Solubilization of Bovine Corpus-Luteum Adenylate Cyclase in Lubrol-PX, Triton X-100 or Digitonin and the Stabilizing Effect of Sodium Fluoride Present in the Solubilization Medium

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1. Adenylate cyclase activity of the washed 600g sediment of bovine corpus-luteum homogenate was solubilized by Lubrol-PX, Triton X-100 and digitonin. Digitonin was the least destructive of NaF-stimulated activity. 2. NaF, present in the solubilization medium together with MgSO₄, increased the percentage yields of soluble activity from untreated 600g sediment and 600g sediment which had been preincubated with p[NH]ppG (guanosine 5'-[βγ-imido]triphosphate). The stabilizing influence of NaF was most marked with digitonin. However, the highest specific activities of soluble enzyme were obtained with Lubrol-PX as solubilizing agent, since digitonin solubilized more membrane protein than does Lubrol-PX, and less of the activity of the digitonin-dispersed 600g sediment was recovered in the 105000g supernatant. 3. p[NH]ppG also has a stabilizing effect when present during the solubilization, but less so than NaF. 4. Both NaF and MgSO₄ alone have a stabilizing effect during solubilization. The greatest amounts of soluble activity were obtained with both agents present in the solubilization medium, there being a synergistic effect.

Soluble adenylate cyclase has been prepared from a variety of tissues by using the detergents Lubrol, Triton and digitonin. However, the yields of soluble activity, expressed as a percentage of the activity seen in the particulate preparation, have been quite variable. We have proposed (Young & Stansfield, 1978) that the success of solubilization of adenylate cyclase from its membrane locus in bovine corpus luteum depends on the state of activation, or the relative proportions of several possible states of activation, of the enzyme in the tissue preparation used. Indeed, it has been shown for a variety of tissues that activity states of adenylate cyclase which are more stable to solubilization can be produced *in vitro* by exposure of the particulate enzyme to activatory agents. For example, preincubation of the particulate enzyme with p[NH]ppG (Pfeuffer & Helmreich, 1975; Bennett & Cuatrecasas, 1976; Mahaffee & Ontjes, 1977; Vauquelin *et al.*, 1977; Young & Stansfield, 1978) or with NaF (Mahaffee & Ontjes, 1977; Vauquelin *et al.*, 1977) has been shown to increase the amount of activity solubilized by detergent. Although preactivation of the particulate luteal adenylate cyclase with p[NH]ppG increased the

absolute amount of activity solubilized by Lubrol-PX, the percentage yields were still low (Young & Stansfield, 1978). Preincubation of the luteal enzyme at 37°C with NaF resulted in an overall loss of activity (Young & Stansfield, 1978) and was, therefore, considered to be an unsuitable preliminary to solubilization.

The investigations described in this paper examined the possibility of activating and stabilizing the luteal adenylate cyclase during the solubilization procedure by the inclusion of NaF or p[NH]ppG in the medium, both with and without preactivation of the particulate adenylate cyclase by p[NH]ppG. The effects of three detergents, Lubrol-PX, Triton X-100 and digitonin, were studied.

Experimental

Materials

ATP (disodium salt) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. p[NH]ppG (tetralithium salt) was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Lubrol-PX (12 A9) was a gift from ICI, Alderley Park, Macclesfield, Cheshire, U.K. Cyclic AMP-binding protein (bovine adrenal gland), Triton X-100, digitonin and all other

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

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chemicals (analytical grade if available) were obtained from BDH, Poole, Dorset, U.K.

Methods

The washed 600g ($r_{av.} = 23$ cm) sediment of bovine corpus-luteum homogenate was prepared as described previously (Young & Stansfield, 1977). p[NH]ppG-activated 600g sediment was prepared by incubation with 0.1mM-p[NH]ppG for 20min at 37°C in 40mM-Tris/HCl buffer (pH 7.5) containing 6mM-MgSO₄, and then washed twice with p[NH]ppG-free buffer at 4°C.

Adenylate cyclase activity of the 600g sediment was solubilized in either Lubrol-PX (10g/l), Triton X-100 (10g/l) or digitonin (10g/l) in 40mM-Tris/HCl buffer (pH 7.5) containing either 6mM-MgSO₄ or 1mM-MgSO₄/1mM-EDTA, and either with or without NaF (see the Results and Discussion section and legends for variable additions). The washed 600g sediment was resuspended in the detergent solution at a concentration equivalent to 75mg wet wt. of tissue per ml, rehomogenized and then gently shaken for 0.5h at 4°C. The detergent-dispersed preparation was centrifuged at 105000g ($r_{av.} = 5.74$ cm) for 1.5h at 4°C and the supernatant (soluble enzyme) was assayed for adenylate cyclase activity.

Adenylate cyclase activity was assayed, as described previously (Young & Stansfield, 1977, 1978), in the presence of 40mM-Tris/HCl (pH 7.5), 6mM-MgSO₄, 1mM-ATP and 6.7M-caffeine (see legends for variable additions). The total assay volume of 1ml contained 600g sediment, or soluble extract thereof, equivalent to 37.5mg wet wt. of tissue. Assays of soluble enzyme activity contained detergent at a concentration of 5g/l as a carry-over from the solubilization procedure. Incubations were carried out at 37°C for 10min. All assays were performed in duplicate. The mean and range of duplicate values are shown.

The cyclic AMP produced was measured by a competitive protein-binding technique (Young & Stansfield, 1977) 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.

Protein was measured by the method of Lowry *et al.* (1951) after treatment of the tissue fraction with 1M-NaOH and 3min 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 effects of detergents and other buffer constituents on the protein measurements.

Table 1. Solubilization of adenylate cyclase activity in the presence and absence of NaF: a comparison of three detergents, Lubrol-PX, Triton X-100 and digitonin. Thirteen tissue fractions were prepared from the washed 600g sediment of bovine corpus-luteum homogenate: (a) 600g sediment resuspended in 40mM-Tris/HCl (pH 7.5) containing 6mM-MgSO₄; (b) 600g sediment dispersed in 40mM-Tris/HCl (pH 7.5) containing Lubrol-PX (10g/l) and 6mM-MgSO₄; (c) the 105000g supernatant of (b); (d) 600g sediment dispersed in 40mM-Tris/HCl (pH 7.5) containing Lubrol-PX (10g/l), 10mM-NaF and 6mM-MgSO₄; (e) the 105000g supernatant of (d); (f), (g), (h) and (i), as for (b)-(e) but with Triton X-100 (10g/l) instead of Lubrol-PX; (j), (k), (l) and (m), as for (b)-(e) but with digitonin (10g/l) instead of Lubrol-PX. The tissue fractions were assayed for adenylate cyclase in both the absence (basal activity) and presence of 10mM-NaF. 37.5mg wet wt. of corpus luteum gave rise to 1.09mg of 600g-sediment protein. Yield is expressed as the percentage of activity solubilized from the particulate fraction.

Detergent	Solubilization medium	Activity before centrifugation at 105000g (pmol of cyclic AMP/10min)		Activity in 105000g supernatant (pmol of cyclic AMP/10min)		Yield of soluble activity (%)		Specific activity of soluble enzyme (pmol of cyclic AMP/10min per mg of protein)	
		+NaF (10mm)		+NaF (10mm)		Basal		Basal	
		Basal	+NaF (10mm)	Basal	+NaF (10mm)	Basal	+NaF	Basal	+NaF
Not solubilized						(100)	(100)	—	—
Lubrol-PX	—	(a) 72 ± 2	(a) 540	(c) 115 ± 5	(c) 152.5 ± 2.5	159.7	28.2	1150	1525
Lubrol-PX	+	(b) 120 ± 5	(b) 165 ± 5	(c) —	(e) 290*	159.7	53.7	—	2599
Triton X-100	—	(f) 100	(f) 127.5 ± 2.5	(g) 100	(g) 112.5 ± 7.5	138.8	20.8	370.4	416.7
Triton X-100	+	(h) —	(h) 237.5 ± 2.5*	(k) —	(i) 225*	—	41.7	—	803.5
Digitonin	—	(j) 102.5 ± 2.5	(j) 190 ± 10	(k) 92 ± 8	(k) 122.5 ± 2.5	127.8	22.7	545.4	723
Digitonin	+	(l) —	(l) 500*	(m) —	(m) 330*	—	61.1	—	1941

* Those tissue fractions prepared in medium containing 10mM-NaF were assayed only in the presence of 10mM-NaF, taking into account the NaF carried over into the assay (5mm).

Results and Discussion

Solubilization of luteal adenylate cyclase activity in Lubrol-PX, Triton X-100 or digitonin and the effect of NaF present in the solubilization medium

Table 1 shows the results of solubilization experiments with three detergents, Lubrol-PX, Triton X-100 and digitonin, both with and without NaF present in the solubilization medium.

Basal activity was enhanced, in this preparation, 1.67-fold, 1.39-fold and 1.42-fold by dispersion in Lubrol-PX, Triton X-100 and digitonin respectively. Dufau *et al.* (1977) found that the adenylate cyclase activity of rat testis and ovary is also enhanced more by Lubrol-PX (10g/l) than by Triton X-100 (10g/l). However, detergent treatment also increases NaF-stimulated activity of rat gonadal tissues (Wilkinson & Stansfield, 1974; Dufau *et al.*, 1977), whereas the bovine luteal adenylate cyclase shows a marked loss of NaF-stimulated activity in the presence of detergents. The recoveries of NaF-stimulated activity after detergent dispersion, but before centrifugation at 105 000g, were 35.2, 30.6 and 23.6% for digitonin, Lubrol-PX and Triton X-100 respectively (Table 1, column 4). Thus digitonin is the least destructive of NaF-stimulated activity. Neer (1973, 1974) found that the recoveries, as soluble enzyme, of NaF-stimulated activity present in control (detergent-free) homogenates of rat renal medulla were lower in the presence of Triton X-100 (10g/l) than in the presence of Lubrol-PX (10g/l), being approx. 29 and 40% respectively.

After dispersion of the bovine luteal 600g sediment in digitonin, Lubrol-PX or Triton X-100 in the presence of 10mM-NaF, 92.6, 52.7 and 44% respectively of the NaF-stimulated activity seen in the detergent-free control remained. Thus NaF, present in the solubilizing medium, has a stabilizing influence at 0–4°C which is most marked with digitonin. Of

the activity remaining after detergent dispersion in the presence of NaF, 66, 102 and 94.7% was recovered in the 105 000g supernatant for digitonin, Lubrol-PX and Triton X-100 respectively. The corresponding yields of soluble protein in the 105 000g supernatant were 15.6, 10.1 and 25.2% respectively. Thus, although digitonin is the least destructive of NaF-stimulated activity, particularly when NaF is included in the solubilization medium, it solubilizes more membrane protein than does Lubrol-PX, and less of the adenylate cyclase activity of the digitonin-dispersed 600g sediment is recovered in the 105 000g supernatant. Hence the specific activity of soluble adenylate cyclase is lower with digitonin dispersion than it is with Lubrol-PX dispersion. Triton X-100 solubilizes the largest amount of membrane protein and the specific activity of the enzyme solubilized by this agent is the lowest.

It is possible that the activity states of adenylate cyclase arise from variable association within a multicomponent system and that the various complexes have different susceptibilities to disruption by detergent. The differential solubilization of activity by the three detergents studied, and the differential stabilizing effect of NaF in the presence of these detergents, perhaps suggest that the detergents dissociate the membrane constituents, and the components of the adenylate cyclase complex, in particular, in different ways and to different extents.

Solubilization of luteal adenylate cyclase in the presence of p[NH]ppG

Table 2 shows that inclusion of 0.2mM-p[NH]ppG in the solubilization medium increased the yield of soluble activity, although less so than when NaF was included. The activity was greater when solubilization was performed in the presence of p[NH]ppG followed by assay in the presence of 0.1mM-p[NH]ppG (235 ± 5 pmol of cyclic AMP/

Table 2. *Solubilization of adenylate cyclase in the presence of p[NH]ppG (0.2mM): a comparison with NaF (10mM)*
Four tissue fractions were prepared from the washed 600g sediment of corpus luteum homogenate; (1) 600g sediment resuspended in 40mM-Tris/HCl (pH 7.5) containing 6mM-MgSO₄; (2) 105 000g supernatant of 600g sediment dispersed in 40mM-Tris/HCl (pH 7.5) containing 6mM-MgSO₄ and Lubrol-PX (10g/l); (3) as in (2) but with 10mM-NaF present in the solubilization medium; (4) as in (2) but with 0.2mM-p[NH]ppG present in the solubilization medium. The tissue fractions were assayed for adenylate cyclase, both in the presence and absence of NaF (10mM) or p[NH]ppG (0.1mM).

Variables of solubilization medium	Tissue fraction	Adenylate cyclase activity (pmol of cyclic AMP/10min)		
		Basal	+NaF (10mM)	+p[NH]ppG (0.1mM)
Not solubilized	(1) 600g sediment	168	3200 ± 200	2600 ± 200
None	(2) 105 000g supernatant	94 ± 2	138 ± 7	125 ± 5
10mM-NaF	(3) 105 000g supernatant	—	605 ± 15*	—
0.2mM-p[NH]ppG	(4) 105 000g supernatant	—	255 ± 5†	235 ± 5†

* Assayed in the presence of 10mM-NaF, taking into account the 5mM-NaF carried over from the solubilization.

† Assayed in the presence of 0.1mM-p[NH]ppG carried over from the solubilization procedure.

10min) than when solubilization was carried out in the absence of p[NH]ppG followed by assay in the presence of 0.1 mM-p[NH]ppG (125 ± 5 pmol of cyclic AMP/10min), indicating that p[NH]ppG, like NaF, has an activating and a stabilizing effect on the adenylate cyclase complex at 0–4°C. This result is consistent with our previous observations (Young & Stansfield, 1978) and with the observations of Mahaffee & Ontjes (1977).

Solubilization of luteal adenylate cyclase by Lubrol-PX in the presence of NaF after preactivation with p[NH]ppG

We have shown previously (Young & Stansfield, 1978) that preincubation of the 600g sediment with p[NH]ppG results in a progressive decrease in the activation due to 10mM-NaF during subsequent assay, and that NaF-inhibited forms of the enzyme are produced which are manifest as net NaF inhibition during assay after 15–20min of preincubation. The yields of soluble enzyme activity derived from the p[NH]ppG-activated 600g sediment increase with increasing time of exposure to p[NH]ppG, until such time as the particulate preparation exhibits inhibition by NaF during assay; the particulate enzyme is not maximally activated by p[NH]ppG at this time. It was decided, therefore, to examine the effect of NaF on the solubilization of activity from 600g sediment which had been submaximally activated by p[NH]ppG.

The results shown in Table 3 demonstrate that

(i) the amounts of soluble activity are greater with p[NH]ppG preactivation than with NaF present in the solubilization medium and (ii) a combination of p[NH]ppG preactivation and NaF present in the solubilization medium gives the highest amount of soluble enzyme activity, despite the fact that the p[NH]ppG-activated soluble enzyme is inhibited by NaF (10mM) present during the assay.

Furthermore, the results confirm that: (i) solubilization of the native (unstimulated) enzyme by Lubrol-PX in the absence of NaF results in a much reduced NaF-stimulated activity and, for this preparation, a slightly enhanced basal activity, (ii) NaF, present in the solubilization medium, increases the yield of soluble activity and (iii) preactivation of the 600g sediment with p[NH]ppG increases the amount of soluble activity, but not the percentage yield with respect to the 600g-sediment activity.

Solubilization of the native (unstimulated) 600g sediment in the presence of 10mM-NaF (Table 3) resulted in activity which was not inhibited during assay by 10mM-NaF relative to 5mM-NaF carried over from the solubilization medium. However, some soluble enzyme fractions prepared in the presence of NaF did show inhibition by 10mM-NaF during assay (results not shown). Thus pretreatment with NaF, like pretreatment with p[NH]ppG (Young & Stansfield, 1978), can give rise to 10mM-NaF-inhibited forms of the enzyme. Manganiello & Vaughan (1976) found that fat-cell adenylate

Table 3. *Solubilization of p[NH]ppG-activated 600g-sediment enzyme in the presence and absence of NaF (10mM)*

Six tissue fractions were prepared from the washed 600g sediment of corpus-luteum homogenate: (1) 600g sediment resuspended in 40mM-Tris/HCl (pH7.5) containing 6mM-MgSO₄; (2) the 105000g supernatant of Lubrol-PX-dispersed 600g sediment; (3) the 105000g supernatant of Lubrol-PX-dispersed 600g sediment with 10mM-NaF present in the solubilization medium; (4) p[NH]ppG-treated 600g sediment resuspended in 40mM-Tris/HCl (pH7.5) containing 6mM-MgSO₄; (5) 105000g supernatant of Lubrol-PX-dispersed, p[NH]ppG-treated 600g sediment; (6) 105000g supernatant of Lubrol-PX-dispersed, p[NH]ppG-treated 600g sediment with 10mM-NaF present in the solubilization medium. The tissue fractions were assayed for adenylate cyclase both with and without NaF.

Tissue fraction	Description	Pretreatment of 600g sediment	Additions to solubilization medium containing Lubrol-PX (10g/l) and 6mM-MgSO ₄	Adenylate cyclase activity (pmol of cyclic AMP/10min)		
				No further additions	5mM-NaF*	10mM-NaF
1	600g sediment	None	Not solubilized	136	—	1160 ± 40
2	105 000g supernatant	None	None	152 ± 12	—	214 ± 18
3	105 000g supernatant	None	10mM-NaF	—	412 ± 44	406 ± 12†
4	600g sediment	0.1 mM-p[NH]ppG for 20min at 37°C	Not solubilized	2200 ± 40	—	2320 ± 160
5	105 000g supernatant	0.1 mM-p[NH]ppG for 20min at 37°C	None	764 ± 12	—	572 ± 20
6	105 000g supernatant	0.1 mM-p[NH]ppG for 20min at 37°C	10mM-NaF	—	960	764 ± 4†

* NaF carried over from the solubilization procedure.

† NaF added to give a concentration of 10mM during the assay, taking into account NaF carried over from the solubilization procedure (5mM).

Table 4. *Solubilization of luteal adenylate cyclase: a comparison of the effects of 6 mM-MgSO₄ and 1 mM-EDTA/1 mM-MgSO₄ in the solubilization medium, both in the presence and absence of NaF (10 mM)*

Five tissue fractions were prepared from a single preparation of washed 600g sediment: (1) 600g sediment resuspended in 40mM-Tris/HCl (pH7.5) containing 6mM-MgSO₄; (2) the 105000g supernatant of 600g sediment dispersed in 40mM-Tris/HCl (pH7.5) containing Lubrol-PX (10g/l) and MgSO₄ (6mM); (3) the 105000g supernatant prepared as in (2) but with 10mM-NaF included in the solubilization medium; (4) the 105000g supernatant of 600g sediment dispersed in 40mM-Tris/HCl (pH7.5) containing Lubrol-PX (10g/l), EDTA (1mM) and MgSO₄ (1mM); (5) the 105000g supernatant prepared as in (4) but with 10mM-NaF included in the solubilization medium. The tissue fractions were assayed both without the addition of NaF and with 10mM-NaF. The assays of fractions (4) and (5) contained, in addition, 0.5mM-EDTA/0.5mM-MgSO₄ which was carried over from the solubilization procedure.

Tissue fraction	Description	Solubilization medium: 40mM-Tris/HCl; Lubrol-PX (10g/l) plus		Adenylate cyclase activity (pmol of cyclic AMP/10min)		
		MgSO ₄ or EDTA/MgSO ₄	NaF (10mM)	Basal	5 mM-NaF*	10 mM-NaF
1	600g sediment	Not solubilized	—	136	—	1160 ± 40
2	105000g supernatant	6 mM-MgSO ₄	—	152 ± 12	—	214 ± 18
3	105000g supernatant	6 mM-MgSO ₄	+	—	412 ± 44	404 ± 12†
4	105000g supernatant	1 mM-EDTA 1 mM-MgSO ₄	—	144	—	128
5	105000g supernatant	1 mM-EDTA 1 mM-MgSO ₄	+	—	180 ± 4	186 ± 2†

* NaF carried over from the solubilization procedure.

† NaF added to give a concentration of 10mM during assay, taking into account NaF carried over from the solubilization procedure.

cyclase which had been preincubated with NaF was inhibited by NaF present during the assay.

Effect of Mg²⁺ on the solubilization of luteal adenylate cyclase by Lubrol-PX

Solubilization procedures published by various laboratories have been quite variable with regard to the inclusion of Mg²⁺ and/or EDTA in the buffered detergent solution. We have attempted to clarify the effect of these agents during the solubilization of luteal adenylate cyclase by Lubrol-PX.

Table 4 compares the effects of 6mM-MgSO₄ and 1mM-MgSO₄/1mM-EDTA on the solubilization of adenylate cyclase by Lubrol-PX, both in the presence and in the absence of 10mM-NaF. Both MgSO₄ and NaF alone had a stabilizing effect during solubilization and the greatest amounts of soluble activity were obtained with both agents present in the solubilization medium, there being a synergism of effects which suggests that either Mg²⁺ and F⁻ interact through components of the adenylate cyclase complex or that Mg/F complexes are responsible for the stabilization of activity.

After preincubation of the 600g sediment with p[NH]ppG, the amounts of activity solubilized by Lubrol-PX were greater with 1mM-MgSO₄/1mM-EDTA than with 6mM-MgSO₄ present in the solubilization medium (data not shown). However, NaF, present in the solubilization medium, increased

the amount of soluble activity when 6mM-MgSO₄ was also present but not when 1mM-MgSO₄/1mM-EDTA was present.

Stability of solubilized adenylate cyclase activity

The adenylate cyclase activity solubilized from luteal homogenate 600g sediment by Lubrol-PX was stable at 4°C for at least 18h. 93–110% of the activity assayed in the presence of NaF was recovered irrespective of whether the 600g sediment had been (i) pretreated with p[NH]ppG, (ii) solubilized in the presence of 5mM-NaF or (iii) subjected to a combination of (i) and (ii). At 23°C 54–60% of the soluble activity was lost during the same period of time.

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