [1]. This inhibition seemed to involve covalent bonding of (−)NCA to the dopamine receptor and was analogous to that exhibited by phenoxybenzamine at the noradrenaline receptor [1]. (−)NCA has also been shown to inhibit selectively the binding of [^{3}H]-*N*-*n*-propylnorapomorphine to striatal tissue [2]. Since [^{3}H]-apomorphine labels several classes of dopamine receptor [3], the effect of (−)NCA on the binding of this ligand has now been investigated.

Materials and methods

L-(−)-[8,9-^{3}H]-Apomorphine (22.3 Ci/mmole) was obtained from New England Nuclear (Boston, MA). The drugs, domperidone and (+)-butaclamol were a kind gift from Janssen Pharmaceutical Ltd. and Ayerst Laboratories Ltd., respectively. (−)*N*-(Chloroethyl)-norapomorphine hydrochloride [(−)NCA] was obtained from Research Biochemicals Incorporated (Wayland, MA) and solutions ("Tris-Ions" buffer containing 0.1% ascorbic acid) were prepared daily. 'Fisofluor 1' was obtained from Fisons Scientific Apparatus (Loughborough, Leics).

Preparation of particulate fraction for binding. Male Wistar rats (200–250 g) were killed by decapitation. The dissected striata (45–55 mg) were homogenised (20 mg tissue/ml) in 50 mM Tris-HCl (pH 7.4), diluted four times and centrifuged at 17,000 *g* at 4° for 20 min. The pellet was resuspended in Tris-buffer at 4 mg tissue/ml, centrifuged again at 17,000 *g* at 4° for 20 min and the pellet resuspended in 50 mM "Tris-Ions" buffer [4].

[^{3}*H*]-*Apomorphine binding.* Apomorphine binding was measured as follows: 200 μl fresh (<2 hr) tissue suspension and 100 μl of drugs dissolved in "Tris-Ions" buffer (0.1% fresh ascorbic acid) were pre-incubated for 3 min at 37°. Incubations for 10 min at 37°, were initiated by addition of 200 μl [^{3}H]-apomorphine (1 nM) and stopped by dilution with 2.5 ml "Tris-Ions" buffer at 4°, followed by rapid filtration under vacuum through Whatman GF/B filters. Assay tubes were rinsed twice with 2.5 ml "Tris-Ions" buffer at 4° and the filters washed with this buffer. The filters were dried and the radioactivity was counted by liquid scintillation spectrophotometry in 9.0 ml 'Fisofluor 1' at 40% efficiency.

Results and discussion

Specific binding of increasing concentrations of [^{3}H]-apomorphine was saturable and displayed a high affinity (K_d = 7.0 ± 2 nM) and a maximum binding capacity of 214 ± 39 fmol/mg protein, in agreement with previously reported values [4, 5]. Binding of [^{3}H]-apomorphine was inhibited by (+)-butaclamol as reported previously [4, 5], allowing specific binding to be defined as the excess over the blank values obtained in the presence of 10 μM (+)-butaclamol and which accounted for 55% of total binding. This specific binding could be further characterized as being sensitive to domperidone (40%) and domperidone-insensitive (60%) at a concentration of 1 μM. The specific binding of 1 nM [^{3}H]-apomorphine, in the presence of increasing concentrations of domperidone, was inhibited in a biphasic manner with a plateau formed between domperidone concentrations of 300 nM and 10 μM (Mitchell *et al.* in preparation). This allowed two distinct classes of [^{3}H]-apomorphine binding sites to be distinguished, as previously described by Sokoloff *et al.* [4]; one class (termed Class I binding sites) having a high affinity for domperidone (IC_{50} = approx. 100 nM), the other (termed Class II binding sites) having a low affinity for domperidone (IC_{50} = approx. 50 μM). For a typical experiment the values obtained were: 1630 dpm (total binding), 1158 dpm (in the presence of 1 μM domperidone) and 740 dpm (in the presence of 0.1 μM (+)-butaclamol).

[^{3}H]-Apomorphine binding to the domperidone-sensitive class of binding sites (Class I) was inhibited by (−)NCA, with 100% inhibition occurring at 3 μM (Fig. 1). In comparison, [^{3}H]-apomorphine binding to the domperidone-insensitive class of binding sites (Class II) was not significantly inhibited at this concentration, significant inhibition only occurring at 10 μM (−)NCA with 100% inhibition at approximately 25 μM. Thus (−)NCA selectively inhibits [^{3}H]-apomorphine binding at domperidone-sensitive sites (Class I) but has relatively little effect on [^{3}H]-apomorphine binding at domperidone-insensitive sites (Class II). The inhibition of (+)-butaclamol-insensitive binding was unaffected by concentrations of (−)NCA up to 30 μM (Fig. 1).

On the basis of drug selectivity and kainate lesions, Sokoloff *et al.* [4] have proposed that the [^{3}H]-apomorphine Class I binding sites which show a high affinity for domperidone and apomorphine (IC_{50} = 5 nM) should be termed

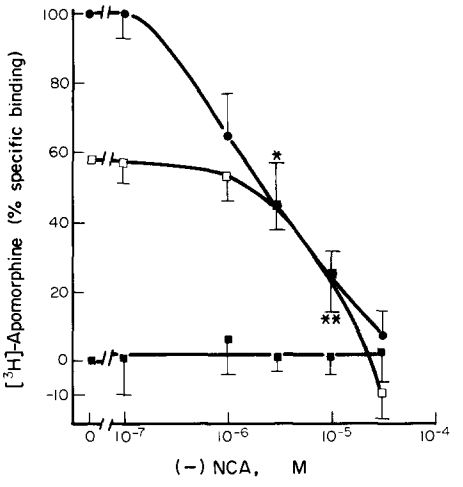

Fig. 1. Inhibition of [^{3}H]-apomorphine binding to a striatal particulate fraction. The tissue was incubated with 1 nM [^{3}H]-apomorphine and total specific binding was measured with increasing concentrations of (−)NCA (●), as well as in the presence of 1 μM domperidone (□) or 0.1 μM (+)-butaclamol (■). Values represent the means ± S.E.M. of 3–7 independent experiments (duplicate determinations in each). *Specific binding was significantly inhibited ($P < 0.005$) by 3 μM (−)NCA compared to specific binding with 0.1 μM (−)NCA. **Specific binding, in the presence of 1 μM domperidone, was significantly inhibited ($P < 0.01$) by 10 μM (−)NCA compared to specific binding with 0.1 μM (−)NCA.

D-2 receptors whilst Class II binding sites which are poorly recognised by domperidone ($IC_{50} = 10\ \mu M$) should be termed D-3 receptors. Labelling of D-1 receptors is not thought to be involved in [^{3}H]-apomorphine binding studies at nanomolar concentrations, because the recognition sites of the dopamine-sensitive adenylate cyclase are stimulated by micromolar concentrations of dopaminergic agonists [6]. Nevertheless, the proposal that the two classes of binding site labelled by [^{3}H]-apomorphine represent distinct dopamine receptors must be regarded with caution. Since (−)NCA can selectively inhibit binding of [^{3}H]-apomorphine to Class I binding sites, the use of (−)NCA as a selective irreversible antagonist at D-2 receptors may provide an important biochemical and pharmacological tool to help clarify the present confusion regarding multiple subtypes of dopamine receptor.

In summary, the novel aporphine (−)NCA, appears selectively to inhibit binding of [^{3}H]-apomorphine to domperidone-sensitive binding sites (Class I) which probably represent D-2 receptors, in rat striatal tissue.

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Acknowledgements—M. W. Goosey is a Parkinson's Disease Research Fellow. The authors would like to thank Dr. R. Mitchell for helpful discussions.

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Biochemical Pharmacology, Vol. 31, No. 22, pp. 3694–3697, 1982.
Printed in Great Britain.

0006-2952/82/223694-04 $03.00/0

Effects of oxygen tension and reducing agents on sensitivity of *Giardia lamblia* to metronidazole *in vitro*

(*Received* 10 *March* 1982; *accepted* 17 *May* 1982)

Metronidazole [Flagyl; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is one of the most important drugs used in the treatment of giardiasis [1]. Nonetheless, there are few *in vitro* studies of the metronidazole sensitivity of the causative agent, the flagellated protozoan *Giardia lamblia* [2–4], and the mechanism of killing of *Giardia* by this drug is not known. Metronidazole is highly specific for anaerobic organisms—both prokaryotic and eukaryotic. Trichomonads (e.g. *Trichomonas foetus*, a parasite of cattle, and the human pathogen *Trichomonas vaginalis*), as well as susceptible bacteria, reduce metronidazole to a toxic form via certain low redox potential reactions which are either absent or unimportant in aerobic organisms [5, 6]. Reduction of metronidazole decreases the intracellular concentration of the unchanged drug, creating a concentration gradient which drives its uptake [7]. The cellular target of the reduced metronidazole has not been positively identified.

The uptake of radioactive metronidazole by *T. foetus*, *T. vaginalis* and *Entamoeba invadens* (a pathogen of carnivorous reptiles) was strongly inhibited under aerobic conditions [6]. Oxygen competes with metronidazole for the electrons necessary for reduction. Killing of *T. foetus* was related to the amount of drug taken up; higher drug concentrations were required for killing under aerobic conditions [6].

It is likely that a similar reduction-driven uptake and activation mechanism is responsible for the sensitivity of *G. lamblia* to metronidazole. If so, killing of the parasite should be decreased under aerobic conditions. This has not been tested previously. Test conditions must be selected to avoid killing of *G. lamblia* by prolonged exposure to atmospheric concentrations of O_2 [8]. We have shown recently that this parasite tolerates exposure to O_2 for up to 8 hr (without growth) [8]. Thus, it is possible to examine the sensitivity of *G. lamblia* to metronidazole during shorter exposure to aerobic conditions. In this report, metronidazole sensitivity was measured with a routinely isolated strain [9] as well as a strain of *G. lamblia* recently isolated from a patient who had repeatedly been treated unsuccessfully with metronidazole [10].

Methods

Giardia lamblia Portland-1 strain [9] (PO; American Type Culture Collection No. 30888) was obtained from Drs. G. Visvesvara [11] and G. Healy (Center for Disease Control, Atlanta, GA). The WB strain (ATCC No. 30957) was isolated in this laboratory from duodenal fluid of a 27-year-old male with symptomatic giardiasis of 2.5-years duration despite four courses of metronidazole and three courses of quinacrine therapy [10].

Trophozoites were grown axenically (with subculture twice weekly) to log phase in filter-sterilized TYI-S-33 medium [12] modified by the addition of bovine bile (bacteriological, 500 μg/ml; Sigma Chemical Co., St. Louis, MO), and doubling the L-cysteine concentration (D. B. Keister, manuscript in preparation). Parasites were enumerated with a Coulter Counter (Coulter Electronics, Hialeah, FL).

Metronidazole sensitivity. Metronidazole was a gift of G. D. Searle & Co. (San Juan, Puerto Rico). Two methods of determining the metronidazole sensitivity of *G. lamblia*