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Adenylate cyclase of bovine adrenal cortex plasma membranes

Divergence between corticotropin and fluoride combined effects with forskolin

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The diterpene forskolin maximally stimulated bovine adrenal cortex adenylate cyclase activity 9-fold with a concentration producing half-maximum effect (ED_{50}) of about 4 μM. The effects of forskolin and the fully active corticotropin fragment ACTH (1–24) were additive over nearly the whole range of concentration of both effectors, indicating separate and independent mechanisms of action. By contrast, 10 mM NaF blocked forskolin action in the nanomolar range of the diterpene concentration, while it allowed a partial stimulation by forskolin in the micromolar range. NaF thus reveals a heterogeneity of forskolin action in the adrenal cortex plasma membranes. Moreover, our data suggest that ACTH and NaF activation effects, both mediated by the stimulatory regulatory protein G_s, proceed through different mechanisms.

Adenylate cyclase; Forskolin; Corticotropin; Fluoride; Adrenal cortex

1. INTRODUCTION

Bovine adrenal cortex plasma membranes contain an adenylate cyclase stimulated by corticotropin (ACTH), the physiological hormone regulating steroidogenesis. As in other systems, hormonal activation of the catalytic unit (C) of the cyclase implies hormone binding on its receptor [1] as well as the mediation of stimulatory regulatory GTP-binding proteins (G_s) [2,3] which were demonstrated in this tissue by Glossmann and Gips [4,5]. These regulatory proteins have been shown to consist of three different protein subunits α, β and γ for which an association-dissociation equilibrium is suggested to play a key role in the activation of the enzyme [6,7].

As a tool for investigating the coupling of the different components of the cyclase in the adrenal cortex plasma membranes, we have used forskolin, a diterpene described as a potent general activator of the enzyme [8,9], in particular in adrenals [10–13]. Forskolin acts directly on the catalytic unit and also interferes with the coupling mechanism of C with G_s [14]. In this paper, the

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Abbreviations: R, receptor; G_s, stimulatory regulatory GTP-binding protein; C, catalytic unit of adenylate cyclase; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; BSA, bovine serum albumin

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stimulation characteristics of adenylate cyclase by forskolin were determined either with the diterpene alone or in combination with the fully active synthetic fragment of corticotropin [ACTH(1–24)], or with fluoride, a non-hormonal stimulatory ligand acting via G_s [15]. We demonstrate additivity of forskolin and ACTH(1–24) stimulation of the enzyme, indicating independent mechanisms of action of these effectors. By contrast, we show partially exclusive effects of fluoride with forskolin which suggests that hormone and fluoride may act in a different way through their common mediator G_s.

2. MATERIALS AND METHODS

2.1. *Chemicals*

ATP, GTP, phospho*enol*pyruvate, pyruvate kinase, PMSF, aprotinin and pepstatin were from Sigma. Forskolin was purchased from Calbiochem, NaF from Merck and DMSO from Fluka. ACTH(1–24) was a kind gift from Drs K. Scheibli and R. Andreatta (Ciba-Geigy, Basel).

2.2. *Plasma membrane preparation*

Bovine adrenal cortex plasma membranes were prepared by homogenizing 45 g of the cortex essentially as in [16] except for the buffer which contained 1 mM $NaHCO_3$ (pH 7.5), 1 mM DTE (buffer A), 0.25 M sucrose, 0.1 mM PMSF and 0.2 mg aprotinin and pepstatin per g tissue. The homogenate was centrifuged at 1200 × *g* for 10 min and the resulting supernatant at 15000 × *g* for 20 min at 4°C. The pellet was rehomogenized in 45 ml of the same buffer without the protease inhibitors and layered on a discontinuous sucrose gradient [30.5% (w/w), 39.2 and 47.8% in a volume ratio of 1:1.7:1 and a 'cushion' at 57%, in buffer A]. Centrifugations were run for either 3 h at 25000 rpm in an SW 27 rotor or for 2 h at 47000 rpm in a Ti 14 zonal rotor. The material at the 39.2–47.8% interface was diluted 6-fold in buffer A containing 20 mg aprotinin and pepstatin per g membrane protein. Membranes were harvested by centrifugation (48000 × *g*, 30 min) and resuspended in the last buffer plus 0.25 M sucrose at ~5 mg protein/ml. Aliquots were stored in liquid nitrogen.

This membrane preparation, set up from previous analysis [17], results in an 8.9 ± 2.1-fold enrichment in forskolin-stimulated adenylate cyclase activity relative to the homogenate with a 24 ± 1% yield (means ± SD on 3 preparations).

2.3. *Adenylate cyclase assays*

Adenylate cyclase activity was determined in 40 mM Tris-HCl (pH 7.5) buffer containing 25 mM phospho*enol*pyruvate, 40 U pyruvate kinase/ml, 0.1% BSA, 5 μM GTP, 1 mM DTE, 2.5 mM $MgCl_2$ and 0.5 mM ATP as final concentrations. Concentrations of added stimulators were as indicated in the figure legends. Forskolin was added from stock solutions in DMSO (final cosolvent concentration 1%, v/v). Incubations were performed in 240 μl final volume containing from 3 to 15 μg membrane proteins. After equilibration of the membranes in buffer (2 min at 30°C), the reaction was started by substrate addition and carried out at 30°C for 20 min. It was stopped by immersing the tubes for 70 s in a boiling water bath and the incubates were stored frozen before cAMP determination. Control samples were simultaneously performed.

cAMP was determined in duplicate for each incubate according to [18] using an Amersham assay kit. The deviates were ~4% of the means. It was verified that the activity was linear with time and protein concentration under all conditions tested.

Protein was determined according to [19] as modified in [20].

3. RESULTS

The dose-response curve to forskolin stimulation of bovine adrenal cortex adenylate cyclase extends over a wide range of concentrations of this effector, from 0.01 to 100 μM (fig.1, lower curve). The ED_{50} value is 3.7 ± 0.4 μM (mean ± SD on 3 preparations) and the maximal stimulation factor k is 9.1 ± 1.5 (n = 3). Forskolin efficacy is higher than that obtained for maximal response to ACTH(1–24) (k = 7.3 ± 0.3, n = 2) or for 10 mM NaF (k = 7.1 ± 0.1, n = 2). In this membrane preparation, the dose-response curve to ACTH(1–24) is characterized by an ED_{50} of 0.18 ± 0.10 μM (n = 2) (fig.2, lower curve).

The combined effects of ACTH(1–24) and forskolin were studied by determining the dose-response curves to one activator at a fixed concentration of the other and conversely (figs 1,2). In the

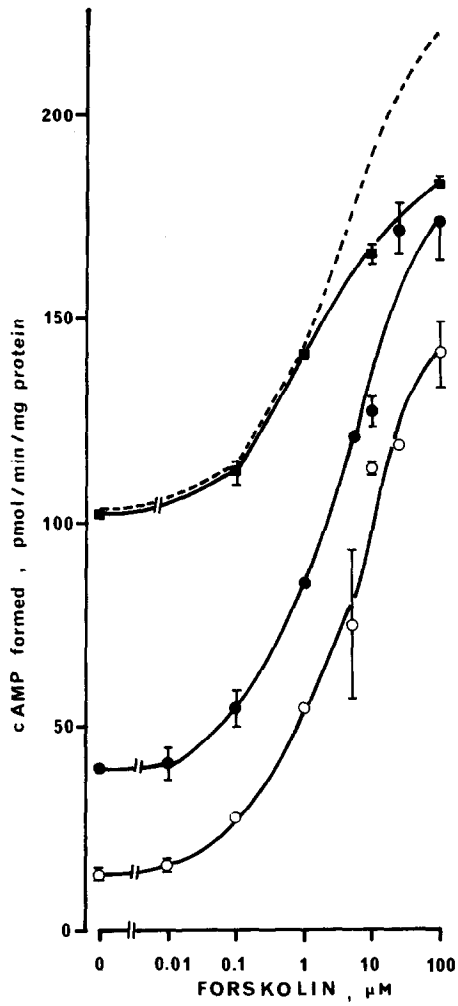

Fig.1. Dose-response curves for forskolin activation of adenylate cyclase activity from bovine adrenal cortex plasma membranes, in the absence (○) or presence of 0.56×10^{-7} M (●) or 0.8×10^{-5} M ACTH(1–24) (■). The bars join the values obtained for duplicate incubations in 1 experiment. The dashed line represents the curve corresponding to complete additivity of ACTH (0.8×10^{-5} M) and forskolin effects.

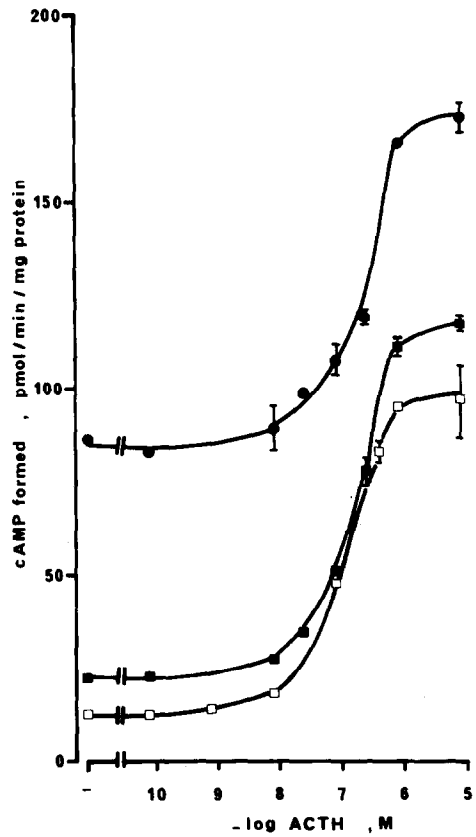

Fig.2. Dose-response curves for ACTH(1–24) activation of adenylate cyclase activity, in the absence (□) or presence of 0.1 μM (■) or 5 μM forskolin (●). The bars join the values obtained for duplicate incubations in 1 experiment. The data are representative of 2 experiments.

case of additivity of the two stimulators' effects, a parallelism between the dose-response curves is expected, the curves being only shifted upward to a higher activity. Such a parallelism is actually observed in fig.2 between the three dose-response curves to ACTH determined in the absence or presence of 0.1 or 5 μM forskolin. Therefore, complete additivity with ACTH effect is demonstrated at these concentrations, where forskolin alone elicited around 10 and 55% of its maximal effect, respectively (fig.1).

Similarly, a parallelism is observed between the dose-response curves to forskolin without or with 0.56×10^{-7} M ACTH (fig.1) corresponding to about 20% of the hormone maximal effect. At 8×10^{-6} M ACTH, where the hormone stimulation is maximal, the two activators add their effects up to a forskolin concentration between 1 and 10 μM. Above that concentration, adenylate cyclase activity further increases with increasing forskolin concentration but in a less than additive way (dashed line in fig.1). The maximum activity value obtain-

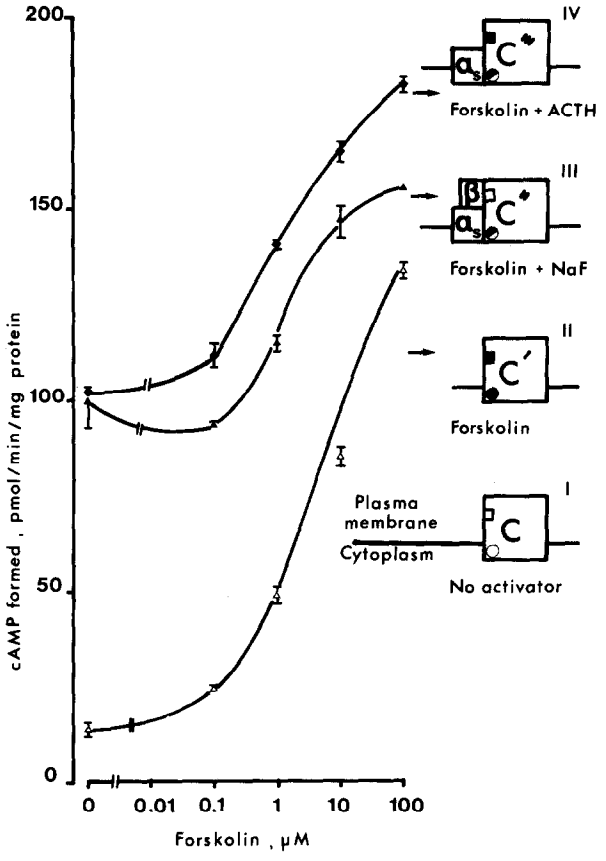

Fig.3. (Left) Dose-response curves for forskolin activation of adenylate cyclase activity in the absence (△) or presence of 10 mM NaF (▲) or 0.8×10^{-5} M ACTH(1–24) (◆). At these concentrations, these last effectors produce their maximal effect. The bars join the values obtained for duplicate incubations in the same experiments. NaF effect is representative of 3 experiments performed on 2 membrane preparations. (Right) Proposed schemes for adenylate cyclase activation in the bovine adrenal cortex plasma membranes; α_s, α-subunit of the stimulatory regulatory protein G_s; β, β-subunit of the regulatory protein G, C, C′, C′′, and C′′′ refer to the catalytic unit in different conformational states. (□, ■) High-affinity forskolin-binding site, either unoccupied or ineffective (open symbol) or fully occupied (closed symbol); (○, ◐, ●) low-affinity forskolin-binding site, unoccupied (open symbol), partially effective (half-filled symbol) or fully occupied (closed symbol). Scheme IV refers to the protein coupling model derived from the experiments of Gilman et al. (i.e. α_s-activated catalytic unit) [6,7] and scheme III to that proposed by Verkman et al. (i.e. $\alpha_s\beta$-activated catalytic unit) [27].

ed at the highest dose of both ACTH and forskolin corresponds to a stimulation factor of 13.4 which can formally be interpreted as resulting from an addition of 100% of the ACTH effect and 70% of the forskolin effect. This slight non-additivity in the high forskolin concentration region increases the apparent potency of forskolin, the ED_{50} value being shifted from 3.7 to $\simeq 1$ μM.

Comparison of ACTH (8×10^{-6} M) and NaF (10 mM) effects on the dose-response curve to forskolin demonstrates a divergence between the behaviour of these two effectors used at concentrations where they have initially similar activation effects on adenylate cyclase activity in the absence of forskolin (fig.3, upper two curves).

NaF and forskolin activation effects are never fully additive whatever the diterpene concentration. NaF appears to block forskolin action in the low concentration range of the diterpene [at 0.01 μM (not shown) and 0.1 μM (fig.3)] where the activity is even slightly depressed as compared to that obtained with fluoride alone. The activity then increases at higher forskolin concentrations, resulting in a maximum activity value which can be interpreted as the addition of 100% of the NaF effect and 43 ± 2% (mean ± SD on 3 expts) of the forskolin effect.

4. DISCUSSION

This report first describes forskolin stimulation characteristics of adenylate cyclase in bovine adrenal cortex plasma membranes. The ED_{50} value (3.7 μM) is significantly smaller than that obtained in other membrane systems in which forskolin action is mostly characterized by an ED_{50} around 10 μM. In addition, its efficacy is among the highest generally obtained in such systems (3–10-fold stimulation over basal activity) [14,21,22]. This high potency and efficacy allows an accurate determination of the catalytic unit activity. Forskolin was used in this work to study the effect of the interaction of C (site of forskolin action) with the other protein components of the cyclase implicated in the stimulation by ACTH and fluoride.

With respect to the combined effects of ACTH and forskolin, the data reported here demonstrate their exact additivity over nearly the whole range

of both effector concentrations. Therefore, these compounds appear to act essentially by independent mechanisms in the adrenal membranes (as illustrated in fig.3, right). The additivity of forskolin and ACTH effects is not complete in the case of high concentrations of both effectors. This may be explained either by a partial impeding effect of ACTH on forskolin action or because the high activation level of the enzyme approaches a maximal turnover that cannot be further augmented. The additivity observed differs from the potentiating effect of forskolin on hormone action described in intact cells [10,22]. The absence of potentiation of hormone stimulation seems to be rather general in isolated membranes [14] except in some instances as for human platelets [23]. It could be suggested that the membrane preparation might remove or alter a cellular component involved in this potentiation.

In contrast with the additive effects of ACTH and forskolin, the NaF (10 mM) and forskolin combination produces less than additive effects on adenylate cyclase activity, whatever the diterpene concentration. In that case, the non-additivity observed at high concentrations of both effectors cannot be due to a maximal turnover of the enzyme since higher activity values have been observed in the presence of both ACTH and forskolin. A correlated observation has been reported for rat cerebral cortical membranes where, in a converse experiment, non-additivity of forskolin and NaF effects was shown on the whole dose-response curve to fluoride [21]. Both experiments converge to indicate interference between the sites of action of NaF and forskolin. Moreover, the variation in degree of this interference with forskolin concentration argues in favour of a heterogeneity of forskolin sites of action in these membranes. In this respect, recent binding studies have shown the existence of low- and high-affinity binding sites of forskolin in rat brain membranes [24], which might also occur in the adrenal membranes. NaF would prevent the stimulation due to forskolin binding on a high-affinity site (nanomolar range) while NaF stimulation would be partially additive with the stimulation due to a forskolin low-affinity binding site (fig.3, right). Such an effect of NaF in the adrenal membranes appears to be at variance with that observed in rat brain and human platelet membranes where NaF was shown to increase high-affinity binding sites for [^{3}H]forskolin [25,26].

Our data show a divergence between hormone and fluoride effects on forskolin-activated adenylate cyclase. We have also demonstrated previously a divergence between the hormone- or fluoride-stimulated adenylate cyclase activity with regard to their temperature dependence [13] as well as to their membrane fluidity dependence (unpublished). These results are in line with recent observations of Verkman et al. [27], derived from target size analysis on intact cells which argue in favour of different mechanisms for hormone and fluoride action. While the hormone would act by promoting the liberation of the α-subunit of G_s which would in turn activate C (general mechanism hypothesized for G_s-mediated effects from studies in detergent solution [6]), fluoride may use the non-dissociated $\alpha\beta$ complex to activate C. Such a model is compatible with our data, the β-subunit then being the component blocking forskolin action on a high-affinity site. A schematic representation of adenylate cyclase activation in the adrenal cortex membranes (fig.3, right) summarizes the proposed conclusions.

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