Stabilization and Solubilization of Bovine Corpus-Luteum Adenylate Cyclase

THE EFFECTS OF GUANOSINE TRIPHOSPHATE, GUANOSINE 5'-[β , γ -IMIDO]TRIPHOSPHATE, SODIUM FLUORIDE AND TRIS/HYDROCHLORIC ACID CONCENTRATION ON ENZYME ACTIVITY

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1. Adenylate cyclase of the washed 600g sediment of bovine corpus-luteum homogenate is stimulated by p[NH]ppG (guanosine 5'-[β , γ -imido]triphosphate), the imido analogue of GTP, and to a lesser extent by GTP itself. Activation by p[NH]ppG is not reversed by extensive washing before assay, but can, however, be reversed by NaF. 2. Both p[NH]ppG and NaF stabilize the enzyme during incubation at 37°C. NaF also causes an irreversible activation, but only of part of the potentially NaF-activatable adenylate cyclase; there are possibly two components of the adenylate cyclase system, which can be distinguished by their response to NaF. 3. Solubilization of the adenylate cyclase activity in the 600g sediment, by using the non-ionic detergent Lubrol-PX, gave variable yields. A relationship between the magnitude of NaF stimulation of the 600g-sediment enzyme and the yield of soluble activity derived from the sediment was recognized. The results suggest that the pre-existing state of the enzyme complex *in vivo* is reflected by the response *in vitro* to NaF and may determine the success with which activity can be solubilized. 4. The absolute yields of soluble activity could be increased by p[NH]ppG preactivation of the 600g sediment. During the development of the maximally active state by preincubation with p[NH]ppG the enzyme passes through a stage in which Lubrol solubilization is increased, but the maximally active state is itself less amenable to solubilization. p[NH]ppG activation causes the appearance of NaF-inhibited states, which appear to be preferentially solubilized by Lubrol-PX.

An understanding of the molecular mechanisms by which hormones activate the membrane-bound enzyme adenylate cyclase requires the reconstitution of a hormone-responsive unit from the solubilized components of the hormone-receptor-transducer-adenylate cyclase complex and other membrane constituents. Purification and characterization of adenylate cyclase, with this end in mind, is complicated by the lability of the enzyme activity during the solubilization procedure. However, neutral detergents, notably Lubrol-PX, have been successfully used to give high yields of soluble adenylate cyclase activity from a variety of tissues (Levey, 1970, 1971; Johnson & Sutherland, 1973; Swislocki & Tierney, 1973; Wilkinson & Stansfield, 1974). The extent to

which the adenylate cyclase activity in the membrane preparation can be recovered in a solubilized form is quite variable, where published data allow such calculations to be made.

We have previously reported both unsuccessful and successful attempts to solubilize the adenylate cyclase activity of rat corpora lutea (Stansfield *et al.*, 1972; Wilkinson & Stansfield, 1974). Our present studies are directed towards solubilization of the adenylate cyclase from the washed 600g sediment of bovine corpus-luteum homogenate by using Lubrol-PX. Initial attempts met with varying degrees of success. Analysis of the data, however, revealed a relationship between the percentage relative activation of the enzyme in the 600g sediment by NaF and the amount (%) of the 600g-sediment activity, both basal and NaF-stimulated, that is solubilized by Lubrol-PX. The results suggest that the degree to which activity is retained, or even enhanced, after

Abbreviation used: p[NH]ppG, guanosine 5'-[β , γ -imido]-triphosphate.

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solubilization may be determined by the pre-existing state of activation of the enzyme achieved *in vivo*. Furthermore, it is possible that the particulate enzyme complex exists in several conformational states which may be amenable to definition by the response of their catalytic components to NaF, both in the intact membrane and subsequent to detergent solubilization, and also by the stability of the enzyme activity to solubilization.

Activation of membrane-bound adenylate cyclase by p[NH]ppG has been demonstrated to protect the enzyme activity during solubilization with Lubrol-PX (Pfeuffer & Helmreich, 1975; Bennett & Cuatrecasas, 1976). Results of experiments in which the 600g-sediment enzyme of corpus luteum was treated with p[NH]ppG before solubilization are presented below.

Stability of the enzyme in the 600g sediment in the presence and absence of p[NH]ppG and NaF, and the effects of the Tris/HCl concentration on enzyme activity, are also reported.

Experimental

Materials

ATP (disodium salt) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. GTP (trisodium salt) and p[NH]ppG (tetralithium salt) were purchased from Boehringer Corp. (London), London W.5, U.K. Lubrol-PX was a gift from I.C.I., Alderley Park, Macclesfield, Cheshire, U.K. Cyclic AMP-binding protein (bovine adrenal gland) and all other chemicals (analytical grade) were obtained from BDH, Poole, Dorset, U.K.

Methods

The washed 600g ($r_{av.} = 23$ cm) sediment of bovine corpus-luteum homogenate was prepared, and adenylate cyclase activity was assayed as described previously (Stansfield & Franks, 1971; Young & Stansfield, 1977). The reaction time course was linear for at least 10 min under all assay conditions used. All assays contained 600g sediment, or soluble extract thereof, equivalent to 37.5 mg wet wt. of tissue/ml of incubation medium, previously shown to be the optimal tissue concentration for 600g-sediment activity (Young & Stansfield, 1977), to obviate the problems caused by the non-linearity with respect to quantity of enzyme typically shown by adenylate cyclases. Caffeine (6.7 mM) was present in all incubations, to minimize interference by cyclic AMP phosphodiesterase.

The cyclic AMP formed was measured by a competitive protein-binding technique (Young & Stansfield, 1977) by using a partially purified cyclic AMP-binding protein from bovine adrenal glands. Standard

assay mixtures used to obtain dose-response curves for cyclic AMP were supplemented with the appropriate concentrations of incubation components to compensate for any effect of these substances on the binding assay.

All incubations were performed in duplicate and data points represent the mean value obtained. The range of the duplicate values is also indicated where they are not essentially identical.

The adenylate cyclase activity of the 600g sediment was solubilized with Lubrol-PX (10 g/litre) in Tris/HCl buffer (see the text and Figure legends for composition). The 600g sediment was resuspended in the Lubrol-PX solution, rehomogenized and then gently shaken for 0.5 h at 4°C before centrifugation. The criterion of solubility used was that the activity should not sediment during centrifugation at 105000g ($r_{av.} = 5.74$ cm) for 1.5 h at 4°C. The maximum concentration of Lubrol-PX carried through into the adenylate cyclase assay was 5 g/litre.

Protein was determined by the method of Lowry *et al.* (1951) after treatment of the tissue fraction with 1 M-NaOH and 3 min immersion in a boiling-water bath. Bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) was used as the standard. The appropriate corrections were made for the effect of Lubrol-PX and other buffer constituents on the protein determination.

Results and Discussion

Activation by NaF

NaF, added to the assay mixture, maximally activated the luteal adenylate cyclase at a concentration of 10 mM (Fig. 1). Increase in the NaF concentration above 10 mM resulted in decreased activation, although activities lower than basal were not observed. Other workers have also demonstrated an optimum concentration of NaF for adenylate cyclase activation (Birnbaumer *et al.*, 1969; Stansfield & Franks, 1971; Sulakhe & Dhalla, 1973).

Basal activity in 38 separate preparations of 600g sediment, assayed under the same conditions, ranged from 21 to 560 (mean 122) pmol of cyclic AMP produced/10 min per mg of protein. NaF-stimulated activity in the same preparations ranged from 60 to 2909 (mean 1067) pmol of cyclic AMP produced/10 min per mg of protein. Stimulation by NaF, relative to basal activity, ranged from 1.74-fold to 52.9-fold (mean 11.3-fold). The mean percentage deviation of individual estimates from the mean of duplicate determinations ($n = 38$) was 7.0% (range, 0–33.3%) and 4.8% (range, 0–20.6%) for basal and NaF-stimulated activities respectively.

NaF inhibition of basal adenylate cyclase activity of some tissues has been reported (Ray *et al.*, 1970; Johnson & Sutherland, 1973; Sapag-Hagar & Green-

Fig. 1. Optimal NaF concentration for the activation of adenylate cyclase in the 600g sediment

The 600g-sediment enzyme derived from 75 mg wet wt. of corpus luteum was incubated for 10 min at 37°C in a total volume of 2 ml with ATP (1 mM), MgSO₄ (6 mM), caffeine (6.7 mM) and Tris/HCl (pH 7.5, 40 mM) and with various concentrations of NaF. Each assay tube contained 2.5 mg of 600g-sediment protein.

Fig. 2. Activation of adenylate cyclase in the 600g sediment by p[NH]ppG; a dose-response curve

The 600g-sediment enzyme derived from 37.5 mg wet wt. of corpus luteum was incubated for 10 min at 37°C in a total volume of 1 ml with ATP (1 mM), MgSO₄ (6 mM), caffeine (6.7 mM) and Tris/HCl (pH 7.5; 40 mM), and with various concentrations of p[NH]-ppG. Each assay tube contained 0.78 mg of 600g-sediment protein.

baum, 1974). Although we did not observe significant NaF inhibition of untreated particulate preparations of the enzyme, in some preparations, which were assayed in the presence of 25 mM-Tris/HCl and 0.5 mM-EDTA (Fig. 4), no activation by NaF was seen.

Activation by p[NH]ppG and GTP

Activation of luteal adenylate cyclase by p[NH]ppG (Fig. 2), included in the assay mixture, was evident at 0.1 μM, half-maximal at 0.8 μM and maximal at 100 μM. These values are similar to those reported for other tissues (Pfeuffer & Helmreich, 1975; Londos & Rodbell, 1975; Lefkowitz, 1975; Lefkowitz *et al.*, 1976).

GTP stimulated the cyclase, but less so than did p[NH]ppG (Table 1) under the same conditions. The larger activation by p[NH]ppG has been observed in other tissues and was originally thought to be due to the resistance of this analogue of GTP to hydrolysis, GTP itself being rapidly degraded by nucleotide phosphohydrolases present in most tissue preparations (Pfeuffer & Helmreich, 1975; Schramm & Rodbell, 1975; Salomon *et al.*, 1975). However, it has been shown that p[NH]ppG is degraded by tissue preparations (Jacobs *et al.*, 1976), and it has been suggested that GTP interacts with a regulatory site

Table 1. Activation of adenylate cyclase in the 600g sediment by GTP and p[NH]ppG

The 600g sediment derived from 37.5 mg of corpus luteum was incubated for 10 min at 37°C in 40 mM-Tris/HCl buffer, pH 7.5, with 1 mM-ATP, 6 mM-MgSO₄ and 6.7 mM-caffeine, and both without further addition and with the addition of GTP (0.1 mM) or p[NH]ppG (0.1 mM). The reaction was started by the addition of the enzyme. The total assay incubation volume was 1 ml and each assay tube contained 1.4 mg of 600g-sediment protein.

Addition	Cyclic AMP produced (pmol)
None	220
GTP	370
p[NH]ppG	1330

to produce a labile pyrophosphate-enzyme complex, whereas p[NH]ppG gives rise to a stable activated p[NH]p-enzyme complex (Cuatrecasas *et al.*, 1975a,b; Jacobs *et al.*, 1976; Bennett & Cuatrecasas, 1976). This theory is no longer tenable, since the pyrophosphohydrolase-stable analogue guanosine 5'-[α,β-methylene]triphosphate has been shown to cause persistent activation of adenylate cyclase (Spiegel *et al.*, 1977).

Fig. 3. Stability of luteal adenylate cyclase in the washed 600g sediment at 37°C in the presence and absence of p[NH]ppG or NaF: cyclic AMP produced (nmol) versus time of preincubation

(a) Effects of p[NH]ppG: ●, when absent from preincubation but present in assay; ▲, when present in preincubation but absent from assay; ◐, when present in both preincubation and assay; ▼, control (absent from both preincubation and assay). (b) Effects of NaF: ◑, when absent from preincubation but present in assay; △, when present in preincubation but absent from assay; ◒, when present in both preincubation and assay; ▼, control (absent from both preincubation and assay). Washed 600g sediment was resuspended in 40mM-Tris/HCl, pH7.5, containing either 10mM-NaF or 0.1mM-p[NH]ppG at a tissue concentration equivalent to 75mg of corpus luteum/ml. Samples (500µl) of each preparation were dispensed into bacteriological test tubes (125mm×16mm) and left for 10min at 0°C. The tubes were then transferred to a 37°C water bath and incubated for various times as indicated on the abscissa. At the end of the preincubation period the tubes were transferred to an ice bath and 5ml of ice-cold buffer [40mM-Tris/HCl (pH7.5)/6mM-MgSO₄] was added. The 600g sediment was then immediately reisolated by centrifugation at 600g for 10min at 4°C and then washed with 2×10ml of the same buffer. The 600g sediment was finally resuspended in 40mM-Tris/HCl (pH7.5) containing 6mM-MgSO₄ and then assayed for adenylate cyclase for 10min at 37°C with 1mM-ATP,

Stability of the 600g-sediment enzyme in the presence and absence of p[NH]ppG or NaF

Figs. 3(a) and 3(b) show the results of stability studies at 37°C of the 600g-sediment enzyme incubated in assay buffer alone, and with the addition of either p[NH]ppG (0.1mM) or NaF (10mM). After the stated preincubation times the 600g sediment was washed extensively (three times) with assay buffer [40mM-Tris/HCl (pH7.5)/6mM-MgSO₄] at 4°C, and then the adenylate cyclase activity was assayed in the usual manner at 37°C for 10min, both with and without the further addition of p[NH]ppG (0.1mM) or NaF (10mM).

Exposure of the 600g sediment to either p[NH]ppG or NaF for 10min at 0°C (0min on Figs. 3a and 3b), followed immediately by extensive washing of the sediment and then assay at 37°C, resulted in an activation of the adenylate cyclase. Other workers have shown that activation of particulate adenylate cyclase by these agents does not occur during low-temperature (0–4°C) preincubation. Cuatrecasas *et al.* (1975a) and Bennett & Cuatrecasas (1976) found no activation by p[NH]ppG during preincubation at 4°C, or when this procedure was followed by washing and further preincubation at 30°C before assay at 30°C. Kelly & Koritz (1971) reported that adrenal adenylate cyclase is not activated by preincubation with NaF at 0°C, although activation was seen when the preincubation was carried out at 15°C, 20°C or 30°C before washing and assay at 30°C. Schramm & Naim (1970) observed activation of rat parotid-gland adenylate cyclase by preincubation at 36°C with NaF and showed that this was not due to carry-over of NaF into the assay incubation. However, our results can only be explained as being due to either low-temperature activation or to membrane-bound activator being carried into the assay incubation despite the extensive washing that we used.

Preincubation of the luteal 600g sediment at 37°C in buffer alone resulted in essentially similar and progressive losses of both p[NH]ppG-activatable (Fig. 3a) and NaF-activatable (Fig. 3b) cyclase in the subsequent 10min assay. Basal adenylate cyclase activity fell by approx. 50% after preincubation for 40min at 37°C (shown in both Figs. 3a and 3b).

When the 600g sediment was preincubated at 37°C in the presence of p[NH]ppG there was a progressive irreversible activation of the enzyme throughout the 40min of preincubation. Although the irreversible activation was not maximal until 40min or more of

6mM-MgSO₄ and 6.7mM-caffeine, and both with and without the further addition of p[NH]ppG (0.1mM) or NaF (10mM). The total assay volume was 1ml and each tube contained 1.12mg of 600g-sediment protein (equivalent to 37.5mg wet wt. of corpus luteum).

preincubation, that portion of the p[NH]ppG-activatable enzyme that was not irreversibly activated before this time was stabilized by the presence of p[NH]ppG and could be activated, either by p[NH]ppG present in the subsequent assay incubation or by prolonged preincubation. Activation was greatest when p[NH]ppG was present both in the preincubation medium and during the assay.

The prolonged preincubation required for p[NH]ppG to achieve maximum activation of the enzyme and the irreversibility of this activation are findings that are also typical of other tissues (Cuatrecasas *et al.*, 1975a,b; Bennett & Cuatrecasas, 1976). However, unlike previous reports (Cuatrecasas *et al.*, 1975a,b; Bennett & Cuatrecasas, 1976), we did not observe a lag phase or an accelerating phase in the p[NH]ppG activation of luteal adenylate cyclase; the reaction time courses obtained with p[NH]ppG, whether present in the assay mixture and/or during a preincubation period, were linear with time for at least 10 min, for both the 600g-sediment enzyme and the soluble enzyme (results not shown).

NaF also caused an irreversible activation, maximal between 10 and 20 min of preincubation. Irreversible activation of adenylate cyclase by NaF has been demonstrated in a number of tissues (Schramm & Naim, 1970; Pastan *et al.*, 1970; Kelly & Koritz, 1971; Perkins & Moore, 1971; Birnbaumer *et al.*, 1972; Severson *et al.*, 1972; Sulakhe & Dhalla, 1973; Johnson *et al.*, 1975; Cuatrecasas *et al.*, 1975b; Manganiello & Vaughan, 1976). However, only part of the total NaF-activatable adenylate cyclase, demonstrable under the assay conditions used, was irreversibly activated. That portion which was not irreversibly activated by NaF, but which was rapidly activatable during assay, was not stabilized by the presence of NaF in the preincubation medium. Thus two forms of adenylate cyclase, differing in their response to NaF, are indicated by these results.

For up to 10 min of preincubation at 37°C, activation of the luteal adenylate cyclase was greater when NaF was added to the assay than when the enzyme was preincubated with NaF before assay in the effective absence of NaF. With preincubation times of 10–40 min the opposite result was obtained. Moreover, the greatest activation was obtained with NaF present in both the preincubation and the assay. These results are in accord with those reported for rat parotid gland (Schramm & Naim, 1970), but are in contrast with those of Perkins & Moore (1971) and Manganiello & Vaughan (1976), who found, for rat cerebral-cortex adenylate cyclase and fat-cell adenylate cyclase respectively, that higher activities could be obtained by preincubation with NaF before assay in the absence of NaF than when NaF was added during the assay. The reason for these differing results

may be found in the different preincubation times and temperatures used and/or a difference in the relative proportions of two forms of the enzyme present in the tissues used.

Solubilization of the adenylate cyclase activity of the 600g sediment

Fig. 4, representing the results obtained with two solubilization and adenylate cyclase assay procedures

Fig. 4. Relationship between the magnitude of NaF stimulation of adenylate cyclase in the 600g sediment and the yield of activity solubilized by Lubrol-PX

Different batches of luteal tissue, as 600g sediment, were assayed for adenylate cyclase activity both with and without 10 mM-NaF. The percentage activation by NaF, relative to basal activity, was calculated for each batch (abscissa). Portions of each 600g sediment were subjected to solubilization by Lubrol-PX and the soluble enzyme was then assayed both with and without 10 mM-NaF. The amounts (%) of both basal and NaF-stimulated activities recovered from the 600g sediment in a soluble form were calculated (ordinate); ●, ○, % of basal activity solubilized; ▲, △, % of NaF-stimulated activity solubilized. Each pair of datum points on a vertical axis thus refers to the same batch of luteal tissue. The two sets of data, denoted by open and closed symbols respectively, were obtained by using different conditions both for solubilization and for the assay of adenylate cyclase in the 600g sediments and soluble preparations: Open symbols denote solubilization in 10 mM-Tris/HCl (pH 7.7)/6 mM-MgSO₄/1 mM-EDTA/Lubrol-PX (10 g/litre) and assays performed in 25 mM-Tris/HCl (pH 7.5)/6.5 mM-MgSO₄/0.5 mM-EDTA/6.7 mM-caffeine/1 mM-ATP. Closed symbols denote solubilization in 40 mM-Tris/HCl (pH 7.5)/6 mM-MgSO₄/Lubrol-PX (10 g/litre) and assays performed in 40 mM-Tris/HCl (pH 7.5)/6 mM-MgSO₄/6.7 mM-caffeine/1 mM-ATP. (See the text for explanation of the horizontal displacement of the closed symbols relative to the open symbols.)

Fig. 5. Effect of Tris/HCl concentration on the basal and NaF-stimulated activities of adenylate cyclase in the 600g sediment

The 600g sediment of corpus-luteum homogenate was washed and resuspended in 10mM-Tris/HCl, pH 7.5, at a concentration of 75 mg wet wt. of tissue/ml. A portion (500 μ l) of this tissue preparation (955 μ g of protein) was then assayed in duplicate for 10 min at 37°C with 1 mM-ATP, 6 mM-MgSO₄, 6.7 mM-caffeine and various concentrations of Tris/HCl, pH 7.5, both with (●) and without (○) NaF (10 mM). ▲, pH of the medium of the NaF-stimulated samples at the end of the 10 min assay.

(see the Figure legend), demonstrates that the extent of solubilization of both basal and NaF-stimulated activities in the 600g sediment is related to the percentage relative activation of the particulate enzyme by NaF.

The lateral displacement of the two sets of data in Fig. 4 indicates that the response of the particulate enzyme to NaF is greater when the assay is performed in 40 mM-Tris/HCl buffer than when 25 mM-Tris/HCl buffer containing 0.5 mM-EDTA is used. The results shown in Fig. 5 demonstrate that NaF activation of the particulate enzyme is indeed dependent on the concentration of Tris/HCl. Except for assays performed in 10 mM- and 20 mM-Tris/HCl, the desired pH was maintained during the assay. In addition, EDTA present in the 25 mM-Tris/HCl incubations further decreases the degree of NaF stimulation in two ways, both by stimulating basal cyclase activity and inhibiting the absolute NaF-stimulated activity (results not shown).

The results in Fig. 6 indicate that it is the Cl⁻ component of Tris/HCl buffer that is important in faci-

Fig. 6. Effect of pH on the adenylate cyclase activity of the 600g sediment; comparison of adenylate cyclase activity in Tris/HCl and Tris/maleate buffers

Portions of 600g-sediment protein (2.21 mg) derived from 75 mg wet wt. of corpus luteum were incubated, in duplicate and in a total volume of 2 ml, with 1 mM-ATP, 6 mM-MgSO₄ and 6.7 mM-caffeine for 15 min at 37°C, both with (●, ▲) and without (○, △) NaF (10 mM) and in either 40 mM-Tris/HCl (●, ○) or 40 mM-Tris/maleate (▲, △) buffers at the pH values indicated on the abscissa.

tating NaF activation, since equimolar Tris/maleate buffer does not allow such a marked activation to occur, although maleate ions do not inhibit basal adenylate cyclase relative to Cl⁻ ions. A possible explanation for the different degrees of NaF activation obtained in these two buffers may be the difference in the chaotropic behaviour of the anions chloride and maleate. Anions may cause alterations of the vicinal water structure, which, in turn, cause structural rearrangements in the membrane, making the catalytic component of adenylate cyclase more susceptible to activation by NaF; in this respect chloride is more effective than maleate.

Johnson *et al.* (1975) have also found that NaF activation of liver-cell adenylate cyclase is potentiated by anions, including chloride.

Basal activity of the luteal cyclase was also enhanced by increasing concentrations of Tris/HCl (Fig. 5), although much less so than the NaF-stimulated activity. Kalish *et al.* (1974) have shown that Cl⁻, Br⁻ and I⁻ ions themselves can activate adenylate cyclase of rat liver and fat-cells, although optimal concentrations of these ions are about 100 times higher than those required for activation by F⁻.

p[NH]ppG-stimulated activity of the 600g-sediment enzyme and also basal and NaF-stimulated activities of the Lubrol-PX-solubilized enzyme are little affected by the Tris/HCl concentration (results not shown).

Although not superimposable because of the different assay conditions used, both sets of data in Fig. 4 show a similar pattern. When the percentage relative activation by NaF of the 600g-sediment enzyme is high the recovery of activity in the 105000g supernatant is low. As the percentage relative activation falls, the yield of solubilized activity increases and may, for the basal activity, reach values greater than 100%; that is, activation occurs as a result of the solubilization procedure. Further decrease in the percentage relative activation by NaF results in decreased recoveries of soluble activity. Also it will be noted that, in general, there is a higher proportion of basal activity solubilized than there is of NaF-stimulated activity.

The activity solubilized (i.e. present in the 105000g supernatant) represented virtually all the detectable activity, both basal and NaF-stimulated, present in the Lubrol-dispersed 600g sediment before centrifugation at 105000g (results not shown).

These observations are taken to indicate a multi-state existence for luteal adenylate cyclase. Variable proportions of at least three activity states are required to explain the relationships described. These three states may be characterized as follows: (a) a strongly NaF-stimulated form, which is labile during solubilization; (b) an NaF-independent form, the activity of which is increased during solubilization; (c) an NaF-inhibited form, which is labile during solubilization. The relative proportions of these three states determine the degree to which the enzyme activity can be solubilized by Lubrol-PX. Thus if state (b) were predominant, solubilization could be associated with an increase in basal activity and the proportions of states (a) and (c) would determine the overall response of the particulate and soluble enzyme to NaF.

MacDonald (1975) has postulated for bovine brain-cortex adenylate cyclase, on the basis of studies with Ca^{2+} , EGTA and Triton X-100, that there are two forms of the enzyme. One form is activated by NaF, an effect enhanced by low concentrations of Ca^{2+} and Mg^{2+} . The other form is totally dependent on Ca^{2+} and inhibited by NaF. Detergent dispersion in Triton X-100 abolishes the NaF-stimulated component but activates the Ca^{2+} -dependent component. Enhancement of the adenylate cyclase activity of other tissues by detergent treatment has also been reported (Øye & Sutherland, 1966; Perkins & Moore, 1971; Johnson & Sutherland, 1973; Wilkinson & Stansfield, 1974; Varimo & Londesborough, 1976).

The response of adenylate cyclase to NaF can be shown to be dependent not only on the assay condi-

tions, such as the presence or absence of potentiating anions, but also on the previous stimulation by hormones *in vitro* or *in vivo*. Conti *et al.* (1976) showed that stimulation of rat ovaries *in vivo* by human choriogonadotropin resulted in a lower response to NaF during subsequent assay *in vitro*, although basal activity remained unaltered. The ovaries also became refractory to further choriogonadotropin stimulation *in vitro* (see also Bockaert *et al.*, 1976; Hunzicker-Dunn & Birnbaumer, 1976). Preincubation of bovine adrenal-cortical adenylate cyclase at 0°C with corticotropin, followed by extensive washing, was shown to result in a stimulation of the enzyme and a lower response to NaF (Kelly & Koritz, 1971). Schramm & Naim (1970) reported a 50% decrease in NaF-stimulated activity of rat parotid gland, measured *in vitro*, after isoprenaline injection. In addition, Sapag-Hagar & Greenbaum (1974) found that NaF (1–10mM) consistently inhibited the adenylate cyclase activity of mammary glands from pregnant rats, but had no effect in mammary gland from lactating rats, and Manganiello & Vaughan (1976) have shown that NaF can inhibit fat-cell adenylate cyclase that had been activated *in vitro* by isoproterenol, cholera toxin or NaF before assay.

Thus it is probable that the state of activation of the adenylate cyclase complex achieved *in vivo* determines the results of subsequent modulation experiments performed *in vitro*. The state of activation, or the relative proportions of several possible states of activation, might be expected to be variable in a tissue, such as the corpus luteum of the oestrous cycle, which is subject to varying hormonal influences throughout its life history. This may afford an explanation for the variable degree of NaF activation that we observe and also the variable success of solubilization of the 600g-sediment activity.

Solubilization of the adenylate cyclase activity of the 600g sediment after preincubation with p[NH]ppG

We decided to see if the stable high-activity state induced by p[NH]ppG is amenable to solubilization. Preincubation of the 600g sediment with p[NH]ppG (0.1 mM) before solubilization increased the yields of soluble activity (Fig. 7). With increasing time of preincubation with p[NH]ppG, up to 15 min, the subsequent yield of soluble activity increased. After 20 min preincubation the yield had fallen, although the 600g-sediment enzyme was not maximally activated at this time (see Fig. 3a).

Associated with the progressive irreversible activation by p[NH]ppG there was a loss of stimulation by NaF. Inhibition by NaF (10mM) became evident between 10 and 15 min of preincubation with p[NH]ppG for the particulate enzyme (Fig. 7a) and between

Fig. 7. Solubilization of the adenylate cyclase activity in the 600g sediment after treatment with p[NH]ppG (0.1 mM) at 0°C and 37°C: cyclic AMP produced (nmol/10 min per 37.5 mg wet wt. of corpus luteum) versus temperature and time of exposure to p[NH]ppG (0.1 mM)

(a) Activity in the 600g sediment assayed both with and without NaF (10 mM); (b) activity in the 105000g supernatant (i.e. soluble enzyme) assayed both with and without NaF (10 mM). Hatching indicates the presence of NaF in the assay. A single preparation of washed 600g sediment was used for this experiment. It was resuspended in 40 mM-Tris/HCl, pH 7.5, containing 6 mM-MgSO₄ and 0.1 mM-p[NH]ppG and then divided into portions that were incubated for various times and temperatures as indicated on the abscissa. After two washes with buffer [40 mM-Tris/HCl (pH 7.5)/6 mM-MgSO₄] at 4°C, each portion of p[NH]ppG-treated 600g sediment was further divided. One portion was resuspended in the same buffer and then assayed in the presence of 1 mM-ATP, 6 mM-MgSO₄, 6.7 mM-caffeine and 40 mM-Tris/HCl, pH 7.5, both with and without NaF (10 mM); these results are shown in Fig. 7(a). A second portion was used to prepare a soluble extract (see under 'Methods'), which was assayed as described for the 600g-sediment enzyme; these results are shown in Fig. 7(b).

Fig. 8. Effect of NaF concentration on the adenylate cyclase activity of the 600g sediment, and on the activity of the soluble enzyme derived from this preparation, after treatment with p[NH]ppG: cyclic AMP produced (nmol/10 min per 37.5 mg wet wt. of corpus luteum) versus concentration of NaF

Washed 600g sediment was incubated for 20 min at 37°C in 40 mM-Tris/HCl, pH 7.5, containing 6 mM-MgSO₄ and 0.1 mM-p[NH]ppG and then washed twice and resuspended in 40 mM-Tris/HCl, pH 7.5, containing 6 mM-MgSO₄. One portion was then taken for adenylate cyclase assay in the presence of various concentrations of NaF and with 1 mM-ATP, 6 mM-MgSO₄, 6.7 mM-caffeine and 40 mM-Tris/HCl, pH 7.5. A second portion was resuspended in 40 mM-Tris/HCl, pH 7.5, containing 6 mM-MgSO₄ and Lubrol-PX (10 g/litre), rehomogenized, gently shaken for 0.5 h at 4°C and then centrifuged at 105000g for 1.5 h at 4°C. The supernatant (soluble enzyme) was assayed as described for the 600g-sediment enzyme. ●, 600g-sediment enzyme (1048 µg of protein/assay tube); ○, soluble enzyme (208 µg of protein/assay tube).

0 and 5 min for the soluble enzyme (Fig. 7b), suggesting that the NaF-inhibited form produced by p[NH]ppG is solubilized preferentially by Lubrol-PX. We have also noted that soluble adenylate cyclase derived from native luteal 600g sediments shows increasing tendency to inhibition by NaF as the relative activation of the particulate enzyme by NaF decreases (results not shown).

Although p[NH]ppG treatment of the 600g sediment was shown to give rise both to a form of the enzyme that is more stable to solubilization and also to an NaF-inhibited form, the results obtained with

the native enzyme (Fig. 4) were not reproduced. It is not clear if the high-activity NaF-inhibitable state of the enzyme complex induced by p[NH]ppG is equivalent to the NaF-inhibited state proposed for the native enzyme, or if activation by p[NH]ppG gives rise to a transient form of the enzyme that does not respond to NaF and the activity of which is enhanced by solubilization.

Fig. 8 demonstrates that preincubation of the particulate enzyme with p[NH]ppG caused a decrease in the concentration of NaF that gave optimal activation of the enzyme, from 10mM (Fig. 1) to approx. 1mM (Fig. 8). A concentration of NaF of 10mM or higher inhibited the p[NH]ppG-activated cyclase. The soluble enzyme derived from p[NH]ppG-activated 600g sediment was not stimulated by NaF at any concentration studied, but was inhibited by NaF at a concentration of 10mM or more. These results also indicate preferential solubilization of the enzyme that is inhibited by NaF (10mM).

Previous reports indicate a variable response of detergent-solubilized adenylate cyclases to NaF (Birnbaumer *et al.*, 1969; Schramm & Naim, 1970; Levey, 1970, 1971; Perkins & Moore, 1971; Forte, 1972; Jakobs *et al.*, 1972; Sulimovici & Lunenfeld, 1973; Johnson & Sutherland, 1973; Swislocki & Tierney, 1973; Wilkinson & Stansfield, 1974; Ryan & Storm, 1974; Neer, 1974, 1976; Queener *et al.*, 1975; Roy *et al.*, 1975), the response ranging from inhibition to activation. Such variation may also arise from the history *in vivo* of the tissue from which the soluble enzyme was prepared and/or the different procedural conditions used by the individual workers.

Conclusions

The effects *in vitro* of modulators of adenylate cyclase activity and the degree to which activity of a particulate fraction can be recovered in a soluble form are influenced by the previous history of the enzyme *in vivo* or *in vitro*.

The response to modulators is also affected by the assay conditions. The effect of Tris/HCl concentration on the extent of NaF stimulation has not generally been considered, despite the report of Johnson *et al.* (1975). Our own experiments on the effect of Tris/HCl concentration were performed to explain, at least in part, the displacement of the two sets of data in Fig. 4. The use of a suboptimal Tris/HCl concentration for the study of NaF-stimulated activity probably explains the different maximal activities obtained with NaF and p[NH]ppG (Figs. 3a and 3b). Qualitatively different results may be obtained if the optimal Tris/HCl concentration for NaF stimulation is used, and this possibility requires further investigation.

Three sets of experimental data presented here (Figs. 3b, 4 and 7) suggest the multi-state nature of the

adenylate cyclase complex. However, it is not yet possible to establish a model for the interconversion of the states indicated by our data or to relate these states to any existing multistate models (Birnbaumer *et al.*, 1974; Schramm & Rodbell, 1975; Cuatrecasas *et al.*, 1975a; Salomon *et al.*, 1975; Rodbell, 1975; Brostrom *et al.*, 1976; Sevilla *et al.*, 1976; Bennett & Cuatrecasas, 1976; Jacobs *et al.*, 1976; Hammes & Rodbell, 1976; Levitzki, 1977). This problem arises partly from differences in the data from which the models are derived and partly from the possibility that the enzyme system is more complex than most models would suggest. For example, few models adequately describe the return to a ground state from the high-activity state or incorporate a refractory state, a possible obligatory pathway for return to the ground state.

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