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MITOCHONDRIAL BENZODIAZEPINE RECEPTORS AND THE REGULATION OF STEROID BIOSYNTHESIS

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INTRODUCTION

The principal site of action of benzodiazepines (BZs)—one that makes this class of drugs the leading anxiolytic compounds—is a domain that allosterically regulates chloride channel gating by γ-aminobutyric acid (GABA) on $GABA_A$ receptors (1–3). Initial studies to identify specific benzodiazepine receptors in the CNS unexpectedly revealed the presence of additional binding sites in peripheral tissues distinct from those associated with $GABA_A$ receptors in the brain (4). Because of its initial identification outside the CNS, this class of recognition sites became commonly known as peripheral-type benzodiazepine recognition sites. These BZ binding sites exist in nearly all mammalian tissues, including the CNS, where their density is either nearly comparable to or greater than that of the BZ recognition sites on $GABA_A$ receptors (5, 6). The term *peripheral-type* is therefore misleading in describing the location of this class of sites, which exhibits a defined binding specificity. Other potential recognition sites for BZs have been reported (7–9); therefore, one should not assume that peripheral-type BZ receptors include these more

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recently identified recognition sites, because they show a binding profile distinct from the class of sites originally discovered by Braestrup & Squires (4).

Peripheral-type BZ recognition sites are found predominantly on mitochondria. This review covers specifically these mitochondrial BZ receptors (MBRs). Although other subcellular structures may potentially contain peripheral-type BZ recognition sites (10, 11), these are not discussed here because their density is much less than that of MBRs. Moreover, the structure and function of these other peripheral-type BZ binding sites is only beginning to be characterized.

For over ten years since their discovery, MBRs were largely an enigma—a drug recognition site with no discernible function. Much of the early work on MBRs has been adequately discussed in prior reviews (12, 13), but considerable progress has since been made concerning the structure, function, and regulation of MBRs. Hence, this review updates these developments and thereby clarifies a possible functional role for these recognition sites.

STRUCTURAL BINDING SPECIFICITY

Before discussing the biological and functional aspects of MBRs, it is important to understand the ligand-binding properties of this class of recognition sites. MBRs were first discovered because they bind the BZ diazepam with nanomolar affinity (4). Shortly thereafter, many BZ derivatives were tested to compare their specificity on MBRs with that on $GABA_A$ receptors. These studies revealed that the two classes of BZ recognition sites display distinct structural specificities (5, 14–16). In rodents, diazepam is rather nonselective, binding with nearly equal affinity to both classes of sites. The 4'-chloro derivative of diazepam designated as Ro5-4864, however, shows a higher affinity for MBRs and very low affinity for $GABA_A$ receptors (17, 18). Clonazepam and flumazenil are selective by virtue of their high affinities for $GABA_A$ receptors and low affinities for MBRs. Another BZ used extensively for the characterization of both receptors is flunitrazepam. This compound was used effectively as a low nanomolar photoaffinity probe for $GABA_A$ receptors (19), but its use to photolabel MBRs has given somewhat ambiguous results because its affinity is two orders of magnitude lower for these sites.

In subsequent years, other classes of organic compounds were found to exhibit high affinity and specificity for MBRs. Some isoquinoline carboxamides (IQCs), which are structurally quite different from BZs, have much greater selectivity for MBRs than for $GABA_A$ receptors (20–22). The IQC derivative PK 11195 is perhaps the most widely used probe specific for MBRs. A comparison of the binding properties between IQCs and BZs is discussed in further detail below.

Other structurally distinct compounds with aromatic groups have been found to bind to MBRs. Quinoline propanamides, similar in structure to IQCs, also bind with low nanomolar affinity. A pair of quinoline propanamide enantiomers, signified as (−)PK 14067 and (+)PK 14068, differ in their binding affinities for MBRs by two orders of magnitude, thus demonstrating stereospecific binding of these receptors (23). These probes provide an especially useful tool to test rigorously for MBR function.

Another important series of compounds used in the study of MBRs is the imidazopyridines. Unlike the quinolines described above, these compounds bind to MBRs as well as $GABA_A$ receptors (24). The highest affinity ligand thus far reported for MBRs is the imidazopyridine alpidem, which exhibits a dissociation constant in the picomolar range, whereas its congener, zolpidem, binds in the high nanomolar range.

The compounds described above were those used predominantly in the study of MBRs, but a long list of others appear also to bind to these recognition sites (although many of them do not exhibit as high of an affinity). This list includes porphyrins (25), dipyridamole (26), thiazide diuretics (27), pyrethroid insecticides (28), carbamazepine (29), lidocaine (30), certain steroids (31), and dihydropyridines (32). In several cases, MBRs are suspected as mediating some of the biological actions of these various agents.

Among the chemical species listed above, BZ and IQC binding have been extensively characterized. There is clear evidence that the binding domains for these two classes of compounds are not identical. Initially it was found that PK 11195 binding is thermodynamically an entropy-driven process whereas that of Ro5-4864 is enthalpy-driven (22). Based on analogies with β-adrenergic receptors, this finding led to an early supposition that PK 11195 and Ro5-4964 act as an MBR antagonist and agonist, respectively. Sufficient examples now exist of these compounds having either similar or opposite actions such that it may not be appropriate to differentiate these two compounds as strictly MBR agonists or antagonists.

Additional evidence demonstrating the inequality of BZ- and IQC-binding domains was obtained via biochemical perturbation. The histidine-modifying reagent diethylpyrocarbonate specifically inhibits binding of PK 11195 by MBRs of rodent origin (33, 34). Ro5-4864 blocks the inactivation by this reagent, thus suggesting that the two ligands bind to different conformational states of the receptor or that the BZ-binding site partially overlaps or is allosterically coupled to the IQC-binding domain. Unsaturated fatty acids lower the affinity for BZs without affecting PK 11195 binding (34, 35), again showing differential sensitivity by the recognition sites of both classes of compounds.

Species-dependent differences have also been found in the binding properties for BZs and IQCs. A low nanomolar affinity for PK 11195 appears conserved among all species in which MBRs have been identified; in contrast,

the affinities for BZs such as Ro5-4864, diazepam, and flunitrazepam are highly variable (36–38). The fortuitous circumstance that rodent species exhibit the highest affinities for BZs permitted the initial discovery of MBRs, hence their classification as a BZ recognition site. Now it is clear that the BZ-binding property of MBRs is at best weakly conserved across species. Previous studies that employed BZs, prior to the establishment of IQCs as alternative probes for MBRs, had suggested that only higher vertebrates possessed MBRs (39). Subsequently, it has been demonstrated with PK 11195 that MBRs are found in vertebrate classes as low as fish (40). Further investigations may yet reveal the presence of MBRs in more primitive eukaryotes.

TISSUE DISTRIBUTION AND SUBCELLULAR LOCALIZATION

Much insight concerning how functional roles for MBRs should be investigated has been gained by determining their tissue distribution. These recognition sites are found in almost all tissues but their levels of expression exhibit a very distinct pattern, which has been reviewed elsewhere (12). Some important points need to be emphasized now for the discussion on the function of MBRs that follows. The most notable finding is that MBRs are most highly expressed in steroidogenic cells such as the adrenal cortex and Leydig cells of the testes (41, 42). As is elaborated below, the involvement of MBRs in steroid biosynthesis accounts for the high expression in these cells.

MBRS are found abundantly in tissues involved with electrolyte transport such as along specific segments of the nephron in the kidney (43), salivary and sweat glands (42), and choriod plexus and ependemya of the brain (44). Aside from these tissues, MBRs are expressed in many other tissues at comparable levels, whereas tissues with relatively low abundance of MBRs include skeletal muscle, gastrointestinal tract, and much of the brain. We emphasize that although these latter tissues have relatively low levels of MBRs, the densities of receptor that can be found are still quite high in comparison with other receptors (>500 fmol/mg of protein).

With regard to the CNS, the brain contains relatively low levels of MBRs in contrast to the spinal cord, where they are quite abundant (6). It appears that MBR expression is predominantly in glial cells (45, 46). The demonstration of glial MBRs may be indicative of the steroidogenic property recently proposed for these cells (47–49).

Subcellular fractionation studies demonstrated that peripheral-type BZ recognition sites are principally associated with mitochondria (hence the term *MBR*), specifically with the outer mitochondrial membrane (50–52). In contrast to these reports, another group has proposed the MBRs are located on

inner mitochondrial membranes (11). This latter study was performed with lung tissue, and it was implied that the disparity in mitochondrial localization may be diverse in different tissues. A more likely explanation may be that MBRs are preferentially localized at contact sites of outer and inner mitochondrial membranes, which typically cofractionate with inner mitochondrial membrane markers.

It is also apparent from histological radioligand autoradiography (42) and subcellular fractionation studies (53) that MBR levels on mitochondria are highly variable; therefore, MBRs do not seem to be constitutive to all mitochondria. Specific examples are the apparent absence of MBRs in chromaffin cells of the adrenal medulla (54) and in certain primary cultures (46).

MOLECULAR CHARACTERIZATION

Early studies to characterize the molecular structure of MBRs included radiation inactivation. It was reported that the target size for the Ro5-4864 binding site was about 34 kd (55), whereas subsequently another laboratory estimated the target size for PK 11195 to be 23 kd (56). Because separate groups performed these studies, it is not explicit that these values are significantly different given the lack of precision associated with radiation inactivation. Nevertheless, these results suggest, but do not prove, that separate proteins may comprise the binding domains for BZs and IQCs.

The first definitive identification of a protein associated with MBRs was made with the development of PK 14105, an IQC photoaffinity probe (57). This probe specifically photolabels a protein of 18 kd that has been identified in all tissues tested that express MBRs (53, 58, 59). This protein has been purified to homogeneity (59–61), and the corresponding cDNA has been cloned from the rat (62), human (63), and bovine species (64). Transfection studies in mammalian cell lines with the receptor cDNA demonstrate expression of BZ and IQC binding with specific affinities for each probe characteristic of the species from which the cDNA was cloned. More recent transfection studies of human MBR cDNA in yeast, which does not normally exhibit recognition sites for MBR ligands, demonstrated expression of binding sites for IQCs, BZs, and quinoline propanamides with affinities that correlated with MBRs in human cell lines (64a). These results provide rather strong evidence that the 18 kd protein comprises the BZ- and IQC-binding domains.

The deduced amino acid sequences of the MBR protein from all three species show moderate conservation in this drug receptor (≈80% identity, 92% including conservative substitutions). It is a very hydrophobic protein containing five potential transmembrane-spanning segments and clearly shows no explicit sequence homology with $GABA_A$ receptors or other ligand- and voltage-gated channels. One other known protein in current databases

(NBRF-PIR Release 27.0, SwissProt Release 17.0, GenBank Release 67.0, EMBL Release 26.0) exhibits notable homology with this 18-kd MBR protein (Figure 1). Interestingly, the gene for this protein, which is termed crtK, is found in the photosynthetic bacterium *Rhodobacter capsulatus* and comprises part of the carotenoid-biosynthesis gene complex (65). The homology observed with crtK (32% amino acid identity, 66% including conservative replacements) probably correlates with the role of MBR in steroid biosynthesis, discussed below. Because the MBR and crtK proteins are distantly related, it is implied that they play similar, but still undefined, roles in the biosynthetic schemes for steroids and carotenoids, respectively.

In addition to the 18 kd protein, there is evidence that another protein of about 30 kd may also be associated with MBRs. Initial studies with flunitrazepam suggesting this (aside from the earlier radiation inactivation studies) indicated the photolabeling of a 30–35 kd protein in kidney mitochondria (66). Although this BZ is a good photoaffinity probe for $GABA_A$ receptors, it has considerably lower affinity for MBRs, thus magnifying the difficulties in detecting specific labeling by the probe. Similar findings were made in one case with [^{3}H]PK 14105 in which a 30-kd protein was specifically photolabeled in addition to the 18-kd protein (61). Another BZ probe has been used to support the existence of a 30-kd protein. An isothiocyanate derivative of Ro5-4864 signified as AHN 086 (67) was reported to label a 30-kd protein in mitochondria of the rat pineal but not that of bovine pineal gland, consistent with the species specificity of BZ binding of MBRs (68). Some caution should be used in considering these findings, as total binding of [^{3}H]AHN 086 was not inhibited by PK 11195 or Ro5-4864. Because relatively high


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Bovine  --------V----l---.....-g-L-AQYtR---F----S----P----R-I-A-I--
   Rat  -SqS----V-L--v---.....-g-M-Ay--R--------S---------R-t-A-I--
 Human  MAPPWVPAMGFTLAPSL.....GCFVGSRFVHGEGLRWYAGLQKPSWHPPHWVLGPVWG
  crtK  .........-SL--fAvyfvacacAqat-Ai-sp-a...--dS-K----v--N-LFpva-S

Bovine  ----------MI--------Ske--------A----------L---T----------
   Rat  ----------II---------e-M-------------------------------M-
 Human  TLYSAMGYGSYLVWKELGGFTEKAVVPLGLYTGQLALNWAWPPIFFGARQMGWALVDLLL
  crtK  ---il-SiSAar-s.g-Amen-l--Lg-AFWAv-I-V-tl-t-----lHRLAgGMLv-V-

Bovine  tG-m----AM--h----p--c----------gm---rM-Q--QvrRS----S-
   Rat  ---v-T---L--hR---p---------------M---y-----s-rR--s--t-
 Human  VSGAAAATTVAWYQVSPLAARLLYPYLAWLAFATTLNYCVWRDNHGWHGGRRLPE
  crtK  LwlSvf--c-lfws-dw-SGlMFv--Vi-VTv-gA--Fs---l-p-ekpitl...
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Figure 1 Sequence homology of the 18 kd MBR and crtK proteins. The amino acid sequence of the human 18 kd MBR protein is compared with the rat and bovine receptor protein sequences and the deduced amino acid sequence from the crtK gene of *R. capsulatus*. *Dots* indicate gaps introduced to maximize alignment between the sequences. *Hyphens* are shown where amino acid residues are identical to those found in the human protein. Conservative or nonconservative substitutions relative to the human protein are represented by *upper* and *lowercase letters*, respectively. For the crtK sequence, the *underlined letters* highlight where no conservative replacements are found with respect to individual MBR proteins of all three species.

nanomolar concentrations of the isothiocyanate probe were required, a substantial level of nonspecific acylation of primary amino groups of proteins will occur; thus the detection of any specifically labeled components might be obscured.

More recent attempts to isolate MBRs in the native state have shown that a 30-kd protein copurifies with the 18-kd protein (69). It was reported in these studies that the 30- and 18-kd proteins were separated and that binding of PK 11195 and Ro5-4864 were found to be associated with the 18-kd protein in agreement with cDNA transfection studies (62, 64). It is possible, however, that the 30-kd protein may influence the binding properties of the 18-kd protein. Present evidence suggests that the 30- and 18-kd proteins are not closely related in sequence because Northern analyses of rat RNA samples probed with the cDNA for the 18-kd protein do not reveal other mRNA species under moderately stringent conditions.

Previously it was hypothesized that the mitochondrial anion channel porin, a protein of about 35 kd located on outer mitochondrial membranes, may correspond to the BZ recognition site. Currently it is hypothesized that due to photolabeling of this protein by flunitrazepam, porin and the 18-kd MBR protein may associate as a complex.

STEROID BIOSYNTHESIS: THE MBR CONNECTION

Leading Evidence

Several lines of independent investigation suggested that MBRs may play a role in the endocrine regulation of steroid biosynthesis. Hypophysectomy causes a dramatic decrease of MBR density in the adrenal gland and testis, which is circumvented by the administration of appropriate pituitary trophic hormones (70–72). It had been shown that administration of diazepam increases plasma testosterone levels in men (73) and plasma corticosterone levels in rats (74), although another study failed to observe a significant effect on plasma testosterone levels in rats (75). The effects on blood steroid levels were believed to be due to the influence of GABAergic regulation on the hypothalamus.

As elaborated above, MBRs are quite abundant in many tissues, but they are extremely abundant in steroidogenic cells. Pertinent to this, it is intriguing that mitochondria carry out the first metabolic step in committing cholesterol to the steroid biosynthetic pathway. These findings, taken at face value, indirectly imply a role of MBRs in steroid biosynthesis.

MBR Function in Steroid Biosynthesis

The first direct evidence supporting the MBR involvement in steroidogenesis was obtained by in vitro studies on decapsulated testes and interstitial cell

suspensions, in which diazepam and Ro5-4864 were shown to stimulate androgen production (76, 77); however, the use of crude testicular preparations limits the validity of these results because Leydig cell steroidogenesis can be regulated by paracrine factors secreted by Sertoli cells (78, 79).

Within a span of six months, four other laboratories reported pharmacological evidence for the involvement of MBRs in steroidogenesis. In the most detailed and conclusive of these studies, nine ligands covering a range of over 10,000-fold in their affinities for MBRs were tested for their potencies to modulate steroidogenesis in the Y-1 adrenocortical and MA-10 Leydig mouse tumor cell lines. The battery of ligands tested included BZs, IQCs, imidazopyridines, and the quinoline propanamide enantiomers PK 14067 and PK 14068. Only those ligands with submicromolar affinities for MBRs increased steroid production. The ligands with highest affinity were also the most efficacious, showing about two- to fourfold stimulations in the Y-1 and MA-10 cells, respectively (80, 81). The K_i to inhibit [^{3}H]PK 11195 binding and the EC_{50} for steroid biosynthesis for this series of compounds were comparable and showed an excellent correlation ($\approx$0.98) in both cell lines. Similar results were observed with rat and bovine adrenocortical cell preparations and with purified rat Leydig cells. Consistent with the species-dependent differences in BZ binding, Ro5-4864 was much less potent and efficacious in bovine adrenocortical cell preparations than in rat adrenocortical preparations.

Parallel studies with bovine adrenal mitochondria (82, 83), human term placental tissue (84), and ovarian granulosa cell lines (85) confirm these results, although a detailed pharmacological study for specificity was not performed. More recently, MBR ligands were also found to stimulate steroidogenesis in mitochondrial preparations from the rat C6 glioma cell line (49), thus providing further support that certain glial cell types may be steroidogenic.

Before discussing the mechanism by which MBR ligands stimulate steroidogenesis, it is first important to appreciate the state of the art describing the biochemistry and cell biology underlying the regulation of this physiological process (see also 86 and 87). It appears that MBRs provide an important missing link in understanding the regulation of steroid biosynthesis.

Steroidogenesis begins with the conversion of cholesterol to pregnenolone, which occurs in the inner mitochondrial membranes. Pregnenolone then leaves the mitochondrion to undergo enzymatic transformation in the endoplasmic reticulum, which in testicular Leydig and ovarian cells will give rise to androgens, estrogens, or progestins. In adrenocortical cells, pregnenolone is metabolized to steroid intermediates that return to mitochondria for the last step in the synthetic pathway ultimately leading to the production of mineralocorticoids or glucocorticoids. Most of these reactions are catalyzed by enzymes of the cytochrome P-450 family.

This biosynthetic pathway is regulated by the tropic hormone adrenocorticotropin (ACTH) in adrenocortical cells and gonadotropins in testicular Leydig and ovarian cells. The peptide hormone binds to its membrane receptor, thus stimulating adenylate cyclase, resulting in an increase of intracellular cAMP. This second messenger then triggers two other responses: changes in protein synthesis and changes in the state of phosphorylation of specific proteins (via protein kinase A) responsible for regulating their function. Figure 2 illustrates in greater detail the chain of intracellular events that follow tropic hormone binding to its receptor. The overall scheme is that cholesterol is transported from its sites of storage or synthesis to the outer mitochondrial membranes and then is translocated to inner mitochondrial membranes, where side chain cleavage of cholesterol takes place via an enzymatic reaction

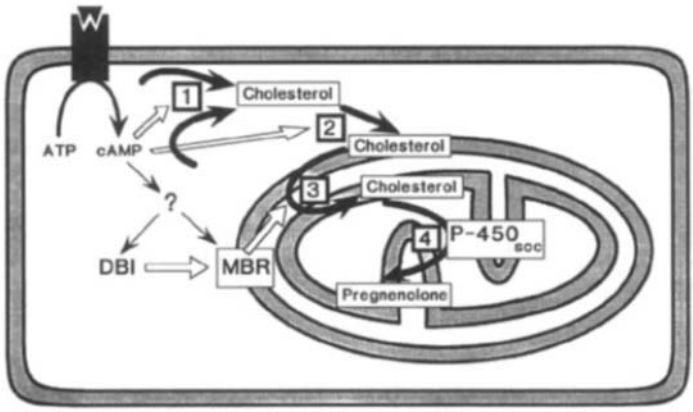

Figure 2 The coupling of MBRs to the physiological stimulation of steroid biosynthesis. This schematic depicts the sequence of events during the stimulation of steroid biosynthesis by tropic hormones that initially bind to a cell surface receptor coupled to the formation of cAMP. The net effect of this stimulation is to increase transport of cholesterol to the side-chain cleavage enzyme ($P\text{-}450_{scc}$) at inner mitochondrial membranes, where cholesterol is converted to pregnenolone. Transport of cholesterol can be considered to occur in four stages as numbered by the *bold arrows*, the first three of which are facilitated (signified by *open arrows*) as a consequence of cAMP elevation or activation of protein kinase A. The first step is liberation of cholesterol from intracellular reserves (hydrolysis of cholesteryl esters or recruitment from membranes), followed by the second step of cholesterol transport to mitochondria, into or around outer mitochondrial membranes. The third step in this scheme is characterized by a translocation of cholesterol from outer to inner mitochondrial membranes, following which the substrate then has access to $P\text{-}450_{scc}$ for conversion to pregnenolone (Step 4). Step 3 is rate-limiting in this entire scheme. This specific step is mediated by MBRs, as shown, and is coupled to the intracellular actions of cAMP by mechanisms incompletely understood but believed to involve diazepam-binding inhibitor (DBI) as a regulator of MBR function. Cycloheximide will completely inhibit the activation of Step 3 at a point downstream of cAMP but prior to MBR involvement, whereas flunitrazepam, by binding to MBRs, will partially block the cAMP-mediated action on Step 3.

catalyzed by the C_{27} side chain cleavage cytochrome P-450 enzyme ($P\text{-}450_{scc}$) that is dependent on an electron transport system comprised of adrenodoxin and adrenodoxin reductase. The processes of cholesterol recruitment and transport to mitochondria (including Steps 1 and 2 of Figure 2), succeeded by its movement within the mitochondria (Step 3), appear to be the points at which cAMP accelerates steroid synthesis. Thus, synthesis of the initial steroid product pregnenolone is controlled by regulating transport of the substrate cholesterol to the $P\text{-}450_{scc}$.

While elucidating the rate-limiting step in steroid biosynthesis, several laboratories noted that the protein synthesis inhibitor cycloheximide blocks the steroidogenic stimulation by tropic hormones. This inhibition occurs at the step of intramitochondrial transport of cholesterol to the inner membrane (88, 89). It is believed that a protein(s) with a rapid turnover rate is required to effect translocation between mitochondrial membranes and that the tropic hormones may act either to increase the level of this protein(s) or to activate this protein by posttranslational modification. A number of candidate proteins have been proposed as potential participants in this scheme (90–92), but the molecular basis for intramitochondrial cholesterol transport has proven to be a difficult puzzle to solve.

Given this background information, the involvement of MBRs in steroidogenesis becomes more apparent. In contrast to the stimulation via cAMP, the stimulation by MBR ligands is not sensitive to cycloheximide and is still observed when the drugs are applied to isolated mitochondrial fractions (81, 93). This suggests that MBRs function in the pathway downstream from the block by cycloheximide. Further evidence shows that MBR ligands do not directly stimulate $P\text{-}450_{scc}$ (81) but mediate translocation of cholesterol from outer to inner mitochondrial membranes (93). Hence, the participation of MBRs in intramitochondrial cholesterol transport has been unequivocally demonstrated, but the specific functional role of MBRs in this process is still not elucidated.

A major concern that arises from these studies is posed by the presence of MBRs in tissues that do not synthesize steroids. An answer to this quandary may be gleaned from studies on another metabolic pathway for cholesterol, that of its conversion to bile acids. A separate set of cytochrome P-450 enzymes participate in this pathway in which sterol 27-hydroxylase is located at inner mitochondrial membranes and, like $P\text{-}450_{scc}$, requires adrenodoxin and adrenodoxin reductase for oxidative activity (94). Sterol 27-hydroxylase does not appear to be used solely for bile acid synthesis, as this enzyme, with its oxidation-reduction protein co-factors, is present in a broad range of tissues besides the liver (95). The significance of these recent findings is under investigation and suggests that other metabolic schemes for cholesterol remain to be elucidated. Noting the close similarities with pregnenolone

synthesis, it seems probable that MBRs may also participate in intramitochondrial cholesterol transport to provide the substrate for the widely expressed sterol 27-hydroxylase.

Modulation of MBR Activity May Participate In Steroidogenesis

An important possibility that cannot be discerned by the previous studies is whether the stimulation of steroidogenesis by polypeptide tropic hormones involves the participation of MBRs. Hormone-stimulated steroidogenesis is not affected by the concomitant addition of high-affinity MBR ligands such as PK 11195, Ro5-4864, and alpidem or by many ligands of lower affinity such as diazepam and zolpidem (81). In discrepancy with these results, another laboratory reported that testosterone production induced by human choriogonadotropin exhibited a 25% increase with Ro5-4864 in crude testicular interstitial cells (77). More recently, two other studies appeared on the effect of BZs on ACTH-stimulated bovine adrenal steroidogenesis. One shows inhibitory effects of diazepam, but at concentrations of 0.1–1 mM (96), which are therefore likely to be nonspecific, and the other shows no effect of diazepam at micromolar concentrations on ACTH-stimulated steroidogenesis (97).

Flunitrazepam has proven especially useful for examining the role of MBRs in the acute tropic hormone activation of steroid biosynthesis. By itself, flunitrazepam acts as a partial agonist in steroidogenesis, but this BZ inhibits hormone- and cAMP-stimulated steroidogenesis in mouse adrenocortical and Leydig cells by 30–60%, with an IC_{50} of about 500 nM, in close agreement with its affinity for rodent MBRs (98). This antagonism by flunitrazepam was found to be mediated through its binding to MBRs because other ligands prevent this inhibition with a rank order of potency characteristic of MBR specificity. The incomplete inhibition occurs probably because flunitrazepam is a partial agonist in steroidogenesis and therefore only partially interferes with the cellular regulation of MBRs in this process. As would be predicted from earlier studies, flunitrazepam inhibits pituitary hormone action on steroidogenesis specifically at the step of cholesterol translocation from outer to inner mitochondrial membranes. Thus far, flunitrazepam is unique in its intrinsic activity to antagonize the stimulation of steroid biosynthesis by pituitary tropic hormones. Nevertheless, this property was fortuitous in enabling the demonstration that MBRs are coupled to the cellular and physiological mechanisms that regulate the steroid biosynthetic pathway.

Concerning MBR function, a polypeptide termed diazepam-binding inhibitor (DBI) was suspected as being a potential endogenous modulator for these recognition sites. DBI was first identified and purified from rat brain based on its ability to displace diazepam from allosteric modulatory sites on

$GABA_A$ receptors (99). Later it was found also to displace ligands from MBRs (100), hence its hypothesized role as an endogenous ligand for these sites. Immunohistochemical studies indicated that DBI exhibited a tissue distribution similar to that of MBRs, and therefore was also found abundantly in steroidogenic tissues (101, 102).

Another laboratory had independently purified a protein from bovine adrenal cortex that stimulated pregnenolone synthesis in mitochondria (91). Sequencing of this protein revealed its virtual identity with DBI (82). Subsequent work by other laboratories has confirmed the role of DBI in steroidogenesis and demonstrated that its primary action is mediated specifically via its interaction with MBRs (103).

Prior to these recent findings in steroidogenesis, there was some question about the concept that DBI is a modulator of MBR activity because micromolar concentrations of this polypeptide are required to displace ligands from MBRs. In contrast, the stimulation of pregnenolone synthesis in mitochondrial fractions is observed at DBI concentrations of 10–300 nM (49, 103). Also, the amount of DBI found in the cells corresponds to a level 2–4-fold greater than that required to obtain a maximal steroidogenic response in mitochondrial preparations. Therefore, in like manner with many other peptides, the displacement potency of DBI for MBR ligands bears little significance to document and support its biological activity.

It now seems that DBI is a primary link in the stimulation of steroid biosynthesis by tropic hormones because flunitrazepam antagonizes DBI stimulation of mitochondrial pregnenolone formation (103) in the same manner as observed on the hormone stimulation of cellular steroid production (98). Moreover, PK 11195 does not stimulate steroidogenesis with optimal doses of tropic hormones on cells or with DBI on mitochondria, thus further supporting a link between DBI and tropic hormone action.

Various endogenous proteolytic processing products of DBI have been identified, including an octadecaneuropeptide (ODN; DBI_{33-50}) (104) and a triakontatetraneuropeptide (TTN; DBI_{17-50}) (105). ODN modulates allosterically the action of GABA on the $GABA_A$ receptor (104) whereas TTN binds preferentially to MBRs (106). The presence of a peptide recognized by ODN antibodies (presumably DBI) has been shown in testicular Leydig cells (107), and TTN-like immunoreactivity was found in adrenocortical and Leydig cell lines (103). TTN stimulates pregnenolone formation by adrenocortical (103) and C6 glioma cell mitochondria (49) with a potency and efficacy similar to that of DBI. On the other hand, ODN is less potent and less efficacious than TTN in activating pregnenolone formation in adrenocortical mitochondria and is completely inactive in C6-2B glioma cell mitochondria.

Because the steroidogenic stimulation by polypeptide hormones seems to involve the interaction of DBI (or its processing products) with MBRs, it is

important to consider whether DBI may be related to the cycloheximide-sensitive factor mediating intramitochondrial cholesterol transport. Acute stimulation of adrenocortical and Leydig cells with tropic hormones does not change DBI levels; however, ACTH replacement in hypophysectomized rats increased DBI levels in the adrenal, within 1–2 hr (108). These data suggest that a more complex mechanism (i.e. proteolytic processing or modification of DBI), involving a cycloheximide-sensitive step, may regulate the interaction with DBI or its processing products with MBRs.

Other potential modulators of MBRs have been previously suggested. Prior to the demonstration that MBRs are coupled to steroidogenesis, it was discovered that ligand binding of MBRs is sensitive to phospholipase A_2 (109). In accordance with this, a protein purified from rat antral stomach was first thought to be an endogenous ligand for MBRs, but it was later found to possess an enzymatic activity indistinguishable from phospholipase A_2 (110). Furthermore, the phospholipase A_2 inhibitor lipocortin I was found to increase the binding affinity for MBR ligands (111). All of these observations are likely due to an effect of unsaturated fatty acids on receptor ligand binding (34), on which, in addition, certain phospholipids and cardiolipin also affect the binding properties of MBRs (35). Whether these observations provide significant clues concerning regulation of MBRs in steroid biosynthesis remains unclear, as they could represent simply a nonspecific biochemical perturbation of the receptor-ligand interaction rather than a potentially important cellular regulatory mechanism of MBR activity. Phospholipase A_2 (112), fatty acids (113, 114), and phospholipid methylation (115, 116) have been implicated as playing some role in steroidogenesis and may therefore correlate with the effects observed on MBR-ligand binding. Perhaps these investigations show relevance with a much earlier study (117) in which different BZs, with a rank-order of potency characteristic of MBR specificity, stimulated phospholipid methylation in C6 glioma cells, a cell line now shown to be steroidogenic.

Endocrine Regulation of Cellular MBR Density

Many laboratories have investigated physiological factors that affect MBR levels in an attempt to gain insight into the regulation of these receptors. These studies have further supported a link between MBRs and steroids. Those described above on pituitary trophic maintenance of endocrine steroidogenic tissues provide a prime example of MBR regulation coordinated with steroidogenic control. In comparison, estrogen (71, 118, 119) and progesterone (120) were also shown to produce changes in MBR density of peripheral and central tissues, but the magnitude of these effects was not as pronounced. In the kidney, MBR levels appear to be regulated by mineralocorticoids (121) and possibly other steroids (120). These findings suggest the

interesting possibility that in spite of the role that MBRs play in steroid biosynthesis, steroids exert some control over MBR levels in a variety of target tissues.

Pertinent to this possibility, the role of stress is another aspect that has been studied by diverse laboratories. Relatively rapid changes were observed in kidney and olfactory bulb MBR densities of rats subjected to an acute swim stress (122). Exposure of rats to cold temperatures reduced MBR levels in brown adipose tissue by 50%, and this effect was prevented by sympathetic denervation (123). In platelets and lymphocytes of patients suffering from epilepsy or anxiety, MBR levels are reduced, but treatment with carbamazepine or the anxiolytic BZ diazepam, respectively, restores the density to normal levels (124, 125). In related studies, MBR levels were reduced in certain stress paradigms (126–128), whereas during examinations normal students exhibited an increase in platelet MBR density (129). The relationship that stress has in these experimental and clinical models is undoubtedly complex. Clearly, one could surmise that determinants including emotional state, hypothalamic regulation of peripheral organs, glucocorticoid secretion, and a multitude of other factors can contribute either directly or indirectly to MBR expression. It is clear, however, that different tissues respond in different ways to stress (124, 127, 130) in which steroids are implicated as mediating some control over MBR expression in a variety of tissues.

POSSIBLE RELATIONSHIPS OF MBRs WITH IN VIVO STUDIES AND THE CNS

Steroids have clearly been shown to produce multiple effects in the brain and alter behavior independently of their roles as feedback modulators in hypothalamic-pituitary regulation. This ability has been extensively studied for steroids derived from adrenals (131) and gonads (132), showing that peripheral endocrine organs modulate CNS activity. Moreover, as was described above, evidence is now accumulating that certain glial cells may be steroidogenic. An important possibility to consider in light of this discussion is whether drugs that bind to MBRs affect CNS function by altering steroid biosynthesis. It is still too early to verify this hypothesis, but some interesting findings pertinent to this concept have been made.

Another level of complexity is becoming apparent in attempting to understand the pharmacological profile of BZs. It is well established that conductance through GABA-operated chloride channels is potently enhanced by certain reduced metabolites of progesterone (133–135). A quite definitive structure-activity profile has been established for this steroid-$GABA_A$ receptor interaction (136), and two of these steroids have been identified at significant levels in rat brain (137). Pregnenolone sulfate, another steroid

found prominently in the brain, is antagonistic to the GABA action on chloride channels (138, 139).

These developments concerning $GABA_A$ receptor modulation imply that a reciprocal link may exist between $GABA_A$ receptors and MBRs. The GABAergic system is known to influence pituitary hormone secretion (140), which is in turn linked with MBR function in the regulation of peripheral steroidogenic tissues. Conversely, regulation of MBR activity will mediate changes in steroid biosynthesis, which may occur in peripheral tissues or in glial cells of the CNS, and this may in turn feed back to modulate $GABA_A$ receptor function. Clearly, at this stage this model is highly speculative, but it illustrates the order of complexity which may exist based on recent developments on the pharmacological profiles of BZs.

The possibility that MBRs may mediate some of the neuropsychotropic actions of BZs is therefore a point worthy of consideration. Furthermore, inasmuch as steroid modulation of the GABAergic system is well documented, it seems that other molecular systems in neurons are also sensitive to steroids, a possibility that would only further expand the realm that the pharmacology of MBRs might infringe upon.

In attempts to explore the pharmacological significance of MBRs and their possible involvement in drug action, a number of in vivo studies have been performed with Ro5-4864 and PK 11195. Before discussing these studies it should first be noted that in vivo mechanisms of action of MBR ligands may be difficult to correlate with the functions determined by in vitro methods discussed above. The common link between $GABA_A$ receptors and MBRs with steroids is one point of complexity. Moreover, there is a likelihood that MBR ligands may act at additional sites, other than at MBRs, which could contribute in a major way to the pharmacological actions of the drugs. For example, Ro5-4864, contrary to the actions of most BZs, is convulsant, but this action is apparently not due to its interaction with MBRs, as PK 11195 does not exhibit a similar activity or antagonize the effect of Ro5-4864 (141). More recently, it was suggested that Ro5-4864 may also bind to certain subtypes of $GABA_A$ receptors (17) to down-regulate GABA gating of chloride channels and thereby induce the convulsant actions (18).

Some of the behavioral studies in which MBRs may mediate CNS activity include sensitivity of DBA/2J mice to audiogenic seizures (142), an increase in proconflict response of rats (105, 143), and the development of contingent tolerance to carbamazepine in amygdala-kindled seizures (144, 145). In each of these paradigms, Ro5-4864 produced effects that were antagonized by PK 11195 but not by flumazenil, the antagonist of BZ action on $GABA_A$ receptors. Similarly, intracerebroventricular injection of the DBI processing product TTN induces a proconflict action that is antagonized by PK 11195 but not by flumanzenil, whereas the converse is true for the proconflict action follow-

ing injections of ODN (105). These findings correspond well with the MBR specificity for TTN and $GABA_A$ receptor specificity for ODN and suggest that both receptor types contribute to this behavioral model in rats. The basis for the opposite actions of PK 11195 and Ro5-4864 in these studies compared with their similar actions on steroidogenesis in cell cultures or in isolated mitochondrial preparations is not known and is subject to further investigation.

In studies focusing on hypothalamic-endocrine regulation by MBR ligands, Ro5-4864 was found to increase secretion of corticotropin-releasing factor in rats by a mechanism antagonized by PK 11195 (146). This was clearly an effect in the CNS, but the basis for this action was unclear. PK 11195, on the other hand, stimulated ACTH and corticosterone release; however, dose-response curves showed that an increase in plasma corticosterone levels was observed at doses of PK 11195 lower than those required to elevate ACTH release. This finding is probably indicative of the direct action of this drug on steroidogenesis mediated by MBRs.

In vivo studies on the mouse immune system have shown that PK 11195 and Ro5-4864 increase the humoral immune response, but ligands selective for $GABA_A$ receptors did not produce a similar effect (147, 148). Subsequent studies demonstrated that macrophage secretions of tumor necrosis factor, interleukin 1, and interleukin 6 were markedly reduced by these selective MBR ligands (149). It was not clear whether this effect was mediated directly by MBRs in the macrophages or whether elevated glucocorticoid levels induced the macrophage response. The latter possibility would be in accordance with the previous study by Calogero et al (146) showing effects of these MBR ligands on ACTH release.

OTHER FUNCTIONS INVOLVING MBRs: PARALLEL CORRELATIONS WITH STEROIDOGENESIS

Following is a concise account of some other cellular functions in which the involvement of MBRs was implicated. Particularly considered is how MBR mediation of steroidogenesis or intramitochondrial cholesterol transport may pertain to these other functions.

Mitochondrial Respiratory Control and Cell Growth

After it was determined that peripheral-type BZ recognition sites are located predominantly on mitochondria, it was hypothesized that they play a role in intermediary metabolism. This possibility was examined by two laboratories, which reported that ligands binding to MBRs alter mitochondrial respiratory control (150, 151). This effect was characterized as an increase in state IV and a decrease in state III rates of respiration and was observed in mitochondria

from steroidogenic and nonsteroidogenic tissues. Subsequently, another laboratory has reported similar findings with AHN 086, but the effects with this ligand were clearly shown to be independent from its interaction with MBRs (152).

Cellular proliferation, supposedly mediated by altering mitochondrial physiology, is another activity in which MBRs are believed to play a role. With specific regard to the role of MBRs in steroidogenesis, the Nb 2 lymphoma cell line is especially noteworthy. Nb 2 cells display a mitogenic response to prolactin that is potentiated at low nanomolar levels of PK 11195 and Ro5-4864 but is unaffected by clonazepam (153). These findings are intriguing in light of the implications that prolactin has in steroidogenesis. Prolactin receptors are abundant in steroidogenic cells, but an explicit role of prolactin in steroid biosynthesis is not yet clear (154). It is likely that the interplay between prolactin and MBRs in the Nb 2 cell line is related to the role of MBRs and prolactin in steroidogenesis.

In a similar study, nanomolar concentrations of PK 11195 or Ro5-4864 increased cell growth and thymidine incorporation into DNA of C6 glioma and Swiss 3T3 cells (155). These findings may be related to more recent investigations in which these MBR ligands, again at nanomolar concentrations, altered mitochondrial morphology and stimulated mitochondrial replication in C6 glioma cells (156).

Several studies have noted differences in MBR levels in proliferating tumorigenic cells. Malignant carcinomas of the colon (157) and ovaries (158) express threefold greater levels of MBRs compared to normal tissues, whereas in renal carcinomas MBRs were undetectable (159). Similar observations were made in analyzing a number of glial tumors (160).

The connection between MBR function and mitochondrial respiration and/or cell proliferation is still too vague for interpretation. It is possible that steroids produced following MBR activation may directly regulate cell growth. This possibility is certainly not valid for cells that lack P-450$_{scc}$, but products dependent on sterol 27-hydroxylase, as alluded to above, may play important and as yet unfathomed roles in cell proliferation. It is also feasible that the role of MBR in intramitochondrial cholesterol transport may change the lipid distribution of mitochondrial membranes, thereby altering physiological dynamics of mitochondria. Clearly, this is only speculative, and further investigations are needed to examine the relationships of MBR function with these other aspects of mitochondrial activities.

Porphyrins

In a search for putative endogenous ligands of MBRs, it was discovered that porphyrins competitively displace ligands from MBRs (25, 66). Among many porphyrins tested, protoporphyrin IX, mesoporphyrin IX, deuteroporphyrin

IX, and hemin exhibited the highest potencies for MBRs, whereas catabolic porphyrin metabolites were relatively ineffective (161). It is unlikely that porphyrins act to regulate steroidogenesis because pregnenolone formation in mitochondrial fractions is stimulated by protoporphyrin IX with an insignificant maximal efficacy, only one-tenth that observed with PK 11195 or Ro5-4864 (V. Papadopoulos and K. E. Krueger, unpublished data).

If there is biological importance to the MBR specificity for porphyrins, consideration of the cellular pathway for hemin synthesis should arouse interest. Protoporphyrin IX is synthesized on the endoplasmic reticulum and then transported into mitochondria, where, at the inner mitochondrial membrane, it is coordinated with an iron atom to form hemin by the enzyme ferrochelatase (162). This scheme is reminiscent of steroid biosynthesis, in which MBRs have already been shown to play an important role in intramitochondrial cholesterol transport. Perhaps the interaction of protoporphyrin IX with MBRs is correlated with the possibility that the receptor participates in intramitochondrial transport of this metabolic substrate as well. Further work is still required though to substantiate whether there is physiological significance to the interactions of porphyrins with MBRs.

SUMMARY AND FUTURE DIRECTIONS

The functional and pharmacological importance of MBRS has been gaining greater appreciation with recent developments regarding roles in mitochondrial function. The involvement of MBRs in steroid biosynthesis is now established from the work of several laboratories. In this metabolic pathway, MBRs mediate intramitochondrial cholesterol transport; however, it is still not clear whether MBRs comprise part of the cholesterol translocator or function in an ancillary or regulatory role in this transport process. Moreover, this transport process, as activated physiologically by pituitary tropic hormones, is coupled to MBRs.

Identification and molecular cloning of the MBR protein has also provided significant insight into these recognition sites. Several laboratories have now verified that an 18-kd protein is clearly identified as a principal component required for binding MBR ligands. This protein shows salient similarity with another protein presently included in databases, the crtK product of the carotenoid-biosynthesis gene cluster in *R. capsulatus*. Certainly this finding reinforces the role of MBRs in steroidogenesis and suggests that a series of related proteins might be found throughout prokaryotic, plant, and animal species. It is likely, however, that other proteins are functionally associated with the 18-kd MBR protein, but their identification has proven difficult. Identification of such associated proteins, or other proteins resembling MBR and crtK, may provide more explicit information on the specificity or poten-

tially diverse functions of MBRs. There is significant evidence that DBI or one of its processing products, following tropic hormone activation of cells, acts as a modulator of MBR activity in steroidogenesis. The involvement of MBRs has also been implicated in several other cell metabolic processes requiring mitochondrial participation. Although a common molecular mechanism remains to be elucidated, these other actions are not inconsistent with the fundamental role these recognition sites play in steroidogenesis.

It is apparent that drugs that bind to MBRs in peripheral steroidogenic tissues are fivefold less efficacious than the physiological pituitary tropic hormones. When steroids are released into the circulation, pharmacological manipulation of MBRs may be of little consequence. A different situation exists in the CNS, where glial cells may need only to secrete small amounts of steroids to affect $GABA_A$ receptor function in neighboring cells. Based on this supposition, MBR ligands may prove amenable for pharmacological intervention to regulate glial neurosteroid synthesis in the brain and indirectly modulate function of $GABA_A$ receptors of neuronal circuits adjacent to the steroidogenic glial cells.

The role of MBRs in steroid biosynthesis clearly should attract further interest regarding the pharmacological actions of BZs and possibly the alternative therapeutic treatments of certain endocrine disorders. Furthermore, the potential involvement of neurosteroid synthesis by glial cells in the modulation of synaptic communication or other neuronal functions could open new avenues in developing novel drugs for the therapy of anxiety disorders with a specificity more discrete than drugs used presently, which act at both $GABA_A$ receptors and MBRs.

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