Pergamon

0960-0760(94)E0062-P

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 4–6, pp. 385–389, 1994
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0960-0760/94 $7.00 + 0.00

The Pharmacology of Neurosteroidogenesis

E. Costa*, J. Auta, A. Guidotti, A. Korneyev and E. Romeo

Fidia Georgetown Institute for the Neurosciences, Georgetown University Medical School, 3900 Reservoir Road, N.W., Washington, DC 20007, U.S.A.

In adrenal cortex and other steroidogenic tissues including glial cells, the conversion of cholesterol into pregnenolone is catalyzed by the cytochrome $P450_{scc}$ located in the inner mitochondrial membrane. A complex mechanism operative in regulating cholesterol access to $P450_{scc}$ limits the rate of pregnenolone biosynthesis. Participating in this mechanism are DBI (diazepam binding inhibitor), an endogenous peptide that is highly expressed in steroidogenic cells and some of the DBI processing products including DBI 17–50 (TTN). DBI and TTN activate steroidogenesis by binding to a specific receptor located in the outer mitochondrial membrane, termed mitochondrial DBI receptor complex (MDRC). MDRC is a hetero-oligomeric protein: only the subunit that includes the DBI and benzodiazepine (BZD) recognition sites has been cloned. Several 2-aryl-3-indoleacetamide derivatives (FGIN-1-X) with highly selective affinity (nM) for MDRC were synthesized which can stimulate steroidogenesis in mitochondrial preparations. These compounds stimulate adrenal cortex steroidogenesis in hypophysectomized rats but not in intact animals. Moreover, this steroidogenesis is inhibited by the isoquinoline carboxamide derivative PK 11195, a specific high affinity ligand for MDRC with a low intrinsic steroidogenic activity. Some of the FGIN-1-X derivatives stimulate brain pregnenolone accumulation in adrenalectomized–castrated rats. The FGIN-1-X derivatives that increase brain pregnenolone content, elicit antineophobic activity and antagonize punished behavior in the Vogel conflict test in rats. These actions of FGIN-1-X are resistant to inhibition by flumazenil, a specific inhibitor of BZD action in $GABA_A$ receptors but are antagonized by PK 11195, a specific blocker of the steroidogenesis activation via MDRC stimulation. It is postulated that the pharmacological action of FGIN-1-X depends on a positive modulation of the GABA action on $GABA_A$ receptors mediated by the stimulation of brain neurosteroid production.

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INTRODUCTION

Diazepam binding inhibitor (DBI) is an endogenous peptide, endowed with multiple biological actions that is present in brain, in endocrine steroidogenic tissues, and in several other organs (liver, spleen, kidney, etc.). DBI was first purified from rat brain by monitoring the peptide ability to displace [^{3}H]diazepam specifically bound to high affinity recognition sites located in crude synaptic membranes [1]. The peptide name derives from the technology used for its purification and the first biological activity that was investigated was the ability of DBI to mimic the action of diazepam on γ-aminobutyric acid (GABA) receptors. However, these experiments revealed that DBI is a negative modulator of the GABA gating of the Cl^- channels present in $GABA_A$ receptors [2]. This action was inhibited by flumazenil, the specific antagonist of molecules acting either as negative (β-carboline) or as positive (diazepam) allosteric modulators of GABA action on $GABA_A$ receptors by binding in the ectodomain of these receptors [2]. Several DBI fragments were sequenced [3] and the complete aminoacid sequence of DBI was deduced from its cDNA isolated and sequenced with recombinant technology [4, 5]. Another important biological action of DBI and of its processing product DBI 17–50 (TTN) was detected using mitochondria suspensions prepared from primary astroglia cultures of rat cerebellum. DBI or TTN in concentrations 10^{-8} M activate steroidogenesis in this subcellular preparation [6]. $GABA_A$ receptors are hetero-oligomeric integral membrane proteins that possess two important modulatory centers. One, located in the $GABA_A$ receptor ectodomain, can be activated or inhibited by anxiolytic or anxiogenic compounds, respectively. They stabilize one of two

Proceedings of the XVI Meeting of the International Study Group for Steroid Hormones, Vienna, Austria, 28 Nov.–1 Dec. 1993.
*Correspondence to E. Costa.

structural conformations of this center, bringing about positive or negative modulation of the GABA action on $GABA_A$ receptors, respectively. Anxiolytic compounds (benzodiazepines, BZDs) positively modulate and anxiogenic compounds (β-carbolines, DBI) negatively modulate GABA action on $GABA_A$ receptors [7]. The action of both classes of drugs is inhibited by flumazenil, suggesting that the allosteric modulatory center continually oscillates between the two conformational states and that the stabilization of one of the two states by specific drugs brings about opposite phenomenological responses. The other allosteric modulatory center of $GABA_A$ receptors is located in the $GABA_A$ receptor transmembrane domain and this center can also be positively or negatively modulated by specific steroids that were termed neurosteroids [8]. Steroid hormones can modify behavior by two main mechanisms; the first is related to their action on DNA transcription and these behavioral changes usually have a long latency time and endure for several hours or days, the second class of behavioral changes induced by steroids has a rapid onset and relates to a synaptic modification. The steroids that elicit this rapid synaptic modification were termed neurosteroids and include 3α-hydroxy-5-α-pregnan-20-one (allopregnanolone) and 3α-21-dihydroxy-5α-pregnan-20-one (THDOC) acting as positive allosteric modulators of GABA action and $GABA_A$ receptors and pregnenolone and dehydroepiandrosterone sulfates acting as negative allosteric modulators of GABA action and $GABA_A$ receptors. While the allosteric modulatory efficacy of BZDs acting in the receptor ectodomain differ according to the structural diversity of $GABA_A$ receptors [9], neurosteroid modulation is much less dependent on $GABA_A$ receptor structure diversity [10]. Moreover neurosteroids, similarly to barbiturates, can operate $GABA_A$ receptor channels in the absence of GABA [8] whereas BZDs and other anxiolytics acting on the $GABA_A$ receptor ectodomain not only require GABA for their action but their maximal modulatory efficacy never surpasses the maximal response elicited by GABA in the same receptor channel. Thus, BZDs which cannot control channel gating in the absence of GABA do not elicit anesthetic activity while neurosteroids, which like barbiturates can gate channels in the absence of GABA, can cause anesthesia.

MITOCHONDRIA DBI RECEPTORS AND NEUROSTEROIDOGENESIS

The discovery of specific recognition sites for anxiolytic drugs in brain synaptic membranes [11] was followed by the discovery of a second class of BZD recognition sites which, because they were discovered in peripheral tissues, were called peripheral BZD recognition sites [12]. However almost immediately it was recognized that BZD binding sites of the peripheral type were also located in brain and were predominantly located on outer mitochondrial membranes [13, 14], thus providing an important criterion to study their biological function [6]. DBI has been proposed by several laboratories to act as a putative endogenous ligand for the BZD recognition sites on mitochondria. Hence to avoid the ambiguities connected with the putative physiological function of a drug receptor, we have termed this receptor, mitochondrial DBI receptor complex (MDRC) [15]. This receptor also binds imidazopyridines (alpidem), several isoquinoline carboxamides [17, 18] and with great specificity, a new class of 2-aryl-3-indoleacetamides [19]. Though BZD recognition sites on $GABA_A$ receptors and MDRC mutually bind a number of drugs, these binding sites occur in structurally different proteins. Moreover isoquinoline carboxamides and 2-aryl-3-indoleacetamides bind exclusively to MDRC. Evidence from several laboratories suggests that a 18 kDa protein constitutes the drug binding domain of MDRC [20]. Moreover this protein fails to show a significant homology to the various α subunits of $GABA_A$ receptors where the BZD binding site is located. Thus from a pharmacological stand point one can propose that the ligands to MDRC may possess a unique pharmacological profile depending on MDRC function. It is necessary here to repeat that MDRC is a hetero-oligomeric integral membrane protein and that it may include other subunits besides the 18 kDa protein mentioned above; these proteins are now being studied in our and other laboratories.

MDRCs are present in various tissues but their density varies more than 100-fold among mitochondria prepared from various tissues. The highest density is in adrenal cortex and Leydig cells, where MDRC accounts for 0.2% of the mitochondrial protein. The MDRC abundance in steroidogenic tissues indirectly suggests that this protein may have a role in the production of steroids. In the mitochondrial inner membrane is located the cytochrome $P450_{scc}$ which catalyzes the conversion of cholesterol into pregnenolone, the parent compound of every steroid that functions in mammalian tissues. But the rate limiting step in steroidogenesis is not the catalytic rate of $P450_{scc}$ but very likely a facilitation of the translocation of cholesterol from outer to inner mitochondrial membranes. Via such a mechanism pituitary tropic hormones appear to stimulate steroid production in target organs. The molecular mechanisms that operate in such a facilitation of cholesterol translocation in mitochondria are not completely understood but the study of MDRCs poses some interesting possibilities for future progress. When a battery of MDRC ligands ranging by four orders of affinity for this receptor were tested on the Y-1 adrenocortical and MA 10 Leydig cell lines [21], their steroidogenic potencies correlated very closely to their affinities to MDRC ($r = 0.98$). Furthermore similar results were observed in rat and bovine adrenocortical cells [21] and rat Leydig cells [22]. Also in rat glioma C6-2B MDRC was reported to be

functioning in steroidogenesis [15]. The question then arises whether drugs that stimulate steroidogenesis in isolated mitochondria in cell lines or in primary cell cultures are also capable of activating steroidogenesis *in situ* in the brains of living animals.

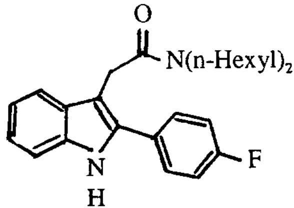
ROLE OF DBI AND MDRC IN STEROIDOGENESIS *IN VIVO*

The protein synthesis inhibitor cycloheximide can block the steroidogenesis elicited by ACTH at the level of cholesterol transport in mitochondria [23]. It is currently believed that one or more proteins are required to affect the cholesterol translocation to the inner mitochondrial membrane. We have contributed evidence in support of the view that ACTH stimulates the processing rate of DBI in adrenal gland and that the blockade of ACTH induced steroidogenesis by cycloheximide may result from inhibition of specific DBI processing regulated by ACTH [24]. In these experiments, we have also shown that in hypophysectomized rats 4′Cl diazepam, a MDRC agonist, stimulates adrenal steroidogenesis in a manner inhibited by PK 11195, a MDRC partial agonist with strong inhibitory activity. PK 11195 also blocks the action of ACTH indicating that the steroidogenic activity of ACTH involves MDRC and activation of DBI processing. In normal animals, the adrenocortical steroidogenic action of 4′Cl diazepam is not readily seen because the presence of pituitary ACTH appears to prevent the steroidogenic actions of MDRC agonists in adrenals.

This evidence prompted us to study whether in adrenalectomized–castrated rats it was possible to detect in brain an increase of pregnenolone accumulation elicited by MDRC agonists. The results of these experiments were reported *in extenso* [25] and are summarized in Tables 1 and 2.

With the use of adrenalectomized and castrated male rats it was demonstrated that FGIN-1-27, an agonist of MDRC that *in vitro* has a specific high affinity for this receptor [19], can elevate the brain biosynthesis of pregnenolone, the parent compound of body steroids. These data confirm previous reports on the existence in rat brain of an independent steroid biosynthesis probably generating neurosteroids [26]. The effects of FGIN-1-27 on brain pregnenolone synthesis are rapid, and when the drugs are given intravenously the peak effect occurs in 5–10 min. After MDRC receptor stimulation in rats receiving trilostane the rapidity by which pregnenolone is synthesized in brain is similar to that observed in adrenal glands of hypophysectomized rats receiving a MDRC agonist [24]. Similarly to adrenal gland also in brain PK 11195 inhibits pregnenolone synthesis stimulated by a MDRC agonist [25]. However the doses of PK 11195 that block FGIN-1-27 stimulated biosynthesis of pregnenolone fails *per se* to decrease brain pregnenolone content of adrenalectomized–castrated rats. This may indicate that either the basal turnover of brain pregnenolone may be low or that pregnenolone is rapidly converted to unknown metabolites.

Table 1. Dose dependence of FGIN-1-27[a] induced increase in pregnenolone content in cerebellum and forebrain of adrenalectomized–castrated rats

Treatment (μmol/kg, p.o.)		Pregnenolone (ng/g)	
		Cerebellum	Forebrain
Vehicle		10 ± 2.0	9 ± 1.5
FGIN-1-27	200	10 ± 2.4	7 ± 2.0
	400	18 ± 3.8*	19 ± 4.0*
	800	26 ± 7.2*	25 ± 6.5*

[a](*N,N*-di-*n*-hexyl 2-(4-fluorophenyl)indole-3-acetamide).
Rats were treated with different doses of FGIN-1-27 or vehicle by oral gavage, 1 h after treatment with trilostane (600 μmol/kg i.p.) and sacrificed 1 h later. Data are the mean ± SE of $n = 5$. Duncan's multiple range analysis indicates that the drug effect was significant ($P < 0.05$). *The effect of FGIN-1-27 at 400 and 800 μmol/kg p.o. differed from vehicle treatment $P < 0.01$.

The presence of a high density of MDRC and pregnenolone in rat olfactory bulb together with the evidence that MDRC stimulation more effectively increases steroid synthesis in this brain structure suggests that MDRC plays an important role in the regulation of steroid synthesis. In brain, the steroid synthesis does not appear to be regulated by ACTH although it may be under hormonal and/or transsynaptic control probably via a signal transduction involving adenylate cyclase stimulation. However, this regulation can be bypassed via direct MDRC agonists. These findings prompt one to evaluate whether there could be a pharmacology dependent on neurosteroidogenesis stimulation via MDRC activation.

Table 2. Time course of FGIN-1-27[a] effect on pregnenolone content in the forebrain of adrenalectomized–castrated rats

Treatment	Pregnenolone (ng/g)		
	30′	60′	120′
Vehicle	7 ± 2.0	7 ± 1.5	8 ± 1.6
FGIN-1-27 (800 μmol/kg, p.o.)	8 ± 1.8	14 ± 2.5*	17 ± 2.8*

[a](*N,N*-di-*n*-hexyl 2-(4-fluorophenyl) indole-3-acetamide).
Rats were administered FGIN-1-27 or vehicle 1 h after treatment with trilostane (600 μmol/kg i.p.) and sacrificed at the times indicated. $n = 4$. Mean ± SE. Duncan multiple range analysis indicates that FGIN-1-27 is statistically effective ($P < 0.05$)*. The effect of FGIN-1-27 at 60 and 120 min differed from vehicle $P < 0.01$.

Such a possibility is facilitated by the finding that in the endocrine systems which are directly controlled by the pituitary, the role of drugs stimulating MDRC does not appear to be appreciable as long as the pituitary function is operative. The reason for this refractoriness is not at present known.

BEHAVIORAL RESPONSES ELICITED BY MDRC AGONISTS

In the assessment of the behavioral actions related to the stimulation of brain MDRC, we have relied on a class of compounds synthesized and characterized in our institute [19, 27, 28] which are derivatives of 2-aryl-3-indoleacetamides. While we suggest that the interested reader consults the original papers on the structure–activity relationship of FGIN-1-X compounds [19, 27, 28], it is sufficient here to say that the high affinity of these compounds for MDRC depends on the following chemical characteristics: (1) the dialkylation of the amide, (2) the chain length of such alkyl substitution, and (3) the halogenation of aryl groups appended to the indole nucleus. These derivatives with nM affinity for MDRC do not bind to $GABA_A$ or $GABA_B$ receptors and to the following receptors: glycine, glutamate (AMPA, kainate or NMDA subtypes) dopamine, serotonin, opiate, cholecystokinin, β-adrenergic, cannabinoid and sigma receptors. FGIN-1–27 (for the structure see Tables 1 and 2) enters the brain and shares some pharmacological actions of THDOC and allopregnanolone which are classic neurosteroids [28]. FGIN-1–27, administered to rats, delays the onset of isoniazid induced convulsions which are due to a decrease in brain content of GABA caused by this glutamic acid decarboxylase inhibitor [19, 27]. However, FGIN-1–27 does not inhibit bicuculline convulsions because this MDRC agonist fails to prevent the isosteric inhibition of $GABA_A$ receptors. Because FGIN-1–27 acts on MDRC its action is blocked by PK 11195, the isosteric antagonist of MDRC. In contrast, the anticonvulsant actions of THDOC and allopregnanolone that derive from the positive allosteric modulation of GABA action via the allosteric modulatory center of $GABA_A$ receptors, are not blocked by PK 11195. Hence, these findings suggest that the anticonvulsant action of FGIN-1–27 can be considered to be due to an indirect positive activation of the modulatory center of $GABA_A$ receptor from the neurosteroids that are presumably produced in higher amounts in the brain of rats receiving FGIN-1–27. In the elevated plus maze test FGIN-1–27 inhibits neophobia in a manner that is antagonized by PK 11195 but not by flumazenil. This selective antagonism of the FGIN-1–27 action strongly indicates that this compound does not bind to any of the two modulatory centers that are located on $GABA_A$ receptors but presumably affects the steroid specific modulatory center of $GABA_A$ receptor via an activation of neurosteroidogenesis [19, 27, 28].

This hypothesis is supported by experiments with the conflict test according to Vogel [27] using thirsty rats. In these experiments, FGIN-1–27 acts as an anticonflict agent with a threshold dose of 5.7 μmol/kg, i.v. in a manner that is inhibited by PK 11195 (2.1 or 4.2 μmol/kg, i.v.) (Table 3), but not by flumazenil [27]. Neither flumazenil nor PK 11195 inhibit the anticonflict action of THDOC in the Vogel test, thereby confirming that the action of FGIN-1–27 may be mediated via MDRC activation [27]. FGIN-1–27 increases corticosterone concentrations in adrenal cortex of hypophysectomized rats in a manner that is inhibited by PK 11195, but fails to increase adrenal corticosterone in sham operated rats [28]. Since the antineophobic action of FGIN-1–27 was also evident in adrenalectomized–castrated rats, one can conclude that the action of FGIN-1–27 is related to brain neurosteroidogenesis stimulation [28].

We are not sure in which cells the increase of brain steroidogenesis takes place. Our present working hypothesis is that it may take place in specialized glia cells in which DBI and MDRC are coexpressed.

CONCLUSION

The data reported in this paper represents compelling evidence that the drugs that selectively stimulate MDRC elicit a pharmacologic profile that can be attributed to an indirect action on receptors located in $GABA_A$ structures that are the site of action of neurosteroids. These experiments support the idea that MDRC selective agonists should be tested in therapy of phobic states and in conditions of endocrine unbalance leading to a deficit in the production of neurosteroids (dysphorias of female patients associated with the progesterone surge?).

Table 3. The anticonflict activity of FGIN-1–27 is blocked by PK 11195 in the Vogel conflict test in thirsty rats

Treatment (μmol/kg, i.v.)	Water drinking—electric shock suppressed behavior (licking periods in 3 min)
Vehicle	9.2 ± 0.8
FGIN-1–27	
(2.9)	13 ± 3.5
(5.7)	17 ± 2.5*
(11.7)	19 ± 2.2*
(17.2)	23 ± 3.2*
FGIN + PK 11195	
(17.2) + (4.2)	10 ± 1.8**
(17.2) + (2.1)	11 ± 1.5**

Each value is the mean ± SE of at least 10 rats. PK 11195 was injected 5 min before FGIN-1–27. FGIN-1–27 was injected 10 min before test. $^*P < 0.05$ compared to vehicle treated rats; $^{**}P < 0.05$ for PK 11195 + FGIN-1–27 treated compared to FGIN-1–27 (17.2 μmol/kg) treated rats. Duncan multiple range test. For details on methods, see Ref. [27].

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