Stimulation of Adrenal Mitochondrial Cholesterol Side-chain Cleavage by GTP, Steroidogenesis Activator Polypeptide (SAP), and Sterol Carrier Protein

GTP AND SAP ACT SYNERGISTICALLY*

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Several factors are known to stimulate cholesterol side-chain cleavage in isolated adrenal mitochondria, including steroidogenesis activator polypeptide (SAP), GTP, and sterol carrier protein (SCP). All of these reportedly function at the level of the translocation of cholesterol to the inner membrane wherein side-chain cleavage to form pregnenolone occurs. We have investigated the activating effects of these factors alone and in combination. Under conditions where exogenous cholesterol is provided and multiple turnovers of a transport system are required, GTP stimulated steroidogenesis in isolated mitochondria and in adrenal homogenates, and this effect was enhanced by a GTP regenerating system. SAP alone had little effect under these conditions, but synergized with GTP to stimulate cholesterol metabolism. A truncated SAP analog and a variant from the C terminus of the minor heat-shock protein GRP78 had similar effects, but an unrelated peptide had no effect. GTP stimulated side-chain cleavage with the same EC₅₀ in both resting mitochondria (from dexamethasone-treated rats) and in activated mitochondria (from ether-treated rats), but SAP effects were most apparent in resting mitochondria. In contrast, SCP stimulation was additive with other factors, suggesting an independent mechanism of action. While the data are consistent with biological roles for these factors, the relatively small magnitude of the in vitro effects may indicate that cell disruption and mitochondrial isolation disrupt important structural or other features which are necessary for the full expression of the steroidogenic response.

ACTH stimulates cleavage, resulting in an increase in adrenal glucocorticoid production within minutes or less. In addition to rapid activation, ACTH has long-term (hours to days) effects on gene expression of steroidogenic enzymes, and therefore regulates the steroidogenic capacity of the tissue; the present studies relate to the short-term regulation by ACTH. Several features of the mechanism by which ACTH acutely regulates side-chain cleavage are well characterized. ACTH binds to its receptor on the plasma membrane and activates adenylate cyclase by conventional transmembrane signalling mechanisms, resulting in the generation of cytosolic cyclic AMP. While the exact mechanisms of cyclic AMP-mediated stimulation have been the focus of recent investigations, it seems clear that the locus of action of the cycloheximide-sensitive "labile protein" mediator is within the mitochondrion (8). Both steroidogenically activated and non-activated mitochondria can be isolated (9), depending on the pretreatment of the cell or animal in such a way as to activate or inhibit steroidogenesis. Specifically, stress (including treatment with ether, referred to as "ether stress") can be used to stimulate the release of endogenous ACTH from the pituitary to promote steroidogenesis (10), and dexamethasone treatment can be used to block ACTH release and therefore steroidogenesis.

The acute cycloheximide-sensitive control of cholesterol side-chain cleavage does not seem to involve regulation of the activity of cytochrome P450 cm itself. Rather, several lines of evidence point to intramitochondrial cholesterol "translocalization" (usually equated with movement of cholesterol to the inner membrane or to cytochrome P450 cm) as the regulated mitochondrial step (1, 11-15). Activation may involve a barrier to cholesterol utilization imposed by the structure and transport systems of the mitochondrion. In one interpretation (1), the mitochondrial intermembrane space provides an aqueous barrier that minimizes the access of the water-insol-

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1 The abbreviations used are: ACTH, adrenocorticotropic hormone; SAP, steroidogenesis activator polypeptide; SCP, sterol carrier protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; FPLC, fast-protein liquid chromatography.
uble substrate to the inner membrane wherein cytochrome P450c, is uninduced. Such a mechanism may account for the high rate of side-chain cleavage of more water-soluble derivatives of cholesterol (22-3S-hydroxycholesterol and 25-hydroxycholesterol) (16-18) which are expected to bypass the normal mitochondrial cholesterol transport systems. A number of candidates for the mediator(s) which regulate the androgenic response to ACTH have been identified during the past decade, based upon their ability to stimulate cholesterol side-chain cleavage in isolated adrenal mitochondria. Sterol carrier protein (SCP) augments cholesterol side-chain cleavage, reportedly by stimulating the movement of cholesterol to the inner mitochondrial membrane (19). However, SCP may not mediate the action of cyclic AMP, since its level does not change with ACTH or stress, nor does it decline acutely with inhibition of protein synthesis (20). Pedersen, Brownie, and colleagues isolated (21) and sequenced (22 a 3.2-kDa polypeptide (30 residues), termed steroidogenic activator polypeptide (SAP), which stimulated side-chain cleavage activity in isolated adrenal mitochondria from cycloheximide-treated rats. The level of SAP increased rapidly with ACTH or cyclic AMP treatment, and its production was blocked by cycloheximide (21, 23). The structure of SAP is almost identical with the C-terminal 29 amino acids (residues 608-636) of the 78-kDa minor heat-shock protein, glucose-regulated protein 78 (GRP78). The latter, differing in only 2 residues (24-28). The peptide represented by this sequence is referred to herein as GRP78(608-636), and may represent the physiologically relevant form of SAP released by proteolysis from GRP78. In addition, we recently reported that GTP (but not other nucleotides) stimulates cholesterol side-chain cleavage in isolated rat adrenal mitochondria, and data implicate cholesterol translocation as the affected step (27). GTP binding proteins participate in a variety of intracellular transport/ trafficking processes (28), and we have proposed that GTP participates in this manner in cholesterol translocation to or within the adrenal mitochondrion. Despite the activation of steroidogenesis by most of these factors, the magnitude of most of the effects is relatively small, and in some cases has proven difficult to reproduce. The present studies were undertaken to test whether two or more of these factors, all of which are reported to act to stimulate cholesterol translocation, might interact synergistically to stimulate mitochondrial cholesterol side-chain cleavage. The results indicate that SAP and GTP modulate steroidogenesis synergistically, but that SCP acts additively with the other factors.

EXPERIMENTAL PROCEDURES

Materials—Cholesterol was purchased from Applied Science Laboratories (Deerfield, IL), and pregnenolone was obtained from Steraloids (Winston, NH). Antiserum specific for pregnenolone was purchased from Radioassays Systems Laboratories, Inc. (Carson, CA). [7-3H]Pregnenolone (13 Ci/mmol) was from Du Pont-New England Nuclear. Cyanoketone (2-cyano-4,4,17-cis-trimethyl-17-5-hydroxyan- drost-5-en-3-one) was a gift of the Sterling-Winthrop Research Institute (Rensselaer, NY). Adenylate kinase, dioxiodo ETDA, GTF, ATP, sodium lactate, phosphoethanolamine, Hepes, oxaloacetic acid, 5,5'-dithiobis(nitrobenzoic acid), and activated charcoal were purchased from Sigma. Dichloromethane, diethyl ether, and perchloric acid were from Mallinckrodt, Inc. Trichloroacetic acid (TCA) was from Pierce Chemical Co. Dexamethasone 21-phosphate (sodium salt) was from Baxter. Sep-Pak cartridges (C18) were purchased from Millipore Corporation. SCPs, isolated from bovine liver according to (29), was a gift from Dr. Richard Crain. SAP and the SAP analogs N-acetylsAP (11-30) and GRP78(608-636) were synthesized using solid-phase methods, purified by HPLC, and the structures confirmed as described previously (30). The SAP sequence was IVQPIIKLYGSGGPPPTGEEDTSEKDEL, differing from SAP by containing a serine substitution for aspartic acid and missing one lysine.

Treatment of Animals and Isolation of Mitochondria—Female rats (Sprague-Dawley, 160-230 g) were ether-stressed 15 min prior to sacrifice by decapitation, or were injected intraperitoneally with diethylstilbestrol phosphate (0.01 mg body weight) (26). Adrenals were removed and dissected free of surrounding fat, and mitochondria were prepared as described previously (15), except that the buffer used for homogenization and centrifugation steps consisted of 0.25 m sucrose, 1 mM EDTA, 25 mM Hepes, and 10 mg/ml bovine serum albumin, at pH 7.0. After isolation by differential centrifugation, mitochondria were resuspended in the same buffer but with bovine serum albumin, recenterifuged, and finally resuspended in incubation buffer (125 mM KCl, 5 mM MgCl2, 10 mM KH2PO4, 25 mM Hepes, 0.2 mM EDTA, at pH 7.0). Changes in mitochondrial integrity under incubation conditions were assessed in representative samples as described previously (27) by release of the matrix marker enzyme malate dehydrogenase and the intermembrane space marker enzyme adenylate kinase. After 60 min of incubation with malate, mitochondria from both ether-stressed and diethylstilbestrol-treated groups retained approximately 90% of malate dehydrogenase activity and 80% of adenylate kinase activity, indicating that the preparations were largely intact during prolonged incubations. No significant difference in specific activity for either marker was seen in mitochondria from the two pretreatment groups. Citrate synthase was measured by the appearance of the free sulfhydryl group of the CoASH released from malonate-CoA, quantified with 5,5'-dithiobis(nitrobenzoic acid) (31). Protein was assayed according to the Bio-Rad method (32).

Mitochondrial Incubations and Pregnenolone Assay—When the GTP-regenerating system was used, incubation buffer containing 5.4 mM acetate phosphate, acetate kinase (8 units/ml), GTP (75 pmol unless otherwise stated) was incubated for 5 min at 37°C along with 10 μM cyanoketone and the indicated concentrations of activating factors (SAP or SCP). Mitochondria (0.5 mg/ml final) were then added along with cholesterol (0.2 mM final, added from a concentrated stock in ethanol to give a final ethanol concentration of less than 0.5%), and the preincubation in a total volume of 50 μl was continued for an additional 5 min prior to the addition of L-malate (4 mM final). Aliquots (50 μl) were removed at the indicated times following malate addition, and pregnenolone was quantified by radioimmunoassay, according to (15).

Analysis of GTP by FPLC—Mitochondria from ether-stressed rats were incubated with regenerating system and 1 nM GTP. At specified times, aliquots (50 μl) were transferred into a test tube containing 70 μl of 7.2% perchloric acid and mixed by vortexing. Samples were centrifuged and the supernatant was neutralized with NaOH. Aliquots were analyzed by FPLC using a Pharmacia LKB Biotechnology, Inc. Mono Q HPLC column (5/5 column). Samples were eluted with a linear gradient from 0 to 0.2 M Na2SO4/5 mM Hepes at pH 7.4. Nucleotide concentrations were monitored by absorbance at 254 nm. The GTP concentration was determined from the peak area, and the percentage remaining was expressed relative to the total content of GTP + GDP + GMP + guanosine.

Induction of Steroidogenesis Activator Polypeptide and Quantitation in the Incubation—Purified SAP was iodinated by the chloramine-T method using Na125I, and the product was purified from unreacted SAP by HPLC (30). The major radioactive and UV-absorbing peak on HPLC had an absorbance maximum at 252 nm which is indicative of a monoiodo-thyrosine residue. The iodinated peptide was shifted to a longer HPLC retention time than SAP, as expected for a more hydrophobic molecule (30). Purified 125I-SAP was diluted with nonradioabeled monoiodo-SAP to a specific activity of 8200 mCi/mmol and then incubated with isolated adrenal mitochondria and adrenal homogenate. Aliquots (100 μl) were removed at specific times and added to 900 μl of 10% trichloroacetic acid, mixed by vortexing, and centrifuged (9000 x g x 2 min). The supernatant was loaded onto a Sep-Pak cartridge, which was washed with 5 ml of 0.1% aqueous trifluoroacetic acid and then the radiolabeled material was eluted with 5.5'-dithiothreitol (10% final). The solution was applied to a C8 HPLC column. The column was washed with 40 μl acetonitrile for 1 min (1.2 ml/min flow rate throughout), and the sample was eluted with an aqueous gradient from 14 to 32%. Over 3 min the percentage was raised to 26% (linear) to elute a front-running degradation peak. Over the next 11 min the gradient was increased to 32% in hyperbolic manner in
order to separate SAP and a closely eluting degradation product. Intact $^{125}$I-SAP and its radiolabeled degradation products were detected using a Packard Cobra Auto-Gamma counter. Intact SAP eluted at 17.0 ml in this HPLC system, while two major degradation products resulting from incubation eluted at 5.4 and 14.1 ml.

**RESULTS**

Development of a GTP Regenerating System—We previously described the presence of a GTP hydrolase activity in adrenal mitochondria which complicated the interpretation of experiments at low GTP concentration and/or extended preincubation times (27). In the present studies, we therefore developed a GTP regenerating system consisting of acetyl phosphate and acetate kinase. Using an FPLC method as described under “Experimental Procedures,” we observed that under the incubation conditions used there was a complete disappearance of 1 mM GTP by 60 and 90 min, but that more than half of the GTP remained at these times in the presence of the recycling system (data not shown).

As shown in Fig. 1, in the absence of GTP, mitochondria from ether-stressed rats showed higher side-chain cleavage activity than those from dexamethasone-treated animals. GTP (75 μM) augmented the activity of both preparations. The components of the regenerating system alone had no effect on activity, but augmented the stimulatory effect of GTP. The effect was predominantly on the rate of side-chain cleavage during the 30–90 min portion of the time course, and in separate experiments (not shown), there was a little if any effect on the early phase of steroidogenesis. This result differs from our earlier report (27) in which mitochondria were incubated for 30 min with GTP, and rapid phase steroidogenesis was stimulated within the first 20 min. We suggest that the difference reflects the long preincubation used in the earlier studies, which is likely to result in accumulation of cholesterol in the steroidogenic pool. The short preincubation protocol used in the present study is designed to detect the actual rate of cholesterol movement into the steroidogenic pool rather than cholesterol accumulated there during the preincubation.

Concentration Dependence for GTP Stimulation of Mitochondrial Pregnenolone Synthesis in Ether-stressed and Dexamethasone-treated Rats—Fig. 2 shows the effect of GTP on pregnenolone generation at 60 min following initiation of the reaction. In the absence of GTP, there was an approximately 2-fold difference in cholesterol side-chain cleavage activity in mitochondria from dexamethasone- and ether-treated animals. GTP stimulated pregnenolone generation about 2-fold for both preparations, thus maintaining the initial difference in activities. For both types of mitochondrial preparations, the $K_M$ for GTP was about 10 μM. Thus, the activation state of the mitochondrion does not affect significantly the sensitivity of the system towards GTP. The $K_M$ observed in the presence of the GTP recycling system was more than an order of magnitude lower than the value which we reported previously (27) in the absence of the system, presumably due to depletion of GTP in the earlier case.

GTP Synergy with Steroidogenesis Activator Polypeptide (SAP)—We tested the effects of SAP and GTP alone and in combination on steroidogenesis in mitochondria from dexamethasone-treated rats, both in the absence and presence of a GTP regenerating system (upper two panels, Fig. 3). For these studies, 1 μM SAP was used, since this concentration was previously reported by Pedersen and Brownie (21) to be maximally activating. Our studies differ from those of Pedersen and Brownie in several respects, including animal pretreatment, time of incubation, and the presence of exogenous cholesterol in the incubation, and these differences may account for differences in results obtained (see “Discussion”). To insure that SAP, which is thought to turn over rapidly, had not been completely degraded during the course of the incubation, $^{125}$I-SAP was incubated with mitochondria under the same conditions as the activity measurements. When $^{125}$I-SAP was incubated with isolated adrenal mitochondria or homogenate, two main radiolabeled degradation peaks were observed by HPLC, both of which eluted earlier than intact iodo-SAP suggesting that they are more hydrophilic. SAP degraded slowly in both preparations with a $t_{1/2}$ of about 45 min. Therefore, the small steroidogenic effects seen with SAP were not due to its rapid degradation. Using mitochondria from dexamethasone-treated rats, there was no stimulatory effect of 1 μM SAP on pregnenolone formation in the absence of GTP. GTP alone had a stimulatory effect as shown above. However, SAP, when combined with GTP, consistently caused a 25–90% additional stimulation over that seen with GTP alone. This effect occurred both in the absence and the presence of the GTP recycling system, as shown in the left and right panels, respectively. Although the magnitude of the SAP effect was rather small, the effect was highly reproducible, and was observed in more than 20 experiments. An
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Fig. 2. Concentration dependence for GTP stimulation of pregnenolone synthesis in rat adrenal mitochondria. Conditions and treatment groups are as in Fig. 1. GTP was varied in the presence of a GTP regenerating system as indicated, and pregnenolone was assayed 60 min following initiation of the reaction with L-malate. Error bars represent the standard error from five experiments for the ether-stressed group (ES), and three experiments for the dexamethasone-treated group (DEX).

Identical concentration of a nonrelated synthetic peptide, PKI(5–22) amide with the sequence TTYADFIASGRTG-RRNAI-NH₂ (39), had no effect on pregnenolone formation under any conditions, including the presence of GTP (data not shown). Stimulation by exogenously added SAP was not reliably observed in mitochondria from ether-stressed rats (vide infra).

Because the magnitude of the SAP effect was small, we reasoned that a factor or factors might have been partially lost during the mitochondrial isolation which could participate in activation. We therefore carried out the same experiments on homogenates of adrenals from dexamethasone-treated rats. Qualitatively the same effect was observed (lower two panels, Fig. 3); SAP stimulated steroidogenesis only in the presence of GTP. In this setting, however, the magnitude of the effect was somewhat larger, with the combination of SAP plus GTP producing nearly a 2-fold increase in the rate of pregnenolone synthesis over that with GTP alone.

Pregnenolone formation in homogenates and mitochondria was initially expressed per mg of protein. Since the protein content in homogenates was higher than that in mitochondria, it was not possible to compare directly the activities in the two systems, e.g. to determine whether extramitochondrial fractions might contain an activating factor in addition to SAP. To compare directly the activities in homogenates and isolated mitochondria, two approaches were taken. In one experiment, four dexamethasone-treated rats were sacrificed; two left and two right adrenals from the animals were then used to prepare either homogenates or mitochondria, and the same experiment as in Fig. 3 was carried out. In all groups (no activator, GTP, GTP plus SAP) the total activity was approximately 2-fold higher in the homogenate than in isolated mitochondria. This could have been due either to the presence of an activator in the cytosol, or may have reflected in part the loss of mitochondria during preparation. To further test this possibility, the data in Fig. 3 were normalized to the content of citrate synthase, an enzyme located exclusively in the mitochondrion. When this was done, the specific activity per unit of citrate synthase was 2–3-fold higher in the homogenate than in isolated mitochondria, suggesting that an additional activating factor other than GTP or SAP was present in the homogenate but not in the isolated mitochondria.

We also carried out a concentration dependence for SAP effects on isolated mitochondria, both in the absence and presence of GTP (Fig. 4). No stimulatory effect of SAP was seen in the absence of GTP. In the presence of GTP, SAP stimulated pregnenolone synthesis with half-maximal effects at about 50 nM and with maximal effects in several experiments seen in the range of 0.2 to 1 μM. We also tested the effects of high concentrations of SAP. At concentrations greater than 50 μM, SAP inhibited pregnenolone synthesis both from cholesterol and from 22-hydroxycholesterol (data not shown), suggesting a nonspecific inhibitory effect at high concentrations. The SAP analog GRP78(606–636) was also tested. This peptide stimulated pregnenolone synthesis in the same concentration range and to the same degree as SAP, except that no inhibition at high concentrations was noted.

Additive Effects of GTP and SCP2—SCP2 has been previously reported to stimulate steroidogenesis in isolated adrenal mitochondria (19). In the present studies, we wished to test whether the presence of SCP2 affected the stimulatory activity...
of GTP and/or SAP. We first evaluated the concentration dependence for SCP₂, both in the absence and presence of GTP, using mitochondria from dexamethasone-treated rats. As shown in Fig. 5 and as reported previously (19), in the absence of other factors, stimulation was linearly dependent on SCP₂ concentration, and activity had not begun to saturate by 40 μM. Due to limited amounts of material, the concentration dependence was not extended above this value. As shown above, in the absence of SCP₂, GTP stimulated steroidogenesis by about 30-40%. At each concentration of SCP₂, the amount of extra pregnenolone formed in the presence of GTP was constant, indicating that the stimulatory effects of GTP and SCP₂ are additive. It should also be pointed out that at high concentrations, the magnitude of the stimulatory effect of SCP₂ was larger than that of any of the other factors, alone or in combination (see below).

Combined Effects of GTP, SAP, and SCP₂ on Adrenal Mitochondrial Steroidogenesis—The effects on pregnenolone formation of all three activating factors, alone and in combination, are shown in Figs. 6 and 7. Fig. 6 shows the time course up to 90 min in preparations of mitochondria from dexamethasone-treated animals, and demonstrates that the activity effects are seen at all three time points. The actions on pregnenolone formation of GTP alone, SAP alone, and both GTP and SAP together were generally similar in either the presence or absence of SCP₂. Fig. 7 summarizes data from additional experiments at both 30 min (upper panel) and 60 min (lower panel) following initiation of side-chain cleavage. Solid and open bars show pregnenolone formed in preparations from dexamethasone-treated animals, and ether-stressed animals, respectively. Results from Fig. 7 can be summarized as follows. As observed herein, GTP stimulated pregnenolone synthesis regardless of the pretreatment of the animals. SAP stimulated cholesterol side-chain cleavage exclusively in the presence of GTP in preparations from dexamethasone-treated animals, but had no reliable effect in mitochondria from ether-stressed mitochondria. In neither case did SAP activate in the absence of GTP. SCP₂ augmented pregnenolone formation in both types of preparations. Its effects can best be interpreted as being additive with other factors. When two or more activating factors are present, the differences between the activated and resting mitochondria were minimized, in particular at longer time points. Quantitatively similar results were seen in the presence and absence of GTP and SCP₂ using the NH₄¹-termally truncated peptide analog N-acetyl-SAP¹¹–30 and the GRP78 C-terminal peptide GRP78(606–636). Again, there was no effect of the same concentration of the control peptide, PKI(5–22) amide (33). Similar results using combinations of GTP, SAP, and SCP₂ were also seen in adrenal homogenates from dexamethasone-treated and ether-stressed rats (data not shown).

DISCUSSION

We investigated the steroidogenic activating effects and possible mutually facilitating interactions among three factors that have been implicated as regulators of cholesterol side-chain cleavage activity in adrenal cortex mitochondria. All of these effectors, GTP, SAP, and SCP₂, have been implicated...
as stimuli affecting the movement of cholesterol into the steroidogenic pool. Hence, it seems reasonable that they may affect the same cholesterol translocation "machinery" in steroidogenic mitochondria.

One conclusion from the present studies is that SAP and GTP function synergistically. These results are consistent with an effect on the same process (e.g. some aspect of cholesterol translocation). The mechanism of activation by SAP did not involve an effect on the sensitivity of mitochondria for GTP, since the $K_{m}$ for GTP was not affected by the activation state of the mitochondrial preparations (i.e. pre-treatment of rats with ether versus dexamethasone) or by their preincubation with SAP. GTP produced about a 2-fold enhancement of pregnenolone synthesis regardless of the activation state of the mitochondria.

In our experiments, stimulation by SAP showed an absolute requirement for the presence of GTP. The lack of effect of SAP alone was not due to degradation of the peptide during the incubation, since about half of added $^{3}H$-SAP remained intact under the same conditions. The dependence of the action of SAP on GTP which was seen in at least 20 experiments, appears contrary to an earlier report (21) in which stimulation of pregnenolone formation by SAP was seen in the absence of added GTP. It is possible that these different results may be attributed to differences in experimental conditions, and we offer some possible explanations. Our experiments were designed to detect effects of steroidogenic factors under multiple turnover conditions of cytochrome P450. For this reason, exogenous cholesterol was provided, and the time of incubation was 90 min, long enough to detect stimulated transport of this substrate into the steroidogenic pool. Also, we observed our most consistent responses and the lowest basal activity in mitochondria from dexamethasone-treated animals. In contrast, Pedersen and colleagues used mitochondria from cycloheximide-treated rats ("cycloheximide mitochondria") to detect the stimulatory effects of SAP. These mitochondria have accumulated cholesterol in their outer membranes (12), and this endogenous rather than exogenous cholesterol was utilized as the substrate. In our experience, we have not been able to reliable obtain cycloheximide mitochondria with low basal activities. Although we have not been able to discover meaningful differences in mitochondrial preparative methods, it is possible that our failure to observe SAP effects in this setting may have been due to the higher basal side-chain cleavage activity in our preparations which masked the enhancing effects of SAP. In addition, in the Pedersen studies, the preincubation of mitochondria with SAP may have allowed accumulation of cholesterol in the steroidogenic pool, thus accounting for the rapid burst of pregnenolone synthesis (1–2 nmol/mg of protein within 1–2 min). In our studies, the effects of factors were seen only at longer time points (30–90 min), and the total product formation was considerably higher. The pregnenolone production rates in this setting should reflect the rate of transport of cholesterol into the steroidogenic pool rather than prior accumulation of steroidogenically accessible cholesterol. If one accepts both sets of results, one possible mechanistic explanation is that under Pedersen's conditions it was possible to observe a SAP-stimulated single turnover of the cholesterol translocation system, but the system failed to recycle in such a way as to produce a longer term activation. GTP may be required for the system to recycle in order to transport additional molecules of cholesterol, accounting for our observation of longer term SAP effects.

Despite the encouraging effect of SAP and the observed specificity for SAP-related structures, the magnitude of the SAP effects (20–30% over the GTP rates) were disappointing. It is possible, of course, that the small magnitude reflects a lack of biological significance. Nevertheless, Mertz and Pedersen (23) have demonstrated that this peptide occurs only in steroidogenic tissues, and that its levels and kinetics of formation and disappearance correlate with steroidogenesis under a variety of natural and pharmacologically manipulated conditions. There is thus a strong circumstantial if not a causal case for a role for SAP. We are inclined, therefore, to seek explanations for the apparently small magnitude of the effect, rather than to discount its importance. We feel that it is plausible that the standard biochemical approaches which are used may prevent the full expression of the effect. For example, isolated mitochondria may have been removed from other potential steroidogenic factors present in the cytosol or elsewhere in the cell. In this context, Hall and coworkers (34, 35) have isolated an 8.2-kDa steroidogenic stimulatory protein from bovine adrenal cortex, the structure of which is essentially identical with bovine endozepine, a natural benzodiazepine receptor agonist. Consistent with the interpretation of additional factor(s), the use of homogenates rather than isolated mitochondria resulted in an improvement in the magnitude of the response to SAP, although the effect remained fairly small compared to the response to ACTH seen in intact cells. It may also be that the process of homogenization either damages mitochondria, rendering them less responsive than those in an intact cell, or removes them from a larger structural environment which is necessary for an optimal response. For example, mitochondria reportedly are associated with cytoskeletal elements which are partially destroyed upon homogenization (36, 37). The response to ACTH is reportedly lost after treatment of cells with agents which disrupt the cytoskeleton (38–40). We are currently undertaking approaches which better maintain the mitochondrion within its normal cellular milieu for additional analysis of the actions of SAP.

In contrast to the synergistic activation by GTP and SAP, the effects of SCP₂ appeared to be largely additive with other factors. This protein is known to stimulate a number of cholesterol-related reactions, presumably by transporting cholesterol or its metabolites to relevant enzymes. In addition to its cholesterol translocating effect, this protein is known to promote the transfer of a variety of other lipids in an apparently nonspecific manner (29), and changes in lipid environment may affect steroidogenic activity (e.g. see Ref. 41). The mechanism by which SCP₂ stimulates side-chain cleavage activity in adrenal mitochondria is unknown, but is thought to involve transport of cholesterol to the steroidogenic pool (20). It is difficult to understand how when added exogenously, this 14-kDa protein might enter the mitochondrial intermembrane space and translocate cholesterol from outer to inner membrane. It seems more likely that SCP₂ moves cholesterol from inaccessible sites (e.g. on membrane fragments or nonaccessible regions of the outer membrane) into specialized regions of the outer membrane which may act as conduits for steroidogenic cholesterol. In this context, it has been suggested (1, 42) that cholesterol transport may occur via contact sites between the inner and outer membrane. It should also be noted, however, that in liver, a portion of the SCP₂ is located in the mitochonden, as judged by immunogold electron microscopy (43). Regardless of the mechanism of SCP₂, this factor does not appear to act via the same mechanism as SAP and GTP. SCP₂ therefore may play a role in movement of extramitochondrial cholesterol rather than regulating intramitochondrial cholesterol movement. In summary, GTP and SAP act synergistically, perhaps by both
affecting the cholesterol translocation machinery, while SCP₂ acts by a distinct mechanism.

REFERENCES