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The Role of Endozepine in the Regulation of Steroid Synthesis

Peter F. Hall

Department of Endocrinology, Prince of Wales Hospital, Randwick, NSW 2031, Australia

Abstract

Endozepine has recently been isolated from various steroid-forming organs. The following article explores the role of endozepine in the regulation of steroid synthesis. Steroid hormone synthesis from cholesterol begins in the inner mitochondrial membrane, where cytochrome P450 converts cholesterol to pregrenolone. Scientists thought that ACTH would stimulate this conversion, but experiments showed no such stimulation. However, addition of aminoglutethimide to block side-chain cleavage caused the expected reaction of ACTH to take place. Next the role of protein synthesis on the actions of ACTH was explored. Then endozepine was isolated from bovine fasciculata based on stimulation of pregnenolone production by freshly prepared mitochondria. After further experimentation it was concluded that endozepine is a peptide with at least two groups of actions: It binds $GABA_A$ receptors in the central nervous system, and it increases the mitochondrial synthesis of pregnenolone.

Index Entries: Steroidogenesis; benzodiazepine; adrenal cortex; cytochrome P_{450}.

Introduction

One of the most unexpected discoveries in investigation of the regulation of steroid synthesis has been the recent isolation of endozepine from various steroid-forming organs, beginning with bovine adrenal fasciculata cells (Yanagibashi et al., 1988). Endozepine was discovered by Guidotti et al. (1983) in the central nervous system (CNS) as a peptide capable of inhibiting the binding of benzodiazepines to specific receptors in synaptosomes (Guidotti et al., 1983; Ferrero et al., 1986; Gray et al., 1986). The search for an endogenous valium was a logical outcome of the successful isolation of endogenous morphine or endorphin (Cox et al., 1975). The peptide was given the operational or pharmacological name of benzodiazepine-binding inhibitor (DBI). It is easy to understand why the more euphonious term endozepine has strong support, since this word does not leave the peptide to stand in the shadow of a recently synthesized group of drugs but allows it to assume its place as an important physiological agent. Moreover, the word endozepine is consistent with the name endorphin. Only time will tell whether we must change endozepine for a broader term to include the striking effects of the peptide in steroidogenic cells.

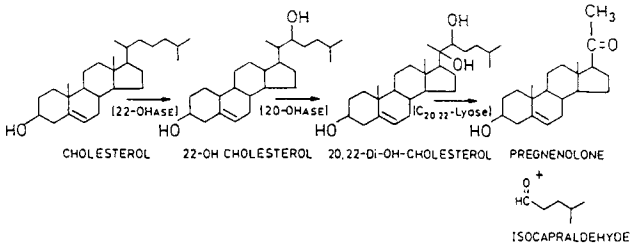
To understand these unexpected actions of endozepine we must consider, however briefly,

the steroidogenic pathway and the regulation of this pathway by trophic stimulation. In what follows, the adrenal cortex and its trophic hormone ACTH will serve as the model for the regulation of the synthesis of steroids, since it was in this structure that the role of endozepine in steroid synthesis was first discovered. The gonads and placenta will receive no more than passing mention, with the assumption that the relevant events are likely to prove very similar to those discussed in the adrenal cortex. It should be added that certain cells in the CNS are capable of synthesizing steroids and that benzodiazepines are capable of influencing this process (Guarneri et al., 1992; Papadopoulos et al., 1992). It is too early to evaluate the importance of this steroidogenic activity but it appears likely that the mechanisms to be discussed here apply to mitochondria from these neural cells.

The Pathway to Steroid Hormones: The Slow Step

The synthesis of steroid hormones from cholesterol begins in the inner mitochondrial membrane where a cytochrome P450 converts this substrate to pregnenolone, the parent substance from which all steroid hormones are formed (Hall, 1966). Pregnenolone leaves the mitochondria to undergo enzymatic transformation in microsomes (Hall, 1984a). The later steps in the pathway diverge to yield the various secreted steroids characteristic of the different steroid-forming organs. Since the conversion of cholesterol to pregnenolone involves the removal of 6 C atoms from the side chain of the substrate, the reaction is referred to as side chain cleavage (scc) and the enzyme that catalyzes the reaction as the side-chain cleavage enzyme or more commonly as P450 scc.

Examination of the relative rates of various parts of the steroidogenic pathway showed that the production of pregnenolone is the slowest step in the pathway (Hall, 1984a, 1985). The synthesis of pregnenolone results from the side chain cleavage of cholesterol. When this reaction is examined in detail, we see that it involves three separate steps. To cleave the C–C bond between C_{20} and C_{22}, P450 scc hydroxylates both of these C atoms and then cleaves the dihydroxy intermediate to give pregnenolone and isocapraldehyde:



Further examination of the reaction revealed that the first hydroxylation at C_{22} is the slowest step in the reaction (Hall and Young, 1968; Burstein and Gut, 1976).

Regulation of the Rate of Steroid Synthesis by ACTH: Mitochondrial Synthesis of Pregnenolone

Introduction

If side chain cleavage is the slow step in the pathway, it was thought likely that ACTH would stimulate the conversion of cholesterol to pregnenolone in vitro by isolated mitochondria prepared from cells incubated with the trophic hormone when compared to those from unstimulated cells using endogenous substrate (cholesterol). When this idea was tested in cultured cells, it was found that the production of pregnenolone by mitochondria from the two sources was the same (Nakamura et al., 1978). However, when side chain cleavage was blocked by addition of the inhibitor aminoglutethimide and adrenal cells were incubated with and without ACTH, mitochondria from the cells treated with ACTH showed greater pro-

duction of pregnenolone (side chain cleavage) than those from unstimulated cells (Nakamura et al., 1978). In these studies the inhibitor was removed by washing the mitochondria so that synthesis of pregnenolone could be measured in the isolated organelles using endogenous cholesterol. It was subsequently shown that inner mitochondrial membranes from cells treated with aminoglutethimide and ACTH showed a greater concentration of cholesterol (per mg protein) than those from cells treated with inhibitor alone (Hall et al., 1979). These findings showed that ACTH accelerates the transport of cholesterol to the inner mitochondrial membrane and does not stimulate side chain cleavage itself. Since the substrate (cholesterol) is located in the cytoplasm it is far removed from the enzyme, which is found in the inner mitochondrial membrane. This separation appears to limit the rate of production of pregnenolone, and since this is the slow step in the synthesis of steroid hormones, the transport process that brings substrate to enzyme could limit the rate of production of the secreted steroid hormones. It is, of course, most unusual for a substrate to be so far separated from its enzyme that a special mechanism is required to bring the two together. Subsequent studies revealed that the cytoskeleton is involved in this process of cholesterol transport (Hall, 1984b). These studies have been reviewed elsewhere (Hall, 1982) and will not concern us here.

Role of Protein Synthesis in the Response to ACTH

Introduction

Before the experiments just described were performed, it was reported that the steroidogenic action of two trophic hormones (LH and ACTH) on their respective target organs (testis and adrenal) is inhibited by puromycin (Hall and Eik-Nes, 1962; Ferguson, 1963). These observations have been repeated with cycloheximide. In fact, inhibition by cycloheximide of a response observed in steroidogenic cells to trophic stimulation has been used as a test of the relevance of the response to the steroidogenic action of the trophic hormones. For example, see Widmaier and Hall (1985). These observations suggested the involvement of protein synthesis in the actions of ACTH and LH on their target tissues. However, numerous attempts to demonstrate increase in the incorporation of various amino acids into total cell protein under the influence of either hormone were not successful (unpublished observations). When it became possible to prepare cells from the steroidogenic organs instead of tissue slices, increase in synthesis of Leydig cell protein was seen with LH when the cells were prepared from hypophysectomized rats (Irby and Hall, 1971).

At this point it was not possible to determine whether inhibition of protein synthesis by puromycin and cycloheximide produced such disruption of cellular function as to abolish the expected steroidogenic response to the trophic hormone or whether, on the other hand, specific proteins synthesized under the influence of the trophic hormones were partly or wholly responsible for the steroidogenic action of these hormones. There matters rested until Garren and coworkers (1965) performed an important experiment in vivo in rats that showed that the inhibitory effect of cycloheximide on the response to ACTH was not exerted on the side chain cleavage step or, for that matter, on the later steps of the pathway (Garren et al., 1965). This observation made a nonspecific effect of the inhibitor unlikely, since such an effect should involve the entire pathway indiscriminately. Moreover, these workers made the prescient suggestion that if cholesterol is indeed the physiological precursor of the steroids measured in these experiments, trophic stimulation must be exerted before side chain cleavage of cholesterol and the only step before side chain cleavage must presumably involve the transport of cholesterol from the cytoplasm to the enzyme (Garren et al., 1965). The studies

in vitro described above were performed after those of Garren et al., but provided confirmation of the findings of these authors by direct measurement of cholesterol transport to mitochondria (Hall et al., 1979). Evidently, ACTH must be responsible for the synthesis of a protein or proteins involved in the response to ACTH and such protein(s) must be specifically associated with the transport of cholesterol to mitochondria. Moreover, two groups of investigators showed that new protein(s) are necessary for the movement of cholesterol from the outer mitochondrial membrane to the inner membrane but not for transport to the outer membrane (Ohno et al., 1983; Privalle et al., 1983). When adrenal cells are incubated with ACTH and cycloheximide, cholesterol accumulates in the outer membrane and moves to the inner membrane when incubation is continued with ACTH but without cycloheximide (Ohno et al., 1983, Privalle et al., 1983). These findings paved the way for attempts to identify specific proteins made under the influence of ACTH-proteins that are somehow involved in cholesterol transport.

Involvement of Specific Proteins

The widespread use of electrophoresis in polyacrylamide gels made it relatively easy to identify specific proteins in cell extracts. A number of proteins, the synthesis of which is stimulated by ACTH and that may be important in expressing the response to this hormone, have been isolated from adrenal cells. Each of these proteins has some claim to a role in the action of ACTH.

30K Protein

Orme-Johnson and coworkers took an approach based on the great resolution provided by two-dimensional polyacrylamide gels (isoelectric focusing followed by electrophoresis in SDS) (Pon et al., 1986). These studies have demonstrated the presence in adrenal mitochondria of a protein synthesized under the influence of ACTH as a 37-kDa precursor that is converted to a 30-kDa phosphoprotein (Epstein and Orme-Johnston, 1991; Orme-Johnson, 1991). The function(s) of these proteins remain(s) unknown.

Sterol Carrier Protein (SCP2)

It was entirely logical for Scallen and coworkers to extend their important studies showing that cholesterol is synthesized from protein-bound precursors (Scallen et al., 1971). These workers isolated sterol carrier protein 2 (SCP2) from adrenal cells and showed that this protein stimulates side chain cleavage in isolated mitochondria (Chanderbahn et al., 1982). SCP2 has been purified and investigated by Ritter and Dempsy (1971), who showed that the amount of the protein in inner membranes of adrenal mitochondria shows a positive correlation with the amount of cholesterol in that membrane (Conneely et al., 1984).

It is important to note that this protein does not promote loading of P450 scc with substrate (cholesterol) (Vahouny et al., 1983). The importance of this observation is discussed later. SCP2 may be involved in binding and/or transport of cholesterol to and/or within the mitochondrion.

Sterol-Activating Protein (SAP)

Pedersen and Brownie (1983) set out to find a protein that stimulates side chain cleavage in adrenal mitochondria that were loaded with cholesterol. In order to enhance such a response to the protein in question they chose to work with adrenal mitochondria from hypophysectomized rats. Rats were treated with ACTH and cycloheximide to load the test mitochondria with cholesterol. The cytosol of adrenals from intact rats was used as the source of the protein, which was isolated on the basis of the production of pregnenolone by the test mitochondria. The procedure yielded a protein of mol wt 2200, which was found to stimulate the synthesis of pregnenolone by mitochondria prepared as described previously (Pedersen and Brownie, 1983). This protein was called sterol-activating protein (SAP) and is highly homologous to the carboxy terminus of a heat shock protein called glucose responsive protein (GRP) (Mertz and

Pedersen, 1989). The protein (SAP) promotes loading of P450 scc with cholesterol *(see the following)*. The significance of this interesting protein in the regulation of steroid synthesis is not yet clear.

Endozepine (8.2K)

Yanagibashi and coworkers (1988) set out to isolate a protein from the cytosol of beef fasciculata cells that is formed under the influence of ACTH and is capable of stimulating side chain cleavage in mitochondria prepared from unstimulated bovine fasciculata cells. It was considered important that the mitochondria required no special preparation, since mitochondria prepared by conventional methods are capable of expressing the stimulating influence of ACTH. A protein of mol wt 8200 was isolated from adrenal cell cytosol by column chromatography and HPLC. The protein stimulates the production of pregnenolone when added to mitochondria from beef adrenal (Yanagibashi et al., 1988). When sufficient amounts of this protein was available for determination of the amino acid sequence, the result was a complete surprise; 8.2K turned out to be des-(gly-ileu)-endozepine, or des-endozepine for short (Besman et al., 1989). More recently, it has been found that the full molecule (endozepine itself) is also present in fasciculata and Leydig cells (unpublished observations).

The identity of 8.2K was established by reference to data bases that revealed that a protein of the same amino acid sequence had been reported in the CNS (Ferrero et al., 1986; Gray et al., 1986; Shoyab et al., 1986) in 1983 and its structure was confirmed by sequencing of cDNA (Mochetti et al., 1986). Endozepine was first isolated on the basis of an assay in which binding of benzodiazepines to synaptosomes was inhibited by the protein (Guidotti et al., 1983). At this time benzodiazepines were known to be anxiolytic compounds and in uptake studies of labeled benzodiazepines various peripheral tissues were used as controls. It came as a surprise that many peripheral tissues showed evidence of specific binding of benzodiazepines (*see* Verma and Snyder, 1989; Snyder et al., 1990). Among such tissues adrenal and testis (Leydig cells) were conspicuous for the density of these binding sites (Synder et al., 1990). In these and other steroid-forming organs, specific binding sites for benzodiazepines have been found in the outer mitochondrial membranes (Anholt et al., 1985; Riond et al., 1989). The same location has been reported in rat kidney cells (O'Beirne and Williams, 1988). This observation led to studies in which preparations of testis (Ritta et al., 1987), ovary (Riond et al., 1989), and placenta (Barnea et al., 1989) were examined as possible target tissues for benzodiazepines.

It soon became clear that these compounds increase the production of steroids by all of these tissues. In some cases, changes in binding of benzodiazepines to these tissues was related to the physiological state of the organ in question in such a way as to suggest an important influence of benzodiazepines on steroid synthesis (Fares et al., 1988; Anholt et al., 1985).

Benzodiazepine Receptors

At this point the receptor for benzodiazepines must be introduced. It is important to distinguish between the relatively small number of studies in steroidogenic cells based on the use of endozepine from those in which benzodiazepines are used. In this latter group there is usually an underlying assumption, namely, that when benzodiazepines are used it is the benzodiazepine receptor that is producing the responses observed. Occasionally, investigators present indirect evidence for this conclusion, but for the most part it is an assumption. It is also important to recall that all receptors may not act like those for steroids in which the active agent is the receptor itself appropriately altered by binding the relevant ligand. In other words, the ligand is not seen as acting itself after using the receptor as nothing more than a port of entry. However, in the present case direct evidence does not seem to have been presented for specific binding of endozepine to benzodiazepine receptors in mitochondria.

Be that as it may, some of the studies quoted earlier clearly showed that benzodiazepines bind specifically to mitochondria of responding cells (Basile and Skolnick, 1986; Snyder et al., 1990). For example, it was clearly shown in a careful study in kidney that the benzodiazepine receptors are located in the outer mitochondrial membrane (O'Bierne and Williams, 1988). In this connection, it should be added that it is now common knowledge that benzodiazepine receptors are of two types, namely $GABA_A$ or central receptors that include a Cl^- channel that, in the presence of benzodiazepines, stabilizes the membrane potential and therefore inhibits neuronal activity and $GABA_B$ or peripheral receptors that act by different mechanism(s). Peripheral receptors are found in numerous tissues throughout the body (Ong and Kerr, 1990). The peripheral receptors also occur in specific parts of the CNS so that the term peripheral is not entirely appropriate. We will be concerned here only with so-called peripheral benzodiazepine receptors. For this reason, we will not consider the action of certain steroids on so-called central receptors (*see* references in Study and Barker, 1981). The peripheral benzodiazepine receptor has been well-characterized (Antkiewicz-Michaluk et al., 1988).

The role of the mitochondrion as the site of the rate-limiting step in steroid synthesis has been established. In view of the action of endozepine on steroid synthesis and the fact that endozepine shares certain of the pharmacological actions of benzodiazepines, the actions of benzodiazepines on mitochondria from steroid-forming cells were studied in greater detail. Endozepine was available at this time in no more than small amounts. By using a battery of benzodiazepines for this purpose it was shown that the so-called peripheral type receptor plays some part in the steroidogenic action of these drugs that act on mitochondria (Basile and Skolnick, 1986; Shoyab et al., 1986; Antkiewicz-Michaluk et al., 1988; Papadopoulos et al., 1990). It is not clear whether the final effector of the benzodiazepines on steroidogenesis is the drug or the mitochondrial receptor or the complex of drug and receptor.

Before the mechanism of action of these agents on steroidogenic mitochondria is considered it will be helpful to return to the major mitochondrial protein involved in steroid synthesis, i.e., P450 scc.

Cytochromes P450

Properties

All cytochromes P450 are heme proteins that use atmospheric or molecular oxygen to oxidize various substrates. The most common type of reaction catalyzed involves hydroxylation of a substrate (Eq. [1]).

$$\mathrm{R{-}H + NADPH + H^+ + {}^{18}O_2 \xrightarrow{[P\text{-}450]} R\text{-}{}^{18}OH + H_2{}^{18}O + NADP^+} \quad (1)$$

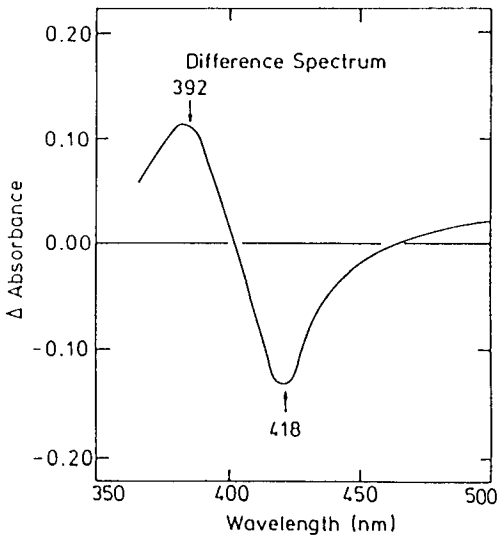
The fate of the two oxygen atoms is revealed when the reaction is performed with $^{IS}O_2$.

Other reactions can be catalyzed by various cytochromes P450 but they are all believed to follow the same mechanism. Each P450 enzyme consists of a heme-binding domain that is highly conserved throughout evolution, a substrate-binding domain that differs in different members of the superfamily and domains for interaction with electron carriers (since to exhibit enzyme activity all cytochromes P450 must be reduced) and other domains for attachment to membranes, because cytochromes P450 are usually membrane-bound. As a result of these common features it is possible to speak in general terms about the enzymatic behavior of this group of enzymes. The following general statements that are reviewed in greater detail in Hall (1987) are important in considering the steroidogenic action of endozepine:

1. Heme is a flat molecule with conjugated double bonds throughout the four pyrrole rings that make up the porphyrin ring system. The presence of iron in the center of the ring system makes the porphyrin a heme. As a result of the structure of heme all compounds containing this moiety show a pronounced absorbance band at approx 420 nm. The exact location of this so-called Soret peak depends on details of the structure of

the particular heme compound including the nature of the protein moiety in the case of heme proteins. The heme of P450 is attached to the protein via a thiolate bond to a specific cysteine residue. The iron can be hexavalent when it is located in the plane of the ring or pentavalent when it is displaced slightly from this plane.

2. The cysteine (thiolate) bond is responsible for those spectral properties that distinguish P450 from other heme proteins, notably the CO–P450 complex. When heme proteins are reduced, they bind CO and this binding is associated with a minor shift in the Soret peak. In the case of all cytochromes P450 this shift is extensive (from 420 to 450 nm). The absorbance peak of reduced P450–CO gives these proteins their name (pigment 450 or P450) and provides a simple, accurate, and nondestructive method of measuring the amount of any P450.
3. The position of the Soret peak is extremely sensitive to the environment of the heme. When a substrate binds P450, the protein undergoes a change in conformation resulting in the iron becoming pentavalent and the Soret peak shifting to 390 nm. The organization of electrons in the iron is said to shift from low spin to high spin. This spectral shift (called type 1) can be measured by difference, i.e., enzyme plus substrate minus enzyme plus solvent:

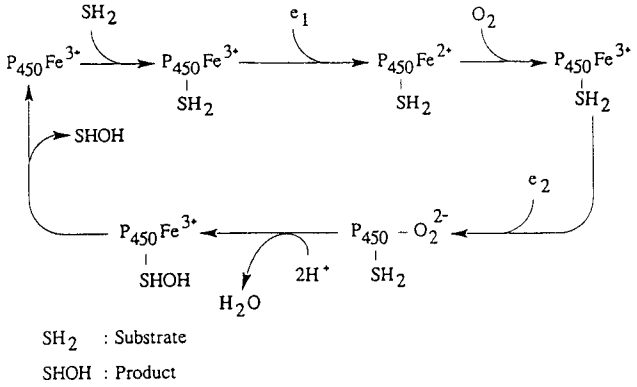


The magnitude of this difference in absorbance (390–420 nm) is directly proportional to the proportion of molecules of P450 that are substrate-bound, i.e. ES, in the terminology of enzymology.

4. The product of a reaction catalyzed by P450 commonly causes the opposite spectral shift (inverse type I), i.e., peak at 420 n*M* and trough at 390 nm.

The P450 Cycle

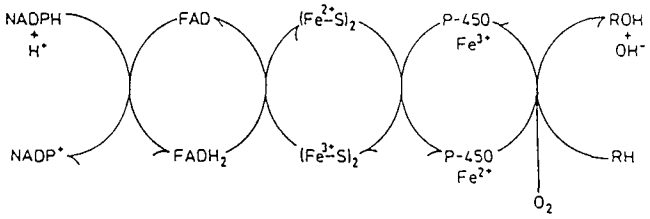
Since P450 acts as an oxygenase it must alternate between oxidized and reduced forms. P450 is autoxidizable and, when reduced by electrons from reduced pyridine nucleotides, the resulting ferrous ion attacks oxygen, thereby activating it to a form that causes monooxygenation (i.e., insertion of one of the two atoms of O_2 into the substrate). The cycle begins with binding of substrate (SH) to P450. The binding causes the first electron (e_1) to reduce the heme iron. The P450 then binds O_2 and the second electron (e_2) reaches the P450–O_2 complex. This activates O_2, which attacks the substrate with the formation of product (SHOH), H_2O, and oxidized P450:



The product is more hydrophilic than the substrate and is accordingly ejected from the hydrophobic active site. In the case of P450 scc, this step must be modified because the intermediates shown above do not accumulate in more than trace amounts. It is therefore likely that they remain in the active site as substrates for the next steps (Hall, 1987). How this modification occurs is not clear.

Electron Transport

Reduction of P450 results from the transfer to the enzyme of two electrons from reduced pyridine nucleotide via two mitochondrial electron carriers, i.e., the flavoprotein adrenodoxin reductase (FAD) and the iron–sulfur protein adrenodoxin ($Fe^{2+}S$):



It is known that adrenodoxin acts as a shuttle between its reductase and P450 (Seybert et al., 1978). Oxidized adrenodoxin has a high affinity for reduced reductase but reduced adrenodoxin has low affinity for reductase so that the complex adrenodoxin reductase-adrenodoxin breaks down when the first electron is passed to adrenodoxin. Reduced adrenodoxin then binds P450. The affinity of this binding is increased if the substrate (cholesterol) is bound to the enzyme (Lambeth et al., 1984). The second electron is passed to P450 by a second molecule of reduced adrenodoxin (Seybert et al., 1978).

Finally, CO provides a means of measuring the rate of reduction of P450. It will be recalled that CO does not bind oxidized P450. P450 is placed in a cuvet with adrenodoxin reductase, adrenodoxin, and buffer saturated with CO. Cholesterol may be added if required. Carbon monoxide is gently bubbled into the mixture to the point of saturation and the spectrophotometer is set to read A_{450} with time. There will be no more than background absorbance. When NADPH is added, the chart recorder shows increase in A_{450} with time. As each molecule of P450 is reduced it forms a complex with CO that (by the definition of P450) absorbs light at 450 nm. The slope of A_{450} as a function of time provides a measure of the rate of reduction of the P450.

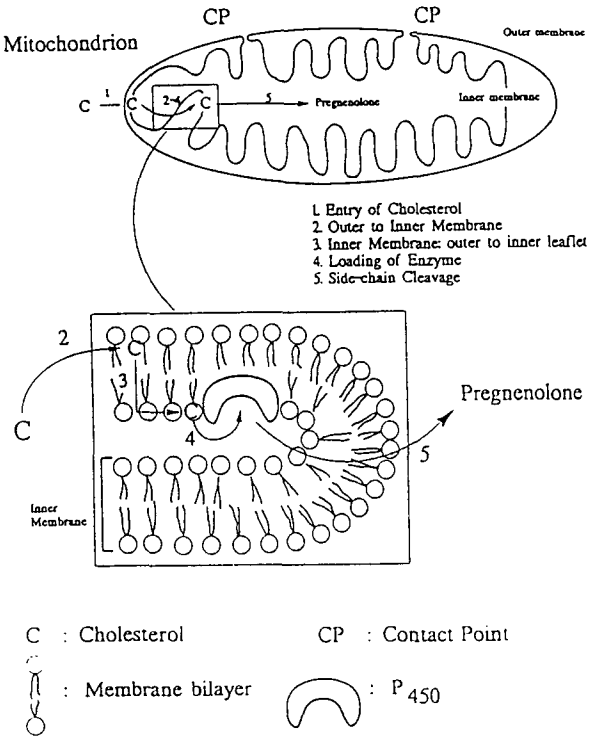
We can now consider the manner in which endozepine influences these events.

The Action of Endozepine on Production of Pregnenolone by Mitochondria

Introduction

Endozepine was isolated from bovine fasciculata by means of an assay based on stimulation of the production of pregnenolone by freshly prepared mitochondria from the same tissue. Since the production of pregnenolone is rate-limiting for steroid hormone synthesis (Koritz and Hall, 1964), this was a logical assay to use during purification of what was at first an unknown protein capable of stimulating steroid synthesis. The assay was used to identify fractions from cell extracts that contained such a protein as purification proceeded (Yanagibashi et al., 1988). As pointed out above, a postmitochondrial supernate was used as the source of endozepine from bovine fasciculata cells.

In considering the role of cholesterol as a substrate for steroid synthesis, it has generally been assumed that this substrate proceeds from cytosol to outer mitochondrial membrane, from outer membrane to the outer leaflet of the inner membrane, from outer to inner leaflet of this membrane, and finally to the active site of P450, where it is converted to pregnenolone. Reasons will be given later for questioning this as the only possible route for entry of cholesterol into the organelle. Since endozepine stimulates the conversion of cholesterol to pregnenolone it must presumably act on one or more of these steps. The question facing investigators was, therefore, which of these steps is (are) stimulated by endozepine:



When studies with liposomes prepared from phospholipids are considered, transfer of cholesterol from one liposome to another shows values for $t_{1/2}$ of the order of minutes (Kimura, 1981). We might equate this movement with that from one bilayer to another and in the present case from outer to inner mitochondrial membrane. On the other hand, movement of cholesterol from one leaflet of a bilayer to the other shows values for $t_{1/2}$ of approx 1.5 h (Kimura, 1981). This latter value is far too slow to account for the action of ACTH on steroid synthesis so this movement must be regulated in vivo. It should be realized that these values for $t_{1/2}$ were made in protein-free liposomes. We have no way of measuring the rate of movement of cholesterol within a single leaflet of a bilayer, although this process is almost certainly extremely fast. Indirect evidence suggests that loading of the enzyme with cholesterol is slow but direct measurements do not appear to have been reported. Clearly, the presence of proteins in mitochondrial membranes is likely to alter the situation found in liposomes by nonspecifically interfering with the movement of cholesterol and possibly as the result of specific interactions between proteins and lipids.

The last step in this pathway involves the catalytic reaction. The studies reviewed earlier suggested that the transport of cholesterol to the active site of the enzyme is slow relative to the cleavage reaction itself. It would appear that among the various steps in the movement of cholesterol one or more is (are) slower than the catalytic step. Since endozepine accelerates the production of pregnenolone from cholesterol it must presumably stimulate at least the slowest of these steps. These steps were examined to determine where endozepine acts as follows:

1. Entry into the mitochondrion. When mitochondria from bovine fasciculata are incubated with and without endozepine, this protein causes no change in the concentration of cholesterol in either membrane. When the incubation medium contains cholesterol, however, endozepine causes considerable increase in the concentrations of cholesterol in both membranes (per mg membrane protein) (Yanagibashi et al., 1988). Clearly, endozepine promotes entry of cholesterol into mitochondria under these experimental conditions. However, it should be pointed out that a suspension of mitochondria in buffered medium may not adequately reflect conditions in the intact cell.
2. Outer to inner membrane. When outer and inner mitochondrial membranes are prepared separately, incubated without and with endozepine (1.0 μ*M*) and then separated by centrifugation, the concentration of cholesterol falls in the outer membrane and increases in the inner membrane with duration of incubation (Yanagibashi et al., 1988). This experiment strongly suggests that one point at which endozepine acts is to increase transport of cholesterol from outer to inner membrane.

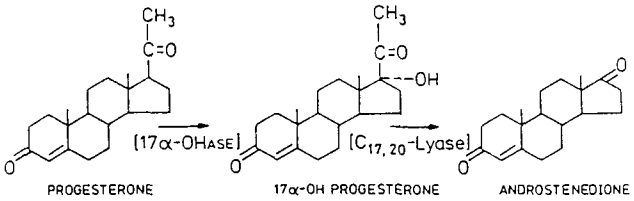
It should be pointed out that in this experiment the amount of cholesterol

leaving the outer membrane is greater than the increase detected in the inner membrane (Yanagibashi et al., 1988). Clearly, some of the cholesterol leaving the outer membrane cannot be accounted for. There are a number of possible explanations for this discrepancy. First, recovery of cholesterol in such studies is often poor because of the promiscuous binding of cholesterol to various surfaces. Second, separation of the coincubated membranes may not proceed quantitatively and the amounts of membrane available in such studies are too small for measurement of marker enzymes to determine whether or not significant contamination occurs in the separated membranes. Third, cholesterol is measured relative to membrane protein. To convert this concentration to amounts of cholesterol per mitochondrion involves further error. These are formidable problems when we consider that only a small proportion of mitochondrial cholesterol is ever converted to pregnenolone—the bulk of mitochondrial cholesterol appears not to have access to the steroidogenic pool (Kimura, 1981). In spite of these reservations, endozepine decreases the concentration of cholesterol in the outer membrane and causes a concomitant increase in the inner membrane.

3. Within the inner membrane. When endozepine is added to inner mitochondrial membrane from bovine fasciculata cells, two important responses are seen, namely, increased loading of P450 with substrate as judged by difference spectroscopy described above and increase in the rate of side chain cleavage (Yanagibashi et al., 1988; Hall, 1990). These experiments were performed with mitoplasts that consist of mitochondria from which the outer membrane has been stripped. This means first that with whatever cholesterol is present in the inner membrane and in whatever distribution it may occur in that membrane, endozepine increases the proportion of molecules of P450 scc that have bound cholesterol (ES). This represents a direct demonstration of increased loading of substrate onto the enzyme by endozepine. Evidently this effect is distinct from the stimulating influence of endozepine on transport of cholesterol from outer to inner membrane, since in this experiment, increase in loading of P450 occurs in the absence of outer membrane, i.e., in mitoplasts.

   Second, endozepine also stimulates side chain cleavage of cholesterol in mitoplasts. It is difficult to perform such experiments in inner membrane alone (i.e., in the absence of the rest of the organelle) for purely technical reasons. Preparation of inner membrane is accompanied by the loss of the electron carriers adrenodoxin and its reductase. The effect of endozepine in such preparations is greatly reduced because the membrane must be supplemented with exogenous electron carriers for side chain cleavage activity to be observed. It is therefore common practice in the field to use mitoplasts as a source of inner membrane, since in these preparations the organization of that membrane is retained.
4. P450 in an aqueous system. The studies already discussed were performed in membrane systems and this is how the side chain cleavage of cholesterol proceeds in vivo. Nevertheless, we learn something from studying the action of endozepine in a reconstituted aqueous system. When homogeneous P450 and electron carriers are incubated in buffer, endozepine stimulates side chain cleavage (Brown and Hall, 1991). Moreover, when cholesterol is added to P450 in an aqueous system, endozepine increases the type 1 difference spectrum, i.e., this protein promotes loading of the enzyme with substrate (Brown and Hall, 1991). This may in turn explain the increase in the production of pregnenolone caused by endozepine in this aqueous system (Brown and Hall, 1991). It is difficult to escape the conclusion that endozepine acts directly on the enzyme.
5. Reduction of P450 scc. It will be recalled that the second step in the catalytic cycle involves the delivery of the first electron to P450. It was also pointed out that this step can be measured by trapping reduced P450 as the P450–CO complex. It has generally been proposed that reduction of P450 is not rate-limiting for the activity of the enzyme. However, when a P450 enzyme catalyzes

more than one successive reaction in which the product of the first step becomes the substrate for the second:



this may not be true because the P450 must undergo two cycles, i.e., it must be reduced twice. In the case of the conversion of progesterone to androstenedione by a microsomal P450, the reaction stops at the 17-hydroxy intermediate in the adrenal but proceeds to androstenedione in the testis (Nakajin and Hall, 1981; Nakajin et al., 1981, 1983). It was shown in this laboratory that the concentration of electron carrier determines the extent of the second reaction (Yanagibashi and Hall, 1987). For microsomal P450 a single electron carrier called reductase is required. The more reductase the more lyase activity. Moreover, when microsomes from the two tissues are studied it turns out that the concentration of reductase is higher in testicular microsomes (two steps) than in those from adrenal (first step only) (Yanagibashi and Hall, 1987). Moreover, antibody to reductase causes testicular microsomes to act like those of the adrenal (Yanagibashi and Hall, 1987).

Side chain cleavage of cholesterol involves three steps and hence three turns of the cycle. When the rate of reduction of P450 scc was measured as described earlier, it was found that reduction was slow and was not increased by endozepine. However, when cholesterol was added, reduction was accelerated and endozepine increased the rate of reduction of the enzyme (Brown and Hall, 1991). It will be recalled that binding of cholesterol to P450 scc facilitates reduction of the enzyme.

6. Cholesterol binding. These various actions of endozepine on the mitochondrial steps in the synthesis of pregnenolone suggest that this agent exerts a number of apparently unrelated effects on the production of pregnenolone. If endozepine were to act as a cholesterol-binding protein these diverse effects could be reduced to a single action. By binding cholesterol, endozepine could shepherd this substrate through the various steps described earlier. To test this idea, fluorescence anisotropy was used with the fluorescent cholesterol analog Δ^7-dehydroesgosterol. This method can be used to avoid the low solubility and low critical micelle concentration of cholesterol that makes the usual methods of measuring specific binding of a steroid to a protein difficult or impossible. Anisotropy is an expression of loss of freedom of rotation of the analog if and when it binds a protein. The results were negative; there is no evidence that endozepine is a cholesterol-binding protein (Brown et al., in preparation).

Mechanism of Action of Endozepine

Can these various actions of endozepine be interpreted in such a way as to provide a useful working hypothesis for the molecular basis of the steroidogenic action of the protein? From the studies described earlier it would appear that the two most likely sites at which regulation by endozepine might occur are the transport of cholesterol from outer to inner mitochondrial membranes and the loading of the enzyme with substrate. Acceleration of either or both of these steps could account for increased production of pregnenolone by mitochondria and hence increased production of steroid hormones by steroid-forming cells. Moreover, the increase in entry of cholesterol into mitochondria produced by endozepine may be secondary to acceleration of later steps in the pathway to inner membrane. We can assume that once cholesterol reaches the inner leaflet of the inner membrane it can move quickly to the nearest molecule of P450. By contrast, the subsequent loading of the enzyme appears to be a possible site of regulation. If endozepine is not a cholesterol-binding protein we must ask whether it exerts one effect on mitochondria or two.

There is no reason to doubt the experimental observations on which the action of endozepine on transport of cholesterol between the two membranes is based (Yanagibashi et al., 1988). However, the mechanism of this action remains unknown and the fact that endozepine does not bind cholesterol removes the most attractive possibility. It has recently become clear that various substances can pass into the interior of the mitochondrion by way of contact points between the two membranes. Moreover, these sites of contact are probably not fixed but may represent points of dynamic, reversible membrane fusion. It has been shown that the number of such sites is related to the metabolic activity of the cells involved (Brdiczka, 1991). It is possible that when adrenal cells are stimulated by ACTH, mitochondrial membranes fuse and admit cholesterol through newly formed contact points. The cholesterol could then gain access to the inner membrane without ever stopping in the outer membrane. Moreover, preparations of both membrane fractions (outer and inner) contain contact points (Brdiczka, 1991) and since it has not been shown that the two membrane fractions can be separated following coincubation in the same way as they were initially separated for the preparation of the two fractions before coincubation, the interpretation of the findings in such experiments may not be straightforward. Nevertheless, endozepine added to intact mitochondria plus cholesterol increases the concentration of the sterol in both membranes (Yanagibashi et al., 1988). However it enters the mitochondrion, cholesterol reaches both membranes in greater amounts when endozepine is present. Moreover, coincubation of the two membranes with and without endozepine results in increase in the concentration of cholesterol in the inner membrane, as discussed previously. We must therefore conclude that endozepine promotes transport of cholesterol to the inner membrane by whatever route this substrate takes. Moreover, this action of ACTH requires the synthesis of new protein (Ohno et al., 1983; Privalle et al., 1983). Since ACTH increases the synthesis of endozepine (Yanagibashi et al., 1988), ACTH may increase transport of cholesterol to the inner membrane by promoting the synthesis of endozepine. There is no evidence to suggest that endozepine causes the formation of contact points.

As far as the effect of endozepine on loading of P450 is concerned, we are on safe ground in accepting this as a direct effect separate from any other action the protein may exert. In the first place, the type 1 spectral shift has been shown by electron paramagnetic resonance (epr) unequivocally to indicate loading of the enzyme with substrate. Incidentally, when endozepine is added to P450 scc without cholesterol, a different spectral shift is observed (unpublished). The occurrence of this different spectral shift clearly indicates that endozepine acts directly on homogeneous P450 in aqueous solution. The type 1 shift is also seen when P450 is added to mitoplasts (Hall, 1991), i.e., it occurs in the inner mitochondrial membrane so that in an organized membrane structure endozepine loads the enzyme with cholesterol and it does this with cholesterol available in the inner membrane, i.e., endozepine does not, under these conditions, require an external source of cholesterol (such as, for example, the outer mitochondrial membrane). Evidently endozepine binds to P450 scc and the result of this binding is enhanced loading of the enzyme with substrate, increase in the rate of reduction of the enzyme, and, hence, increased production of pregnenolone.

The Role of Benzodiazepine Receptor

The studies just reviewed were performed with endozepine—either that extracted from adrenal or brain or the protein genetically engineered by Jens Knudsen (Odense, Denmark). Actions of endozepine in whole cells or mitochondria might be attributed to binding to the benzodiazepine receptor in the outer mitochondrial membrane and, as discussed earlier the receptor, with or without bound ligand, may be responsible for the effects observed (Krueger and Papadopoulos, 1990). Alterna-

tively, endozepine itself may act directly without the intervention of a receptor. In this case endozepine must act by a mechanism different from that used by benzodiazepines. Much will depend on the outcome of detailed studies of specific binding of endozepine to mitochondria and the proof that the sites of binding are the benzodiazepine receptors. Benzodiazepines stimulate transport of cholesterol from outer to inner mitochondrial membranes (Yanagibashi et al., 1989a,b; Papadopoulos et al., 1990). This important observation merits careful attention and raises three crucial points. First, this response to benzodiazepines does not occur in mitoplasts, i.e., mitochondria without outer membranes (Yangibashi et al., 1989b). This in turn raises two points. On the one hand, this means that the inner membrane does not contain benzodiazepine receptors or at least not in such numbers and in such condition as to respond to direct addition of these drugs. Presumably the action of benzodiazepines on the transport of cholesterol must be exerted at the outer membrane. On the other hand, this is quite different from endozepine, which acts in mitoplasts (Hall, 1991) and also acts on soluble P450 where there is no question of receptors being present. The second point to be considered in these studies is related to the first, namely treatment of adrenal mitochondria with digitonin abolishes stimulation of side chain cleavage by benzodiazepines—presumably by destroying an outer membrane receptor. It will be of interest to determine whether or not digitonin abolishes the mitochondrial responses to endozepine. Presumably it does not because endozepine acts on mitoplasts so that unless digitonin exerts additional effects, e.g., on the inner membrane, it should not abolish the effect of endozepine on inner membrane. In any case, we are forced to conclude that the action of endozepine on soluble P450 scc itself cannot involve the benzodiazepine receptor.

The third point raised by the studies of Papadopoulos et al. (1990) is that cycloheximide does not inhibit the responses to PK-11195, which is an agonist of benzodiazepine receptors. This suggests that the inhibitory action of cycloheximide is exerted before the involvement of the receptor and yet cycloheximide inhibits the increase in transport of cholesterol from outer to inner membrane that occurs under the influence of ACTH, as discussed previously (Ohno et al., 1983; Privalle et al., 1983). Clearly, a newly synthesized protein is necessary for this effect and the new protein cannot be the benzodiazepine receptor. Evidently, the effect of ACTH on transport of cholesterol requires synthesis of new proteins(s) (presumably including endozepine), whereas the response to PK-11195 does not. This would suggest that the benzodiazepines stimulate steroidogenesis by a mechanism that differs from the action of endozepine on this process. It should be noted that this does not exclude a role for the benzodiazepine receptor in the response to ACTH. It will be important to determine whether or not endozepine plus benzodiazepines exerts a greater effect than either alone.

These complex and confusing findings can be most readily reconciled by proposing two actions of endozepine. The first results in accelerated transport of cholesterol to the inner mitochondrial membrane and may or may not be exerted via the benzodiazepine receptor. The second action is not shared with benzodiazepines but results from a direct effect of endozepine on P450 scc, which leads to increased binding of substrate, which in turn promotes reduction of P450 scc and hence increased side chain cleavage and increase in the production of steroid hormones.

Summary and Conclusions

Endozepine may be thought of as a peptide with at least two groups of actions. First, the protein binds $GABA_A$ receptors in the CNS where its effect can be described as anxiolytic with, no doubt, other important actions. In this capacity the protein exerts inhibitory effects by stabilizing membrane potentials through interaction with a Cl^- channel associated with

the GABA receptor. Benzodiazepines exert other actions in the nervous system that involve a different receptor not associated with a Cl^- channel. These responses are diverse and are exerted through the type B or so-called peripheral receptor. These actions may lead to the synthesis of steroids by the responding neural cells and may open the exciting possibility of autocrine and paracrine responses to steroids in the CNS. It is not clear that endozepine shares these effects, which occur in the nervous system by way of $GABA_B$ receptors. It may turn out that the action of endozepine in neural cells employs the same mechanism as that used in steroidogenic cells.

The second action of endozepine involves increase in the mitochondrial synthesis of pregnenolone. This response involves increased delivery of cholesterol to the side chain cleavage enzyme in the inner membrane. It is clear that transport of cholesterol to that membrane is regulated by ACTH and LH in their respective target organs. What is not clear is whether the cholesterol proceeds from outer membrane through intermembrane space to inner membrane or whether it is admitted to the interior of the organelle via contact points between the two membranes. Such contact points appear to form reversibly in relation to the relevant metabolic activities within steroidogenic and other cells. By whatever means cholesterol reaches the inner steroidogenic membrane, there is good reason to believe that endozepine accelerates this process of transport. To this point, the action of endozepine is shared by benzodiazepine drugs that act through the peripheral GABA receptors ($GABA_B$). The simplest proposal for the mechanism of this shared effect on cholesterol transport would suggest that endozepine and benzodiazepines converge on a common receptor in the outer mitochondrial membrane, which is then responsible for promoting transport of cholesterol within the mitochondrion. Workers in the field will be relieved if endozepine does act via $GABA_B$ receptors, although this had yet to be demonstrated and moreover, whereas endozepine may mediate the action of ACTH on steroidogenic mitochondria, this does not appear to be the case with benzodiazepines.

Unlike benzodiazepines, endozepine acts on the inner membrane by a second very different mechanism as the protein appears to bind to P450 scc and promotes binding of cholesterol to the active site of the enzyme. This in turn promotes reduction of P450 scc. Reduction of the heme group of the enzyme to the ferrous form enables it to activate oxygen that, in this activated form, attacks the side chain of cholesterol as the abbreviation P450 scc suggests. This results in increased synthesis of pregnenolone. Since the production of this steroid is rate-limiting for the synthesis of steroid hormones, these changes result in increased production of the secreted steroids. Since the effect of endozepine on loading of P450 with substrate is seen with pure enzyme in an aqueous system, no receptor is necessary for this effect.

These two mechanisms, by which endozepine acts on steroidogenic mitochondria, are strange in several respects. For example, promoting movement of cholesterol from membrane to membrane without binding this steroid is not easy to imagine. Again, loading an enzyme with its substrate is an unusual mechanism of regulation. Whether these actions are strange or not, it is hard to see how they can be related to the many other actions of the protein on other peripheral tissues, e.g., those of the gastrointestinal tract. Perhaps the first challenge in the study of endozepine is to find ways in which such diverse effects can be reduced to some common molecular mechanism or mechanisms that will make the many responses seen in the whole animal easier to understand.

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