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Cyclic AMP-independent effects of ACTH on glomerulosa cells of the rat adrenal cortex ☆

Nicole Gallo-Payet [a, b,*], Mylène Côté [a], Alzbeta Chorvatova [b], Gilles Guillon [c], Marcel-Daniel Payet [b]

[a] *Service of Endocrinology, Department of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada, J1H 5N4*
[b] *Department of Physiology and Biophysics, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada, J1H 5N4*
[c] *Unité INSERM U 469, CCIPE, Montpellier, France*

Abstract

The aim of the present paper is to point out the complexity of ACTH action in glomerulosa cells of the adrenal cortex. We demonstrate that the increase in cAMP production induced by ACTH is the result of a balance between activation of adenylyl cyclase and direct modulation of a PDE2 phosphodiestease activity, an effect mediated by inhibition of cGMP content. Moreover, Ca^{2+} is essential for cAMP production and aldosterone secretion, but its exact primary action is not clearly determined. We recently described that ACTH activated a chloride channel, via the Ras protein, which can be involved in steroidogenesis. ACTH also increases tyrosine phosphorylation of several proteins. These data, together with those of phospholipase C activation, indicate that ACTH action in the adrenal is complex, and most certainly not limited to cAMP production, in particular for the low concentrations of the hormone.

Some years ago, cAMP was considered to be the unique second messenger of ACTH action; now it becomes more and more evident that ACTH triggers complex signaling pathways using several second messengers in a closely interacting way. The most predominant point is that these signals are observed for low concentrations of ACTH.

1. Introduction

Although aldosterone secretion by the zona glomerulosa of the adrenal cortex is under multifactorial regulation, ACTH appears to be the most potent stimulus, in the majority of animal models and humans. The stimulating effect of ACTH on steroid secretion ranged from a 10- to 29-fold increase according to experimental models, compared to a 2- to 3-fold increase for the other agents, including angiotensin II. Moreover, in addition to its rapid action on steroidogenesis, ACTH exerts a trophic action, stimulating the synthesis of all steroidogenic enzymes (from StAR to P450 aldo) [1]. The hormone is also a growth factor, stimulating hypertrophy of the zona fasciculata and mitotic activity of glomerulosa cells [2,3]. Indeed, as demonstrated for several G protein-coupled receptors [4], recent studies demonstrate that, in the Y1 cell line, ACTH is able to activate the MAPK pathway [5].

The precise mechanism by which ACTH stimulates steroid synthesis and secretion is still not completely understood. Although it is well known that ACTH increases cAMP production, it is also well known that Ca^{2+} participates in this increase [6,7], probably acting upstream to and downstream from adenylyl cyclase (AC) activation [8]. It is clear that the source of Ca^{2+} is from the extracellular medium; however, the control of influx is probably more complex than previously described [9,10]. Indeed, ACTH increases the amplitude of a L-type calcium current but decreases those of T-type calcium current [11]. Besides these events, previous studies have shown that cGMP [12] and phos-

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* Corresponding author. Tel.: +1-819-564-5243; fax: +1-819-564-5292.

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phoinositides [6,13] pathways may be involved in the action of ACTH. In the present paper, we present evidence that ACTH exerts several actions which are cAMP-independent.

2. Materials and methods

2.1. Chemicals

The chemicals used in the present study were obtained from the following sources: [^{3}H]-Adenine (24 Ci/mmol), [α^{32}P]-ATP (3000 Ci/mmol), [8^3-H]-cAMP (24 Ci/mmol), Biotrak cGMP enzyme immunoessay (EIA) from Amersham (Oakville, Ontario); ATP, cAMP, GTP, cGMP, Adenosine, Guanosine, 5′-nucleotidase *Crotalus* venin and DNAse, A23187 and Ras peptide from Sigma (St Louis, MI, USA); ACTH 1–24 peptide (Cortrosyn) from Organon (Toronto, Canada); aldosterone antiserum from ICN Biochemicals (Costa-Mesa, CA); [^{3}H]-aldosterone (72 Ci/mmol) from New England Nuclear (Boston, MA); creatine kinase, creatine phosphate disodium and EGTA from Boehringer Mannheim (Montreal, Canada); collagenase, Minimun Essential Medium (MEM Eagle medium) and OPTI-MEM medium from GIBCO (Burlington, Ontario); BZA-5B from Genetech (San Francisco, CA); DPC (*N*-phenylanthranilic acid) from Aldrich (USA); Geldanamycine from Calbiochem (USA); H-89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonanide) from Seikagaku America (St Petersburg, FL); IBMX (isolbutyl methylxanthine) from Calbiochem (USA); bovine brain G$\beta\gamma$-subunit from Calbiochem (San Diego, CA); H-Ras(259) azide-free from Santa Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase-conjugated anti-mouse antibody and enhanced chemiluminescence (ECL) detection system from Amersham (Oakville, Canada); anti-phosphotyrosine antibody and monoclonal antibody against MAP-K from UBI (Lake Placid, NY). PD 098059 was a generous gift from Dr David T. Dudley (Parke-Davis Pharmaceuticals Research Division, Warner-Lambett Co, Ann Harbor, MI 48105). The QEHA peptide was synthetized by 'Service de Séquence de Peptides de l'Est du Québec' (Le Centre Hospitalier de l'Université Laval, Qué, Canada). All other chemicals were of A-grade purity.

2.2. Preparation of glomerulosa cells

The zonae glomerulosa were obtained from adrenal glands of female Long Evans rats weighing 200–250 g, and were isolated according to the method described in detail elsewhere [14]. Cells were cultured for 3 days in OPTI-MEM medium supplemented with 2% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Membrane preparation

The tissue was homogeneized with a Polytron homogenizer, in cold buffer containing Tris-HCl (50 mM pH 7.6), EDTA (0.1 mM), DDT (1 mM) and PMSF (0.1 mM). The homogenate was centrifuged at 150 g for 10 min and the supernatant was further centrifugated at 15,000 g for 30 min. The membrane fraction was washed twice in the buffer and frozen at –80°C for subsequent analysis. On the day of the experiment, membranes were washed 3 times in buffer containing Tris-HCl 50 mM pH 7.6, EGTA 2 mM and DTT 1 mM.

2.4. Cyclic AMP determination

Intracellular cyclic AMP accumulation was determined by measuring the conversion of [^{3}H]-ATP into [^{3}H]-cyclic AMP, as previously described [6]. Briefly, cultured cells were incubated at 37°C in OPTI-MEM culture medium containing 2 μCi/ml [^{3}H]-adenine. After 1 h, the cells were washed with cold Hank's buffered saline (HBS: NaCl, 130 mM; KCl, 3.5 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 0.5 mM; $NaHCO_3$, 2.5 mM; HEPES, 5 mM) supplemented with 1 g/l glucose and 0.5% BSA. Cells were incubated in the same buffer with or without 1 mM isobutyl methylxanthine (IBMX) or selective PDEs inhibitors for 15 min at 37°C. The hormones were then added as described previously [6].

2.5. Adenylyl cyclase activity

Membrane preparations were incubated for 10 min at 37°C in Tris-HCl (50 mM, pH 7.6), $MgCl_2$ (5 mM), creatine kinase (0.25 mg/ml), creatine phosphate (1.3 mg/ml), [α^{32}P]-ATP, 10^6 cpm and [^{3}H]-cAMP, 20,000 cpm with or without hormone or IBMX (1 mM), as described by Méry et al. [15]. The reaction was stopped by addition of 500 mM HCl and [α^{32}P]-cAMP formed was separated on Alumina columns.

2.6. Phosphodiesterase activity

Membrane preparations were incubated with or without ACTH in presence or not of PDEs inhibitors in BSA-Tris buffer (EGTA, 1 mg/ml; $MgCl_2$, 10 mM and $CaCl_2$, 0.1 mM, pH, 7.4) as described by Méry et al. [15]. Reactions were performed at 37°C with addition of cAMP (10^{-6} M)+[^{3}H] cAMP for 10 min. The reaction was stopped by addition of IBMX (1 mM), cAMP (0.01 μM), cGMP (0.01 μM), followed by incubation with snake venom (containing 5′-nucleo-

Table 1
Cyclic AMP and cGMP levels in rat adrenal glomerulosa cells[a]

	cAMP production (membranes) nmoles cAMP/mg/15 min		cAMP accumulation (cells) cAMP/ATP (% control)	cGMP (cells) fmol/2.5×10^5 cells	
IBMX	–	+	–	+	–
Control	0.67±0.14	0.91±0.12	0.04±0.01	0.11±0.03	466±91
ACTH (0.1 nM)	1.15±0.17	1.57±0.18	0.19±0.02	1.01±0.11	126±22
ACTH (10 nM)	1.87±0.27	2.32±0.20	1.1±0.15	5.80±0.32	30±6.5

[a] Cyclic AMP and cGMP productions were measured on 3-day cultured of glomerulosa cells, as described in Materials and methods; 250,000 cells were preincubated for 10 min at 37°C with or without the non-selective PDE inhibitor, IBMX (1 mM) before addition of ACTH. For cAMP accumulation, cells were labelled with [^{3}H]adenine and cAMP was measured as the conversion of [^{3}H]-ATP into [^{3}H]-cAMP. Adenylyl cyclase activity was measured in regenerating system consisting of creatine kinase + creatine phosphate and [α^{32}P]-ATP; 30 μg of membrane or 250,000 cells were stimulated as described for cells. [α^{32}P]-cAMP formed was separated on alumina columns. Cyclic GMP was measured by ELISA. Results are the mean ± SEM of three experiments, each in triplicate.

tidase activity) for 20 min at 37°C. The snake venom used in these studies exhibited no appreciable phosphodiesterase activity. The snake venom reaction was stopped by addition of guanosine 0.1 mM, adenosine 0.1 mM and EDTA 0.015 M. [^{3}H]-adenosine formed was separated on QAE Sephadex A25 columns.

2.7. Western blot analysis

Analysis of protein tyrosine phosphorylation were done using whole cell lysates. Cells were incubated for various time periods at 37°C in PBS in the presence of stimuli. After incubation, cells were lysed with SDS 1%/Na_3VO_4 1 mM/staurosporine 100 nM/proteases inhibitors for 40 min at 4°C and centrifugated at 10,000 rpm for 10 min at 4°C. Proteins were separated in 8% SDS-PAGE gels, followed by Western Blot analysis, using a specific antibody against tyrosine phosphorylated proteins or MAP kinase. Immunoreactivity was visualized by chemiluminescence.

2.8. Measurement of aldosterone secretion

Cells were incubated for 2 h in HBS buffer as described previously [14]. Aldosterone content was determined by radioimmunoassay, using specific antisera and tritiated steroid as tracer.

2.9. Electrophysiology

The physiological solutions used for patch clamp experiments were the following: the basic external solution contained (mM): NaCl, 100; $CaCl_2$ 10; tetraethylammonium, 35; $MgCl_2$, 1; CsCl, 5.4; Hepes, 5 and glucose 2 g/l at pH 7.4. The control pipette solution contained (mM): Cs-aspartate, 120; NaCl, 18; $CaCl_2$, 1; EGTA, 11; $MgCl_2$, 2; Hepes, 5; ATP, 3, and GTP, 0.4 at pH 7.2. Cells were used after 1–2 days of culture. Solutions containing hormones or drugs were freshly prepared before each experiment. Experiments were performed at room temperature. Ionic currents were recorded using the whole-cell configuration of the patch-clamp method [11,16], with an axopatch 1B. Analysis were performed with a custom-made program.

2.10. Data analysis

The data are presented as means ± S.E. Statistical analyses of the data were performed using the Student *t*-test.

3. Results and discussion

3.1. ACTH and phosphodiesterase activity

In rat glomerulosa cells, 10 nM ACTH induces a 28-fold increase in cAMP production compared to a 3-fold increase in membrane preparations. However, the threshold is 10 pM in membranes, compared to 0.1 nM in cells. In addition, preincubation with IBMX (a non-selective inhibitor of all PDE) enhanced by 100 % cAMP production from intact cells, but has no effect on membranes (Table 1). These results suggest that differences exist in the early events of ACTH action or in the process of cAMP degradation.

The intracellular concentration of cAMP is partly determined by the activities of one or more adenylyl cyclases isoforms [17,18] and partly by one or several cyclic nucleotide phosphodiesterases (PDE). Four isoforms of PDE are known to hydrolyze efficiently cAMP at physiological substrate concentrations, namely the Ca^{2+}-calmodulin-regulated PDEs (PDE1), that hydrolyzes both cAMP and cGMP, the cGMP-stimulated PDE (PDE2), that hydrolyzes cAMP at a relatively high range of concentrations (10–100 μM),

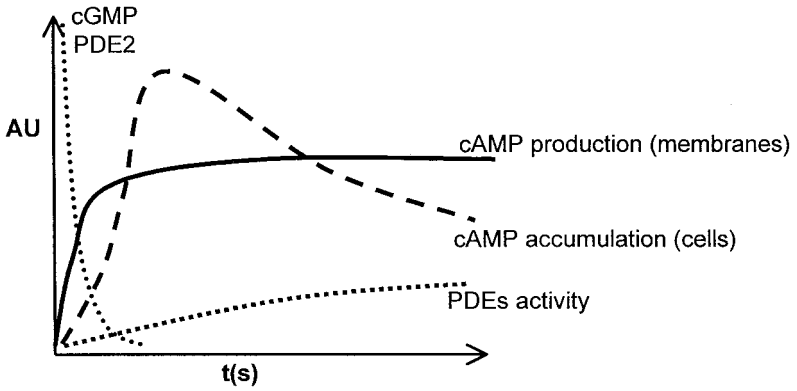

Fig. 1. Model of intracellular time-course regulation of cAMP by PDE2 and cGMP in rat adrenal glomerulosa cells. The time-course of cAMP production in membranes reflects the cAMP production in absence of any PDE activity. The time-course of cAMP accumulation measured in cells can be divided in two phases: one where the cAMP concentration, after a delay, increases rapidly to a peak value, and a second one, characterized by a slow decrease to a plateau. PDE activity controls these two phases. In the basal state, the level of cGMP is high and PDE2 is activated to inhibit the initial burst of cAMP. Thereafter, cGMP is rapidly degraded, inducing a time-dependent loss of PDE2 activity and a rapid increase in cAMP accumulation. Simultaneously, ACTH induces a slow activation of PDE (several forms). This delayed PDE activity is responsible of the slow decrease of cAMP accumulation. The curves were simulated by using a set of two differential equations. One represents the production of ACTH (—) in the absence of PDE activity; the second the cAMP accumulation if PDEs are active. The activity of PDE as function of time (....) was proportional to the cGMP degradation induced by ACTH. The slow activation of PDE (.....) was chosen to obtain the best fit to the experimental data of the cAMP accumulation (AU, arbitrary units).

the cGMP-inhibited PDE (PDE3) which has a high affinity for cAMP and is inhibited by low concentrations of cGMP and finally the cAMP-specific PDEs (PDE4) which has a high selectivity for cAMP [19–21]. A 10 min preincubation with 25 μM Rolipram (a specific PDE4 inhibitor) or 25 μM Ly 195 115 (a specific PDE3 inhibitor) does not affect the production of cAMP induced by ACTH, while preincubation with 25 μM EHNA, (specific PDE2 inhibitor) enhanced the production of cAMP to a level similar to that observed with addition of IBMX (data not shown). Time-course experiments indicate that, in the absence of IBMX, the maximal effect of ACTH on cAMP production occurs after 15 min and then decline to return to the basal level after 40 min of incubation, even in the continuous presence of ACTH. In membranes, the time-dependent increase reaches a plateau after 15 min, without further decrease (Fig. 1). Moreover, preincubation with 25 μM EHNA strongly enhanced cAMP production induced by ACTH. The effect was evident as soon as after 1 min stimulation and this increase in cAMP was maintained at elevated levels for up to 40 min. These results suggest a role for PDE2 in the regulation of cAMP production induced by ACTH. Since PDE2 is a cGMP-stimulated PDE [19,20], we measured ACTH effect on cGMP concentration. ACTH induces a time-dependent and a dose-dependent decrease in the level of cGMP (Table 1). Previous studies demonstrate that the cGMP-PDE2 was the predominant isoform of PDE present in the adrenal cortex [22], with the highest concentration found in the zona glomerulosa [23], at least of bovine origin.

Fig. 1 summarizes the different factors involved in the time-course of cAMP accumulation measured in cells. This profile can be obtained only by the combination of a time-dependent increase in cAMP production with a complex time-dependent degradation by PDE. Activation of adenylyl cyclase, as observed in membrane preparations, follows an exponential time-course and reaches a steady-state value. This time-course reflects the cAMP production in the absence of any PDE activity. However, the time-course of cAMP accumulation measured in cells is quite different; it can be divided in two phases: one in which the cAMP concentration, after a delay, increases rapidly to a peak value, and a second one, characterized by a slow decrease to a plateau. As shown in Fig. 1, PDE activity appears to control these two phases. In basal state, the level of cGMP is high and PDE2 is activated to inhibit the initial burst of cAMP. Thereafter, cGMP is rapidly degraded, inducing a time-dependent loss of PDE2 activity and a rapid increase in cAMP accumulation. Simultaneously, ACTH induces a slow activation of PDE (several forms) either directly by an as yet unknown mechanism or indirectly by increasing cAMP concentration. This delayed PDE activity may be responsible of the slow decrease of cAMP accumulation.

3.2. ACTH and Ras-activated chloride channel

Another interesting feature of ACTH action concerns the implication of chloride ions. Chloride channels play an important role in the maintenance of the cell volume, water transport and cell membrane potential stabilization [24]. Although Ca^{2+}-dependent chlor-

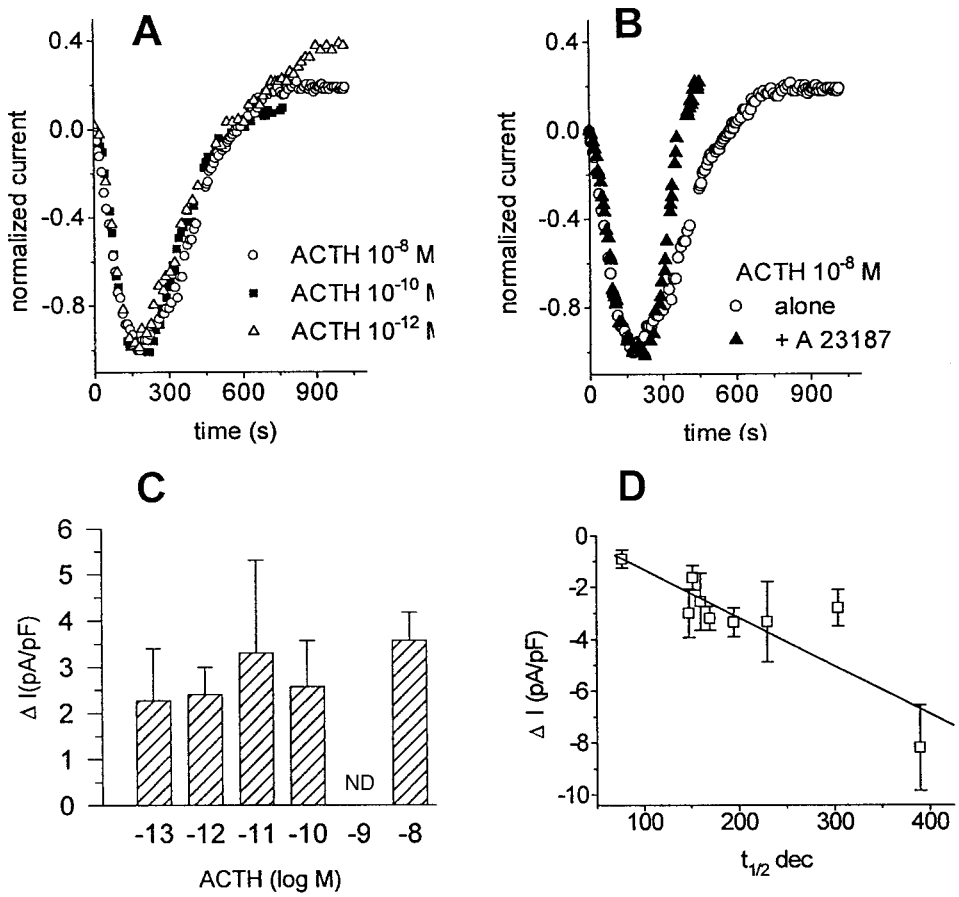

Fig. 2. Relationship between ACTH concentration and induction of a Cl^- current in rat adrenal glomerulosa cells. (A) Normalized current activated by ACTH 10^{-8} M (open circles), 10^{-10} M (filled squares) and 10^{-12} M (open triangles). Normalized current $=(I-I_c)/(I_{max}-I_c)$, where I_c represents the amplitude of the current measured before and I_{max} at maximum of ACTH action. Each trace: one different cell, H.P. −40 mV. (B) Histogram of amplitudes of current induced by ACTH at H.P. −40 mV (I_{max}–I_c), in function of different ACTH concentrations. (C) Effect of A23187 (5×10^{-6} M, pretreatment for 10 min) on normalized current. Chloride current activated by ACTH (10^{-8} M) in the absence (filled triangles) and in the presence (open circles) of calcium ionophore A23187 in the external medium. Same conditions as in A. (D) Relationship between the induced current amplitude and the time necessary for 50% decrease of the current ($t_{1/2}$ dec), as recorded in different experimental conditions, H.P. −40 mV.

ide current increased by Ang II was characterized in bovine fasciculata cells [25] and chloride ions were proposed to be implied in the modulation of testosterone production by Leydig cells [26–28], no information is available to date on their possible implication in the steroid secretion induced by ACTH in adrenal cells.

In our preparation of zona glomerulosa cells, application of ACTH resulted in a transient increase in a current, characterized as a chloride current, since it was abolished by chloride channel inhibitor such as DPC (*N*-phenylanthranilic acid) and its reversal potential followed changes in chloride concentration (data not shown). The observed chloride current was induced by ACTH in concentrations as low as 10^{-13} M (Fig. 2A) and when normalized, showed same kinetics in all tested cases. The amplitude of the induced current was not significantly different across a wide range of concentrations (10^{-13}–10^{-8} M, Fig. 2B); instead it was the number of responding cells that seems to decrease with lower concentrations. These results demonstrates that activation of the chloride current does not follow the increase in cAMP concentration. Indeed, pretreatment of the cells with IBMX and application of forskolin or 8BrcAMP did not trigger the current. Preincubation of glomerulosa cells with the PKA inhibitor, H-89 [8,9], was also ineffective on the amplitude of the current, in contrast with the observation on the PKA-regulated CFTR-channel [29].

When calcium ionophore A23187 was added to the external medium for 10 min, ACTH (10^{-8} M) induced a smaller current (-0.9 ± 0.35 pA/pF, $n=3$) than in control conditions (-3.35 ± 0.56 pA/pF, $n=5$). Normalization of the currents recorded at –40 mV (Fig. 2C) indicated that the increase of intracellular calcium did not affect the current activation, but reduced the time necessary for 50% decrease of the current ($t_{1/2}$ dec), that changed from 194.97 ± 57.63 ($n=5$) in the absence, to 77.1 ± 22.3 s ($n=3$) in the pre-

Table 2
Regulation of aldosterone secretion by ACTH (*$P < 0.01$; **$P < 0.001$, compared to corresponding ACTH-stimulating values)[a]

	Aldosterone secretion (pg/2.5 $\times 10^5$ cells)		
	Control	ACTH (0.1 nM)	ACTH (10 nM)
Control	107.4±24	2231±51	2873±128
DPC (50 μM)	114±7.2	1646±127*	3067±150
BZA (50 μM)	94.8±4.2	1141±52**	2201±93*
U173122 (10 μM)	126±10	1249±179**	1998±134*

[a] Cells were cultured for three days as explained in Materials and methods and stimulated for 2 h in HBS buffer with or without the different stimuli and inhibitors. Aldosterone was determined by radioimmunoassay, using specific antisera and tritiated steroid as tracer.

sence of calcium ionophore. When the amplitude of the chloride current was plotted in function of $t_{1/2}$ dec, recorded in different experimental conditions (Fig. 2D), we obtained a linear relationship. This suggests that a correlation exists between the amplitude and the time-dependent decrease of the current, and that a smaller amplitude of ACTH-induced current in the presence of calcium ionophore could be the result of its faster decrease.

The signaling pathway involved in the activation of chloride current by ACTH was studied and we demonstrated that Ras was involved in the ACTH-induced current. Indeed, ACTH-induced current was abolished in cells pretreated with the permeant benzodiazepine peptidomimetic (BZA-5B), an inhibitor of farnesyl transferase [30], or when a monoclonal antibody against Ras, H-Ras(259), was added to the pipette medium. In agreement with these results, Ras-GTPγS gave rise to a chloride channel. Moreover, inhibition of MAPKK by PD 098059 [31] indicates that the chloride current is not activated by the MAPK, as was postulated for a K^+ current induced by PACAP 38 [32] (data not shown). Interestingly, DPC and BZA decrease more efficiently aldosterone secretion induced by low stimulating concentrations of ACTH than high concentrations, indicating that chloride ions and the Ras pathway could play an important role for low concentrations of the hormone, when cAMP production is not significant (Table 2).

3.3. ACTH and tyrosine phosphorylation

In several cell types, the initial activation of Ras is depending upon the activation of several tyrosine phosphorylated proteins [33,34]. Moreover, we have previously shown that ACTH induced a rapid translocation of microfilaments from the cytosol to the plasma membrane and that Gs proteins were not only localized on the membranes, but were also closely associated with microfilaments [35]. Several actin-associated proteins are activated through Tyr-phosphorylation, thus we examined whether ACTH could modulate the pattern of tyrosine phosphorylation in glomerulosa cells. Indeed, results shown in Fig. 3A indicate that ACTH modulates protein tyrosine phosphorylation of several proteins. However, in contrast to that observed with the Y1 cell line [5], ACTH did not increase $p42^{mapk}$ and $p44^{mapk}$ phosphorylation, suggesting that ACTH effect on growth promoting activity may be indirect, via stimulation of others growth factors (Fig. 3B).

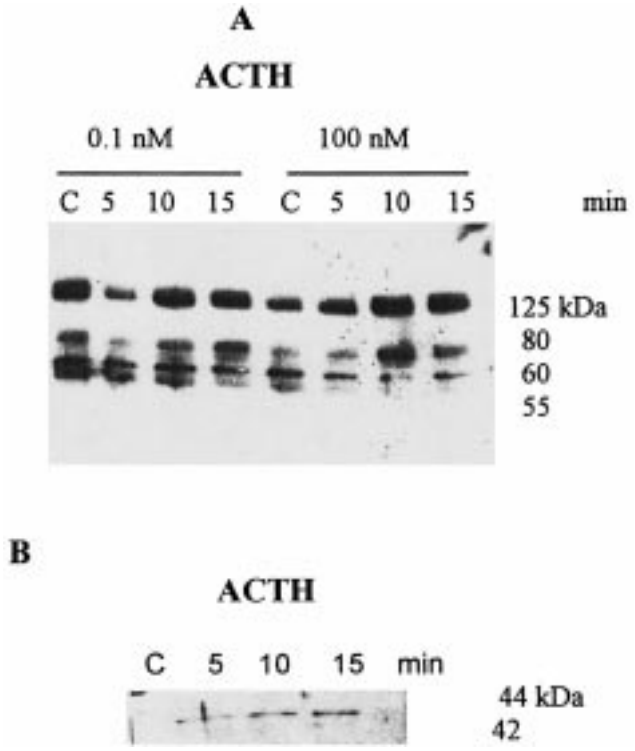

Fig. 3. Western blot analysis of the effects of ACTH on tyrosine phosphorylation (A) and MAP kinase activity (B) in rat adrenal glomerulosa cells. Cells were cultured in 35 mm Petri dishes (1×10^6 cells/dish), as described in Materials and methods. Cells were then stimulated for various durations with 0.1 and 10 nM ACTH; 30 μg proteins from whole cell extracts were analyzed by Western blotting. Phosphotyrosinated proteins were revealed with an anti-phosphotyrosine antiboby (A) and MAPK with an antibody against the phosphorylated form of $p42^{mapk}$ and $p44^{mapk}$ (B). Numbers on the right indicate position of molecular mass markers (kDa).

3.4. ACTH and the phosphoinositide pathway

Previous studies from Farese et al. [13] and from our group [6] have shown that ACTH could stimulate a small increase in inositol phosphate production. We now show that this increase is involved in aldosterone secretion, since preincubation with the phospholipase C (PLC) inhibitor U73122 decreased significantly ACTH-induced aldosterone secretion (Table 2).

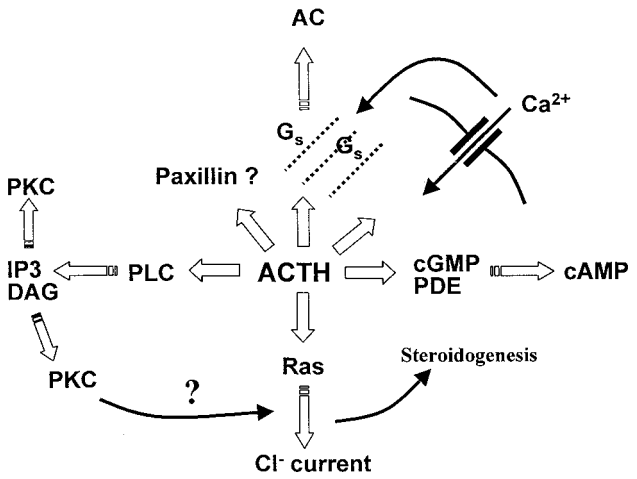

Fig. 4. Model of ACTH action in rat adrenal glomerulosa cells. The figure emphasizes that ACTH acts not only on adenylyl cyclase, but also on the cGMP content to control PDE2 activity and subsequently cAMP concentration. ACTH also activates a chloride channel by a Ras-dependent pathway and stimulates phosphoinositide breakdown for low concentrations. In addition, ACTH induces a rapid effect on microfilaments distribution at the plasma membrane, an effect abolished in a Ca^{2+}-free medium. For more details, see text.

However, this increase in inositol phosphates, which is observed only for low concentrations of ACTH, was not sufficient to release calcium from intracellular stores. It is also not known to date if ACTH stimulated phospholipase via Gq, Gi protein or even $\beta\gamma$ subunits, as described in some models [4].

4. Conclusion

All these results, summarized in Fig. 4, indicate that ACTH action is complex. First, we demonstrated that the increase in cAMP production induced by ACTH is the results of a balance between activation of adenylyl cyclase and direct modulation of a PDE2 phosphodiestease activity. In addition, although it is known that Ca^{2+} is essential for cAMP production and aldosterone secretion, its exact primary action is not adenylyl cyclase, since isoforms detected in rat cells are not Ca^{2+}-sensitive [36]. Moreover, the large increase in intracellular calcium develops slowly, mainly through L-type Ca^{2+} channels, controled by protein kinase A phosphorylation [8–10]. However, the regulation of the Ca^{2+} influx is probably more complex. K^+ channels, for example, are also blocked by ACTH [37,38], and this blockage depolarizes the membrane at potentials where Ca^{2+} channels could be opened and positively regulated by PKA. In addition, we showed that ACTH is able to activate a chloride channel at very low concentrations. This effect is important for steroidogenesis, since aldosterone increase by the low stimulating concentrations of ACTH is blocked by DPC, while the higher stimulating concentrations are not affected (Table 2). It is possible that Cl^- ions could be linked to Ca^{2+} transport in the cell and modulate the action of ACTH, playing a role essentially for low concentration of the hormone (when cAMP production is low). Importantly, this chloride channel is activated by the Ras protein, but not by cAMP-PKA pathway. The mechanism by which Ras is activated remains to be determined. However, activation of protein kinase C resulting from activation of PLC may activate $p21^{ras}$. Indeed, activation of PLC is not suffisient to release Ca^{2+}, but PKC activation may be involved in the activation of the chloride current, as well as AC7 isoform of adenylyl cyclase, present in human cells (data not shown). All these new data were obtained for low concentrations of ACTH, clearly indicating that ACTH action in the adrenal is certainly not limited to cAMP production.

We previously showed that ACTH induced a rapid microfilament translocation to the membrane and that absence of Ca^{2+} from the experimental medium disrupted the microfilament network in such a way that Gs protein is no longer translocated to the membrane [35]. We thus propose that the actin cytoskeleton may be the primary target of Ca^{2+} action. Consistent with these effects, ACTH increases Tyr-phosphorylation of several proteins, actin-binding proteins possibly being some of them. Some years ago, cAMP was considered to be the only second messenger of ACTH action, now it becomes more and more evident that ACTH triggers complex signaling pathways using several second messengers in an interacting way as illustrated in Fig. 4. Besides the variety of ACTH-induced signals, the most predominant point is that these signals are observed for low concentrations of ACTH. Analysis of the interactions between the various signals will be a challenge over the next years to obtain a complete view of the ACTH effects.

Acknowledgements

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