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### ► To cite this version:

Marthe Rizk-Rabin, Sabrina Chaoui-Ibadioune, Anna Vaczlavik, Christopher Ribes, Michel Polak, et al.. Link between steroidogenesis, the cell cycle, and PKA in adrenocortical tumor cells. *Molecular and Cellular Endocrinology*, 2020, 500, 10.1016/j.mce.2019.110636 . hal-03014038

**HAL Id: hal-03014038**

**<https://cnrs.hal.science/hal-03014038v1>**

Submitted on 19 Nov 2020

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1 Link between steroidogenesis, the cell cycle, and PKA in adrenocortical tumor cells

2

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15 Short title: Cell cycle and PKA control steroidogenesis

16 Key words: cell-cycle phases, PKA activity, MAP kinase, steroidogenesis genes, Star, PKA

17 R1A and CA.

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26 **Abstract**

27 Adrenocortical tumors (ACTs) frequently cause steroid excess and present cell-cycle  
28 dysregulation. cAMP/PKA signaling is involved in steroid synthesis and play a role in cell-  
29 cycle regulation. We investigated, by cell synchronization in the different phases of the cell-  
30 cycle, the control of steroidogenesis and the contribution of PKA in adrenocortical cells  
31 (H295R and culture of primary pigmented nodular adrenocortical disease cells). Cells showed  
32 increased steroidogenesis and a maximal PKA activity at G2 phase, and a reduction at G1  
33 phase. *PRKACA* overexpression, or cAMP stimulation, enhanced PKA activity and induced  
34 steroidogenesis in all synchronized groups but is not sufficient to drive cell-cycle progression.  
35 *PRKARIA* inactivation enhanced PKA activity and induced *STAR* gene expression, only in  
36 cells in G1, and triggered cell-cycle progression in all groups.

37 These findings provide evidence for a tight association between steroidogenesis and cell-cycle  
38 in ACTs. Moreover, *PRKARIA* is essential for mediating the function of PKA activity on both  
39 steroidogenesis and cell-cycle progression in adrenocortical cells.

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## 51 **1. Introduction**

52 Adrenocortical tumors (ACTs) are often associated with increased steroid secretion, which  
53 contributes to morbidity. This has been observed for benign tumors, such as those of primary  
54 pigmented nodular adrenocortical disease (PPNAD) (Espiard et al., 2014), macronodular  
55 hyperplasia (Louiset et al., 2013), adrenocortical adenomas (Bassett et al., 2005), and  
56 malignant adrenal cortical carcinoma (ACC) (Nakamura et al., 2015; Abiven et al., 2006).  
57 Even ACCs, which are classified as endocrine-inactive based on routine hormonal  
58 assessment, are often responsible for abnormal steroid secretion, as demonstrated by mass  
59 spectrometry analysis (Arlt et al., 2011). ACT cells also frequently display cell-cycle control  
60 defects (Ragazzon et al., 2011; Stratakis, 2009; Assie et al., 2014). Consistent with this  
61 observation, amplifications and deletions of genes encoding key regulators of the cell cycle  
62 (CDK4, CCNE1, RB1, CDKN2A) have been described in several studies and are more  
63 frequent in ACCs (Assie et al., 2014; De Martino et al., 2013; Ross et al., 2014; Pereira et al.,  
64 2018).

65 It is well established that the effects of steroid hormones, such as glucocorticoids, androgens,  
66 and estrogens, on cell proliferation and differentiation are mediated by the regulation of key  
67 events in the control of cell-cycle progression or arrest (Kullmann et al., 2013; Heisler et al.,  
68 1997; Amanatullah et al., 2002), but little is known concerning steroid-hormone secretion  
69 during the cell cycle in the adrenal gland. The adrenolytic drug mitotane (op'DDD), used in  
70 ACC treatment, decreases cortisol secretion and induces the destruction of the zona  
71 fasciculata and zona reticularis of the adrenal cortex (Cerquetti et al., 2008). Its use has been  
72 shown to be associated with cell-cycle arrest at the G2/M phase and apoptosis (Doghman et  
73 al., 2013; Hescot et al., 2013).

74 Genetic alterations leading to constitutive activation of the cAMP/PKA pathway in various  
75 types of ACTs, such as cortisol secreting adenomas (ACA) or PPNAD, result in steroid over-  
76 secretion (Lodish et al., 2016). The best examples are inactivating mutations of the regulatory  
77 subunit R1A of PKA (*PRKAR1A*), which is mutated in the germline DNA of patients with  
78 PPNAD and Carney complex or tumor DNA in a subset of secreting ACAs and ACCs  
79 (Kirschner et al., 2000;Bertherat et al., 2003;Zheng et al., 2016). In benign adrenal tumors  
80 associated with Cushing syndrome, *PRKACA* somatic mutations also result in constitutive  
81 PKA activity by disrupting the interaction between *PRKACA* and the regulatory subunits of  
82 PKA, including *PRKAR1A* (Beuschlein et al., 2014;Goh et al., 2014;Calebiro et al., 2014;Cao  
83 et al., 2014;Sato et al., 2014). Changes in cAMP signal transduction also play a role in  
84 regulating the cell cycle (Massimi et al., 2017;Gupta et al., 2017;Sun et al., 2009). Activation  
85 of the cAMP/PKA pathway in many cell types inhibits proliferation by preventing cells from  
86 entering the S phase and arresting cells in G1 (Duncan et al., 2006;Hewer et al., 2011). In  
87 contrast, stimulation of the cAMP/PKA pathway may facilitate cell proliferation, such as in  
88 thyroid and pituitary tumors (Medri et al., 1994;Spada et al., 1992).

89 Cyclic AMP exerts its actions primarily through the activation of protein kinase A (PKA).  
90 PKA is composed of two types of regulatory and four types of catalytic subunits. The PKA  
91 regulatory subunits have been studied for their role in tumor formation (Taylor et al., 2013).  
92 *In vivo* and *in vitro* studies have shown that inactivating *PRKAR1A* leads to tumorigenesis  
93 (Kirschner et al., 2005;Nadella et al., 2005). These studies showed that the lack of *Prkar1a*  
94 protein in primary mouse cells *in vitro* causes constitutive PKA activation and  
95 immortalization of primary mouse embryonic fibroblasts (MEFs). At the molecular level,  
96 knocking out *Prkar1a* led to the upregulation of D-type cyclins (Kirschner et al., 2005).  
97 Similarly, we showed that inactivation of *PRKAR1A* confers resistance to apoptosis and  
98 enhances proliferation in the adrenocortical cell line H295R (Basso et al., 2014). *PRKAR1A*

99 inactivation disrupted the cell-cycle checkpoint and cells accumulated at the G2 phase with an  
100 increase in cyclin D and PKA CA activity. These results suggested that there may be a link  
101 between the increase in steroid secretion and cell-cycle dysregulation, which are  
102 concomitantly observed in adrenal tumors.

103 Here, we investigated the cell-cycle-dependent control of adrenal-steroid oversecretion and  
104 the contribution of cAMP/PKA in two cell lines synchronized by pharmacological drugs:  
105 H295R, a human adrenal cortex cell line derived from an ACC (Gazdar et al., 1990) that does  
106 not harbor any mutations in the cAMP/PKA pathway, and primary cultured PPNAD cells,  
107 with inactivating mutations of *PKARIA*. We mimicked the constitutive activation of PKA that  
108 is observed in adrenal tumors with *PRKARIA* inactivating (Kirschner et al., 2000; Bertherat et  
109 al., 2003) or *PRKACA* activating (Beuschlein et al., 2014; Calebiro et al., 2014; Weigand et al.,  
110 2017) mutations using either cAMP treatment, transient silencing of *PRKARIA* by  
111 transfection with an siRNA, or overexpression of one of the catalytic subunits of PKA,  
112 concomitant with the pharmacological drugs.

113 Overall, our data show that the hypersecretion of steroids by the H295R ACC cell line and  
114 PPNAD is controlled by the phase of the cell cycle and that PKA activity plays a role in such  
115 coordination.

116

## 117 2. Materials and methods

### 118 2.1 Cell culture

119 Human H295R adrenocortical carcinoma cells, purchased from the ATCC, were grown as  
120 previously described (Bouizar et al., 2010;Ragazzon et al., 2009;Groussin et al., 2000), in  
121 DMEM Ham/F12 medium (Sigma, St. Quentin Fallavier France) supplemented with 50  
122 units/ml penicillin, 50 mg/ml streptomycin, 2 mmol/L glutamine, 2% Ultrosor G2 (Biosepra,  
123 Fremont, CA), and ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) (Sigma)  
124 at 37°C in an atmosphere of 5% carbon dioxide/95% air. Cells were allowed to attach for 24 h  
125 before use.

### 126 2.2 Primary cell culture of PPNAD

127 Human adrenals were obtained after informed consent from two CNC patients undergoing  
128 surgery for PPNAD. Adrenal tissue collection was approved by the ethics committee of  
129 Cochin Hospital. PPNAD adrenal tissues were obtained during surgery and immediately  
130 immersed in culture medium. Briefly, adrenocortical explants were enzymatically dispersed  
131 as previously described (Cazabat et al., 2014;Bram et al., 2016). Adrenocortical cells were  
132 cultured at 37°C in 5% CO<sub>2</sub> and allowed to attach for four days before use. Cells were  
133 incubated with various pharmacological inhibitors to synchronize the cell-cycle for 24 h with  
134 fresh DMEM medium, as for the H295R cell line.

### 135 2.3 Western blotting

136 Whole-cell protein lysates were obtained by extraction with RIPA lysis buffer containing 50  
137 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% v/v Nonidet P-40 (NP40), anti-  
138 phosphatase, and anti-protease (Roche, Boulogne Billancourt, France) as previously described  
139 (Basso et al., 2014). Western blotting was performed with primary mouse antibodies for R1A  
140 (1/500, BD Laboratories, Rungis, France), R2B (1/1000), PKA catalytic subunits (1/1000)

141 (Santa Cruz Biotechnology, Nanterre, France), and Star (1/1000) (Cell Signaling, St Quentin  
142 en Yvelines, France). We used anti- $\beta$ -actin (1/2500) (Sigma) or GAPDH antibodies (1/1000)  
143 (Santa Cruz Biotechnology) as loading controls. The antigen-antibody complexes were  
144 visualized with appropriate secondary antibodies (Santa Cruz Biotechnology) by  
145 chemiluminescence detection (ECL<sup>TM</sup> kit, Thermo scientific, Saint Herblan France). The  
146 signals were digitized with the Syngene/Gene Tool analysis system. (Ozyme, Montigny Le  
147 Bretonneux, France)

#### 148 *2.4 Cell-cycle distribution*

149 Cell cultures were harvested, rinsed with PBS, fixed in 500  $\mu$ l 70% ethanol, and frozen (-  
150 20°C). Fixed cells were centrifuged, rinsed in PBS, suspended in 500  $\mu$ l PBS containing 50  
151  $\mu$ g/ml propidium iodide (Sigma) and 100  $\mu$ g/ml RNase A (Sigma), and examined by flow  
152 cytometry (FACscan, EPICS XL Coulter, Villepinte France). Data were analyzed using  
153 Multicycle software. All experiments were carried out three times. Each histogram was  
154 constructed from at least 10,000 cells (Rizk-Rabin et al., 2008).

#### 155 *2.5 Cell-cycle synchronization*

156 We used three different inhibitors, each specific for a cell-cycle check point: L mimosine for  
157 G1 (Vackova et al., 2003), aphidicolin for G1/S (Sorscher et al., 1991), and nocodazole for  
158 G2 (Ho et al., 2001) (Sigma). The cell-cycle phases of H295R cells arrested at a specific point  
159 in the cell cycle were well defined in a previous report (Bouizar et al., 2010). Cells were  
160 cultured in 12-well plates at a density of  $150 \times 10^3$  cells/well and then treated for 24 h with L-  
161 mimosine (400  $\mu$ M) or aphidicolin (10  $\mu$ M) or for 24 h or 48 h with nocodazole (10  $\mu$ M). For  
162 cell-cycle release of the H295R cells, following 24 or 48 h of synchronization, cells were  
163 washed three times with PBS and cultured in medium without drugs for 6, 12, 24, 36, or 48 h.  
164 For PKA activation or inhibition, cells were transiently transfected with an siRNA for



165 *PRKARIA*, the control scramble RNA (siS), *PKACA* (in the PCDNA3+ vector, a gift from Dr  
166 D. Calebiro), or the empty vector, or incubated with the PKA inhibitor PKI 14-22  
167 (Calbiochem, Fontenay sous-Bois France ) and cultured with the different drugs for 24 h.

#### 168 *2.6 Transfection and co-transfection*

169 H295R cells cultured in six-well plates were transfected with 100 pmol siRNA *PRKARIA*  
170 (UGAAUGGGCAACCAGUGUdTdT), the siRNA negative control (siS)  
171 (CAGUCGCGUUUGCGACUGGdTdT), 500 ng *PKACA* or PCDNA3+, as empty vector,  
172 using the Effectene reagent (Promega, Charbonnières les Bains, France). Cells were analyzed  
173 24 h after transfection. For luciferase assays, 24-well plates were used. Cells were co-  
174 transfected with the siRNAs or vectors and a luciferase reporter gene driven by Star  
175 promoters. Firefly and Renilla luciferase activities (Promega) were measured as previously  
176 described (Basso et al., 2014;Ragazzon et al., 2009).

#### 177 *2.7 Steroid measurement.*

178 Cortisol and aldosterone levels in cell medium were measured by competitive immunoassays  
179 and revealed by chemiluminescence assays (Laboratory of Hormonology at Cochin Hospital,  
180 France ).

#### 181 *2.8 Protein kinase A assay*

182 The PepTag nonradioactive protein kinase assay kit (Promega) was used to measure PKA  
183 activity, according to the manufacturer's instructions, as described in (Rizk-Rabin et al.,  
184 2008)

#### 185 *2.9 Analysis of RNA by quantitative PCR*

186 Total RNA, extracted from the cell lines, was treated with DNase and further purified using  
187 the RNeasy Mini kit and RNase-free DNase Set (Promega), according to the manufacturer's  
188 instructions. Purified RNA was reverse transcribed with high-capacity cDNA reverse

189 transcriptase (Applied Biosystems, Illkirch France) and expression levels of the target genes  
190 analyzed by quantitative PCR using a LightCycler Fast Start SyberGreen kit (Roche  
191 Diagnostics, Meylan France), according to the manufacturer's instructions. The PCR  
192 conditions for all target steroidogenic and cyclin genes are shown in Table 1. Relative  
193 quantification of target cDNA was determined by calculating the difference in cross-threshold  
194 (CT) values after normalization to PPIA (CYCLO) signals (DDCT method).

#### 195 *2.10 Statistical analysis*

196 Data were analysed by ANOVA, Student's t-test, or regression analysis with Fisher's projected  
197 least significant difference or Scheffe's F test for comparison of the means (StatView 5.0,  
198 SAS Institute, Cary, NC, USA). Data are expressed as the means  $\pm$  S.E.M. Values of  $P = 0.05$   
199 were considered significant. The number of experiments is indicated in each figure legend.  
200 Results of all data for which statistical analysis was performed represent the quantification of  
201 at least three duplicate experiments. Control conditions were set as 1. Significance was set at  
202  $p < 0.05$  (\*),  $p < 0.01$  (\*\*), and  $p < 0.001$  (\*\*\*)).

203

## 204 **3. Results**

### 205 *3.1 Cell-cycle synchronization of H295R and PPNAD cells*

206 We synchronized the cells by treatment with L-mimosine, aphidicolin, or nocodazole,  
207 assessed by propidium iodide staining and cytometry analysis (Figure 1). We obtained similar  
208 results for both cell types. Asynchronous cells treated with vehicle (DMSO) displayed a  
209 typical profile, with a higher percentage of cells in G1 (Figure 1A I,II) . Treatment of the cells  
210 with L-mimosine resulted in the accumulation of cells in G1 and decreased the percentage of  
211 cells in G2 (Figure 1B I,II). Aphidicolin treatment resulted in the accumulation of cells in late  
212 G1, with fewer cells in S and G2 than for the control (Figure 1C I,II) as demonstrated in  
213 (Bouizar et al., 2010). Nocodazole treatment resulted in a decrease in the number of cells in  
214 G1 and the accumulation of cells in G2 (Figure 1D I,II) as assessed in (Bouizar et al., 2010).  
215 All phases of the cell cycle were verified by analyzing the expression of various cyclins to  
216 distinguish between the different check points of the cell cycle (Supplemental Figure 1).  
217 Cyclin D1 mRNA levels increase substantially in cells blocked with L-mimosine and this  
218 increase serves as a marker of initiation of the G1 phase, the level of Cyclin E increases in  
219 Aphidicolin-treated cells and serves as a marker of late G1/S, and increases in cyclin B and D  
220 levels serve as markers of the G2 Phase (Pereira et al., 2018).

### 221 *3.2 Steroidogenesis is higher in the G2 phase in synchronized H295R and PPNAD cells*

222 H295R cells secrete both glucocorticoids and mineralocorticoids, whereas PPNAD secretes  
223 only glucocorticoids. The analysis of steroid secretion showed significantly greater cortisol  
224 secretion by both H295R and PPNAD cells at G2 (Figure 1D III, IV). In H295R cells,  
225 aldosterone secretion was also higher at G2 (Figure 1D III). In contrast, cortisol and  
226 aldosterone secretion was lower in H295R at G1 ( $p < 0.01$ ), whereas there was no difference  
227 for PPNAD (Figure 1A III, IV). Finally, we observed no differences for either cell type at  
228 G1/S (Figure 1C III, IV).

229 We analyzed the impact of cell-cycle synchronization on the expression of genes responsible  
230 for steroidogenesis by real-time RT-PCR. Cells synchronized with nocodazole accumulated at  
231 G2 and showed a significant increase in mRNA levels for all studied steroidogenic enzyme  
232 genes, both in H295R and PPNAD, except for *CYP11A1* in H295R and *CYP17A* in PPNAD  
233 (Figure 2C I, II). In contrast, we only observed a significant increase in *CYP17A* ( $p < 0.05$ )  
234 and decrease in *CYP21A2* mRNA levels in H295R in cells synchronized in G1 (Figure 2B I),  
235 whereas we observed a decrease in *CYP11A1* and *CYP21A2* mRNA levels in PPNAD cells  
236 (Figure 2A II). H295R cells at the early G1/S transition also showed a significant decrease in  
237 *CYP11A1* mRNA levels (Figure 2B I). Star protein expression only increased in cells at G2 in  
238 H295R (Figure 2 III). The increase in Star protein levels at G2 correlated with an increase in  
239 mRNA expression in cultured the H295R and PPNAD cells (Supplemental Figure 2).

240 We verified that the changes in steroidogenesis during the cell cycle resulted from  
241 physiological regulation and were not due to side effects of the drugs by performing similar  
242 analyses on H295R cells that were released from synchronization and cultured in normal  
243 medium to follow their progression through the cell cycle from 0 to 6, 12, 24, 36, and 48 h  
244 after release. The release of cells synchronized at G1 (53.2% G1, 37% S, and 9.7% G2) by L-  
245 mimosine treatment, resulted in an increase in the percentage of cells in S at 12 h after release,  
246 followed by an increase in cells in G2 at 36 and 48 h (Figure 3A I). The expression of  
247 steroidogenic enzyme genes by RT/PCR at various times after release is presented as the fold  
248 change with respect to asynchronous cells at time 0, set to one. There was a progressive  
249 increase of *CYP11B1*, *CYP11B2*, *CYP11A1*, *NR5A1 (SF1)*, and *STAR* mRNA levels from 6 to  
250 12 h, followed by a peak of expression after 36 h, concomitant with entry into G2 (Figure 3B  
251 I and C I).

252 The release of cells blocked in G2 (11.7% G1, 16.8% S, and 71.5% G2) with nocodazole (48  
253 h of treatment) resulted in their progression through the cell cycle (Figure 3A, right). They  
254 then re-entered G2 phase from 36 to 48 h after release (Figure 3A II). There was a progressive  
255 decrease in *CYP11B2* mRNA levels from 0 to 24 h, coinciding with the decrease in the  
256 percentage of cells in G2, followed by a distinct increase from 36 h that reached the initial  
257 level observed at time 0 of the release (Figure 3B II). The expression of *CYP11B1* and  
258 *CYP11A1* mRNA levels did not change from 0 to 24 h and then increased (Figure 3B II).  
259 *NR5A1 (SF1)* and *STAR* mRNA levels increased at 36 h (Figure 3C II).

260 These data confirm that steroidogenic enzyme mRNA levels change during cell-cycle  
261 progression.

### 262 *3.3 PKA and MAP kinase activity in synchronized H295R cells*

263 Cell-cycle progression is often associated with the modulation of PKA and MAP kinase  
264 activity. Thus, we next assessed PKA and MAP kinase activity in synchronized cells. ERK1/2  
265 phosphorylation, reflecting MAP kinase pathway activity, was significantly higher in all  
266 synchronized groups than in asynchronous cells (Figure 4A-C I and Supplemental Figure 3).  
267 MEK phosphorylation was also significantly higher in cells in G2 (Figure 4C I). Moreover,  
268 there was significantly less PKA activity in cells in G1 (Figure 4A II), whereas it was  
269 significantly higher in cells in G2 (Figure 4C II) than in asynchronous cells.

270 Under the same conditions, PKA R1A protein levels were markedly lower in cells at G2  
271 (Figure 4C III), whereas those of PKA R2B and PKA CA were not significantly altered in any  
272 of the studied groups (Figure 4A-C).

273

### 274 *3.4 The effect of PKA activation on the control of the cell cycle and steroidogenesis in* 275 *synchronized H295R cells*

276 The overexpression of steroidogenic enzyme genes at G2 is associated with an increase in the  
277 level of PKA activity. We next investigated whether increased PKA activity effects the phases  
278 of the cell cycle and steroidogenesis in synchronized cells. We thus increased PKA activity by  
279 overexpressing *PRKACA* (Ca) or silencing *PRKARIA* (Figure 5 I-II). PKA Ca was  
280 overexpressed in transfected cells of the asynchronous control and all synchronized groups.  
281 However, it was less highly overexpressed in the nocodazole group, for which the basal level  
282 was much higher than that of the control asynchronous cells (Supplemental Figure 4A). The  
283 expression of the PKA RI protein was reduced in all depleted *PRKARIA* synchronized groups  
284 (Supplemental Figure 4 B). However, the magnitude of the increase in Ca protein levels and  
285 decrease in RIa protein levels depended on the phase of arrest.

286 Overexpression of *PRKACA* (*Ca*) for 24h in H295R cells had no effect on the cell cycle  
287 (Figure 5A I) but increased both the activity of PKA and a luciferase reporter construct driven  
288 by the *STAR* promoter (*STAR-Luc*) in all groups, independently of the phase of cell-cycle  
289 arrest (Figure 5B and C I). *STAR-Luc* activity was the highest in cells in G2 (Figure 5C I).  
290 Similarly, PKA activation by cAMP had no effect on the cell cycle (Supplemental Figure 5).

291 Transient depletion of *PRKARIA* for 24 h was sufficient to increase the percentage/number of  
292 cells in G2 in the asynchronous group and decreased the percentage of cells in G1 relative to  
293 the control (siS, asynchronous cells; Figure 5A II). In L-mimosine treated cells, the  
294 invalidation of *PRKARIA* shifted the cells toward the S and G2 phases and decreased the  
295 percentage of cells in G1 ( $p \leq 0.02$  siR1A vs siS). Simultaneous transfection with siR1A and  
296 aphidicolin treatment for 24 h decreased the percentage/number of cells in G1 ( $p < 0.001$   
297 siR1A vs siS) and subsequently increased those in S ( $p < 0.05$  siR1A vs siS). Simultaneous  
298 nocodazole treatment and R1A silencing increased the percentage of cells in S and decreased  
299 the percentage of those in G2 ( $p \leq 0.05$ ) (Figure 5A II). As expected, *PRKARIA* silencing

300 enhanced PKA activity in all groups (Figure 5B II). Under the same conditions, *PRKARIA*  
301 silencing led to increased activity of the *STAR*-Luc reporter in the asynchronous group ( $p <$   
302 0.05) and L-mimosine treated cells ( $p < 0.05$ ), whereas its activity remained unchanged in the  
303 aphidicolin and nocodazole-treated cells (Figure 5C II).

### 304 *3.5 Effect of PKA inhibition on control of the cell cycle in synchronized H295R cells*

305 We confirmed the above data with the pharmacological inhibition of PKA activity by  
306 treatment with the cell-permeable specific inhibitor Myr-PKI (14-22), which contains a PKA  
307 substrate consensus sequence with the serine replaced by alanine (Wiley et al., 1999). The  
308 major effect of PKA inhibition was observed in nocodazole treated cells (G2), for which there  
309 was a decrease in the percentage of cells in G2 ( $p < 0.001$ ) and an increase in the percentage  
310 of those in S ( $p < 0.02$ ) (Figure 6A). This effect was paralleled by a marked and significant  
311 decrease in *STAR*-Luc activity in the nocodazole-treated cells ( $p < 0.001$ ) (Figure 6B).  
312 Overall, our results show that the PKA R1A subunit mediates the effect of PKA activity on  
313 cell-cycle progression and the activation of steroidogenesis, depending on the phase of the  
314 cell cycle.

315

316 **4. Discussion.**

317 Excess adrenal-steroid secretion can be explained by mutations of various key components of  
318 the cAMP pathway that are also likely to be involved in adrenal tumorigenesis (Bertherat et  
319 al., 2003;Beuschlein et al., 2014;Calebiro et al., 2014;Horvath et al., 2006;Horvath et al.,  
320 2008). Here, we demonstrate that steroidogenesis is modulated according to specific cell-  
321 cycle phases in both an established human adrenal cortical carcinoma cell line, H295R, and a  
322 primary cell culture of PPNAD (harboring *PKARIA* inactivating mutations) from patients with  
323 cortisol excess.

324 Major actors of steroidogenesis (NR5A, Star, and enzymes) were preferentially induced  
325 during G2 (Figures 1, 2 and 3), leading to peak steroidogenesis during the same phase.

326 It is well known that cAMP/PKA and the MAP kinase activity vary during the cell cycle and  
327 that MAP kinase activity is higher during the G2 phase in most cells (Philipova et al., 1998).  
328 However, in most cells, cAMP levels and PKA activity are low during G2 and mitosis and  
329 then become high in G1 (Bolte et al., 2003;Kurokawa et al., 1998;Yu et al., 2008). This  
330 variation is cell-type specific, as the elevation of intracellular cyclic AMP (cAMP) is  
331 considered to be a potent mitogenic signal for a number of cell types, including Swiss 3T3  
332 cells, thyroid epithelial cells, and somatotroph cells of the anterior pituitary (Withers, 1997).

333 Here, we demonstrated that only the level of PKA activity varied notably according to the  
334 stage of the cell cycle in the adrenocortical cell line H295R. PKA activity was high in G2  
335 (Figure 4C II) and lower in G1 (Figure 4A II). Thus, both PKA and MAP kinase were  
336 activated in G2 (Figure 4C, I, II). The change of PKA activity in H295R cells at G2 is a  
337 molecular mechanism in which the cAMP/PKA pathway is specifically involved in the  
338 control of mitosis, as it phosphorylates several substrates involved in its progression. The in  
339 vivo dynamics of PKA activity during mitosis was highlighted by the study of Vandame P et



340 al. (Vandame et al., 2014) using FRET-based biosensors in single living HeLa cells. They  
341 showed that PKA activity is required for the maintenance of chromosome condensation and  
342 that it may also be required during mitosis, because it phosphorylates histone H1.4, resulting  
343 in the dissociation of this protein from the chromatin during mitosis. Moreover, Aurora A  
344 kinase, a key protein for the progression of mitosis, is also a target of PKA. The high PKA  
345 activity in G2 corresponded with a decrease in the level of the regulatory subunit R1A of  
346 PKA (Figure 4C III). One explanation for the decrease in the level of PKA R1A protein is that  
347 the increase in PKA activity at G2 liberates the PKA R1A protein from PKA CA, making it  
348 more accessible to degradation. Another possibility is the consequence of the association of  
349 PKA R1A with the cytoskeleton or microtubules through either low-affinity or highly  
350 dynamic interactions, as shown in BW1J hepatoma cells (Imaizumi-Scherrer et al., 2001).

351 Mutations of PKA signaling components (*GNAS*, *PRKARIA*, *PRKACA*, and  
352 phosphodiesterase genes) in adrenal tumors activate the pathway (Nakajima et al., 2016; Mete  
353 et al., 2018; Drougat et al., 2015). Although these mutations are associated with the  
354 pathogenesis of benign tumors, we tested whether PKA may regulate the cell cycle and  
355 steroidogenesis in a coordinated fashion by activating PKA in the adrenocortical cell line  
356 H295R through overexpression of the PKA catalytic subunit alpha, stimulating the cells with  
357 Bu2cAMP, or silencing *PRKARIA* in combination with cell synchronization. The responses  
358 of the cells to the activation of PKA were different depending on the PKA subunits used.  
359 *PRKACA* overexpression stimulated the activity of a PKA-dependent construct (*STAR-Luc*) in  
360 all groups but did not alter the phase of the cell cycle (Figure 5 A and C, I). The activation of  
361 PKA by wild type *PRKACA* overexpression may not mimic the effect of *PRKACA* mutations,  
362 as Bathon et al (Bathon et al., 2019) recently showed that transfection of HEK293 cells with  
363 various *PRKACA* mutant constructs for 48 h highly increased PKA activity over that of wild  
364 type levels and increased the phosphorylation of the specific PKA substrate histone H1.4,

365 involved in proliferation, relative to that by wild type *PRKACA*. Our result represents the  
366 early effect of PKA activation (24-h transfection of H295R) by wild type *PRKACA*, which  
367 indiscriminately activates substrates and not selected ones. Similarly, 24-h activation of PKA  
368 by Bu2 cAMP did not alter the cell cycle profile, neither of asynchronous nor synchronized  
369 cells. Our results are consistent with those of the study of Liu et al (Liu et al., 2004), in which  
370 the effect of Bu2cAMP on proliferation and apoptosis was not observed at an early time point,  
371 one to two days of incubation, but later, after three days to one week of incubation.

372 As expected and described by Basso *et al.* (Basso et al., 2014), inactivation of *PRKARIA* in  
373 asynchronous cells for 24 h increased both PKA and *STAR*-Luc reporter activity (Figure 5B  
374 and C II) and led to the accumulation of the cells in G2 and a decrease in the number of cells  
375 in G1 (Figure 5A II). In synchronized cells, PKA activity increased after *PRKARIA* depletion.  
376 The increase was greater in cells treated for G1 arrest than those treated for G1/S and G2  
377 phase arrest. In contrast, the effects of the increase in PKA activity on steroidogenesis were  
378 restricted to the cells treated for G1 arrest. In addition, the depletion of *PRKARIA* induced  
379 cell-cycle progression in all synchronized groups, as demonstrated by a significant decrease in  
380 the percentage/number of cells in G1 reflecting progression to the S and then G2 phases  
381 (Figure 5A II).

382 Moreover, PKA controlled progression of the cells through the cell cycle, as the inhibition of  
383 PKA activity by PKI slowed the progression of cells with a high level of PKA activation and  
384 those in G2 (Figure 6).

385 Thus, in our model, the essential and the early determinant of PKA activity as a regulator of  
386 cell-cycle progression is the level of PKA R1A. However, our findings on malignant  
387 adrenocortical cells that harbor different mutations (Tissier et al., 2005;Ragazzon et al., 2010)

388 might not apply to normal adrenocortical cells or benign adrenocortical tumors carrying  
389 alterations in PKA signaling (Bathon et al., 2019).

390 PKA R1A can also interact directly with other specific proteins and modulates a number of  
391 other signaling pathways. It has been demonstrated that PKA and PKA R1A are important for  
392 regulation of the meiotic spindle in mammalian oocytes, in which increased PKA activity is  
393 responsible for maintaining meiotic arrest. On the other hand, the knockdown of *PRKARIA*,  
394 as expected, increased PKA activity but the meiotic arrest of mouse oocytes was not  
395 maintained (Duncan et al., 2006). PKA R1A is also associated with the second subunit of  
396 replication factor C (RFC40) and functions as a nuclear transporter for RFC40, causing G1  
397 arrest of MCF7 cells (Gupte et al., 2005). In the absence of PKA R1A, RFC40 may remain in  
398 the cytoplasm and cells progress toward the G2 and M phases.

399 The role of PKA R1A on the control of cell-cycle progression can be explained by the spatial  
400 regulation of PKA activity by A-kinase anchor proteins (AKAPs). AKAPs serve as scaffold  
401 proteins to tether PKA in close proximity to its substrate, thereby locally regulating its  
402 activity and PKA subunit specificity (Pidoux et al., 2010;Day et al., 2011). Recently, R1A  
403 was shown to be associated with AKAPs, which form a complex with the ribosomal S6  
404 protein kinase RSK1 kinase (Chaturvedi et al., 2009;Chaturvedi et al., 2006). *Prkar1a*  
405 silencing in mouse lung fibroblasts (B82L) decreased the nuclear accumulation of active  
406 RSK1 and increased its cytoplasmic localization. The resulting increase in active cytosolic  
407 RSK1 levels is accompanied by increased phosphorylation of its cytosolic substrate tuberous  
408 sclerosis complex 2 (TSC-2) and the anti-apoptotic factor BAD. As RSK1 shares common  
409 phosphorylation sites with PKA on certain substrates, such as CREB, BAD, and Nur77, part  
410 of the cell response in the absence of PKA R1A may not be due to increased PKA activity  
411 (Chaturvedi et al., 2009)

412 In conclusion, we have provided evidence of a tight association between the phase of the cell  
413 cycle and steroidogenesis in *two* adrenal tumors, *PPNAD and the malignant H295R cell line*.  
414 Enhancing PKA activity clearly appears to be critical for steroid excess, whereas the PKA  
415 R1A subunit is essential for triggering the cell cycle.

416 **Declaration of interest:** there is no conflict of interest that could be perceived to affect the  
417 impartiality of the research reported. All authors declare that there is no conflict of interest.

418 **Funding:** this work was supported by the National Research Agency: grants ANR 14 CE12-  
419 000-02 and ANR 15 CE 12-0017

420 **Acknowledgements:** We thank the members of the FACS Core Facility CYBIO of the  
421 Institute Cochin for their cooperation. The English of the manuscript has been corrected by a  
422 native English-speaking scientific editor of Alex Edelman and Associates.

423

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710 **Figure legends**

711 Figure 1. The effect of cell-cycle synchronization of the H295R cell line and primary PPNAD  
712 cell culture for 24 h on the cell-cycle profile and steroid secretion.

713 Flowcytometry analysis of DNA content by propidium iodide incorporation in synchronized  
714 H295R cells and PPNAD primary culture for **A)** control cultures without treatment and  
715 cultures treated with **B)** L-mimosine, **C)** aphidicolin, or **D)** nocodazole. Histograms of cortisol  
716 and aldosterone content secreted into the cell medium after cell-cycle synchronization are  
717 shown to the right. The results are presented as the means  $\pm$  SEM of three duplicate  
718 independent experiments for H295R and one representative example of two duplicate  
719 experiments for PPNAD. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.001$ .

720 Figure 2. The effect of cell synchronization on steroidogenic enzyme and Star protein  
721 expression, mRNA expression by RT/PCR of steroidogenic enzymes in H295R cells and  
722 PPNAD primary cultures in the **A)** G1, **B)** G1/S, and **C)** G2 phases of the cell cycle. Each  
723 studied steroidogenic enzyme is normalized to the corresponding results in the control  
724 asynchronous cells, which is set to one. For Star protein expression, the results are normalized  
725 against those of control cells and a histogram of the western blot in synchronized H295R cells  
726 and PPNAD primary cultures are shown on the right. The results are presented as the means  $\pm$   
727 SEM of three duplicate independent experiments for H295R and one representative example  
728 of two duplicate experiments for PPNAD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

729 Figure 3. Cell-cycle profile and the kinetics of steroidogenic enzyme expression after cell-  
730 cycle arrest and release of H295R cells. 0 h represents the profile at the end of  
731 synchronization after 24 h of treatment with L-mimosine (I) or 48 h with nocodazole (II).  
732 Each time point is normalized to asynchronous control cells at 0 h of release. **A)** Histogram  
733 showing the percentage of cells in each phase of the cell cycle at each time point, analyzed by

734 flow cytometry, showing the progression in the cell cycle. RT/PCR analysis of **B)**  
735 steroidogenic enzyme and **C)** SF-1 and Star mRNA levels for each time point. The results are  
736 presented as the means  $\pm$  SEM of three duplicate independent experiments. The level of  
737 significance is indicated at the top of each figure.

738 Figure 4. Effect of the different drugs on cell signaling pathways and PKA subunit regulation  
739 in H295R cells. The effects are shown for **A)** L-mimosine, **B)** aphidicolin, and **C)** nocodazole.  
740 MAP Kinase activity is shown by histograms of western blots of pERK/total ERK, and  
741 pMEK/total MEK protein levels normalized to the control, set to one. We spliced the original  
742 full gel images of ERK and MEK, presented in Supplementary Figure 3, to re-order the  
743 samples in a more logical manner. PKA activity was measured by a nonradioactive PKA  
744 assay and is presented as the ratio of free PKA/total PKA. Protein levels of the PKA subunits  
745 R1A, R2B, and Ca in cells treated with the different drugs are shown by histograms of  
746 western blots. The results of the histograms are expressed as the percentage relative band  
747 density/the band density of GaPDH, normalized to the corresponding controls (arbitrarily set  
748 to one) in H295R-treated cells. The results are presented as the means  $\pm$  SEM of three  
749 duplicate independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

750 Figure 5. Effect of PKA activation, by the over expression of *PRKACA* or *PRKARIA*  
751 depletion, on the regulation of the cell cycle, PKA activity, and steroidogenic response  
752 (Star/luc-reporter gene activity) simultaneously with the synchronization of H295R cells. **A)**  
753 Cell-cycle distribution analyzed by flow cytometry. **B)** PKA activity measured by a non-  
754 radioactive assay and presented as the ratio of free phosphorylated PKA/total PKA. **C)** *STAR*  
755 reporter gene activity measured by Luciferase/Renilla. The results are presented as the means  
756  $\pm$  SEM of three duplicate independent experiments normalized to the control of each group,

757 set to one. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 for the over expression of  
758 PKA CA.

759 Figure 6. Inhibition of PKA activity by the PKA inhibitor PKI (5  $\mu$ M) throughout cell cycle  
760 synchronization (24 h) showing that mostly the nocodazole-treated cells were affected. **A)**  
761 Cell-cycle phase distribution. **B)** Star/luc reporter gene activity. The measurement of the PKA  
762 activity is not presented because PKA activity was inhibited and thus no PKA  
763 phosphorylation. The results are presented as the means  $\pm$  SEM of three duplicate  
764 independent experiments. \* p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

765

766 Supplementary Figures:

767 Supplementary Figure 1. Distribution of cyclin mRNA expression in in H295R cells arrested  
768 at various phases of the cell cycle. **A)** Arrested in early G1 by L-mimosine treatment. **B)**  
769 Arrested in late G1/S by aphidicolin treatment. **C)** Arrested in G2 by nocodazole treatment.  
770 The results are presented as the means  $\pm$  SEM of three duplicate independent experiments  
771 normalized to the control of each group, set to one. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

772 Supplementary Figure 2. Star mRNA expression by RT/PCR in H295R cells and PPNAD  
773 primary cultures in the G1, G1/S, and G2 phases of the cell cycle. The results are presented as  
774 the means  $\pm$  SEM of three duplicate independent experiments for H295R and one  
775 representative example of two duplicate experiments for PPNAD. \*p < 0.05, \*\*p < 0.01,\*\*\*p  
776 < 0.001.

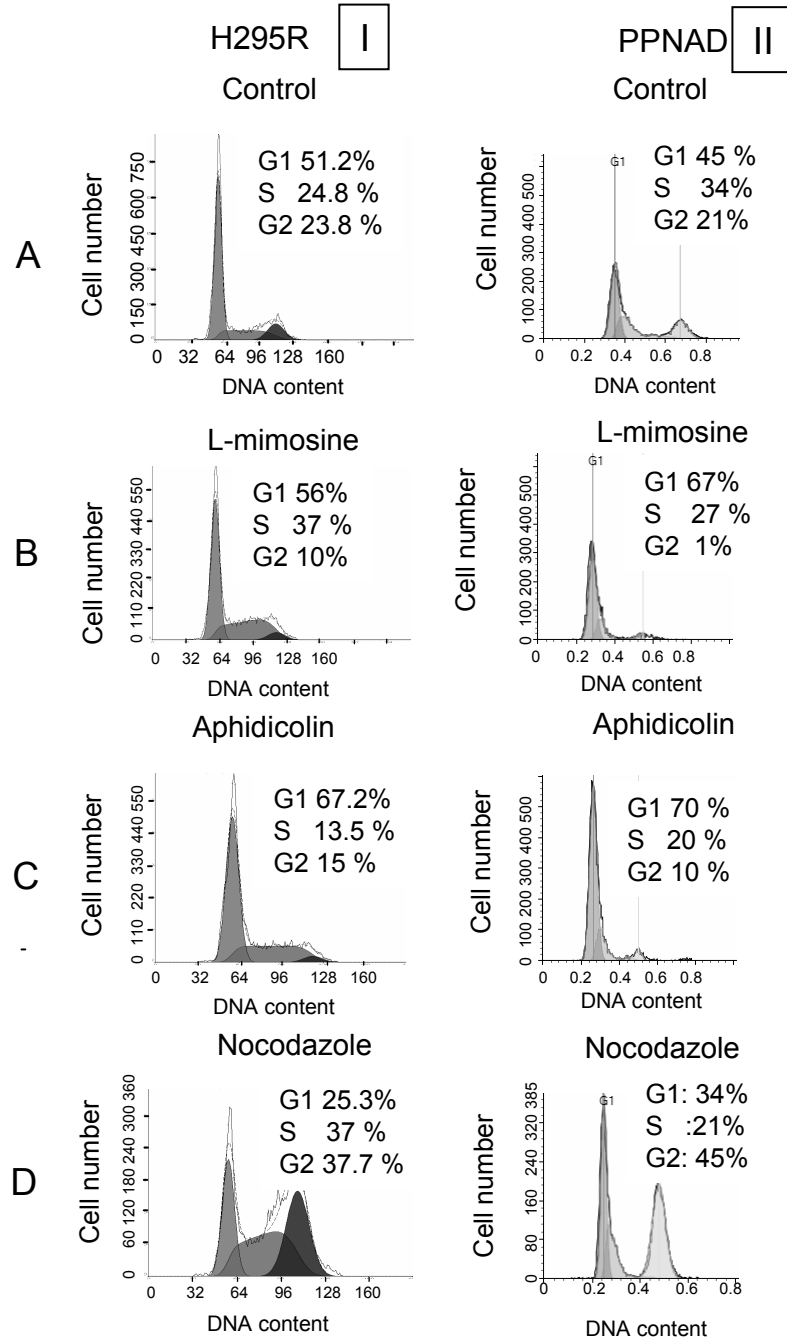
777 Supplementary Figure 3. Image of the full western blot of MAPKinase cell signaling used for  
778 Figure 4. **A)** Image of the full western blot. **B)** The corresponding Histogram, as in Figure 4.

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780 Supplementary Figure 4. PKA CA and PKA R1A protein expression in simultaneous A)  
781 overexpression of wildtype *PRKACA* or B) depleted *PRKARIA* and cell-cycle  
782 synchronization. Results are normalized to the control vector or sis, set to one. The  
783 comparison is inter group (Vector vs Ca or SiS vs siRI ) for each group. The results are  
784 presented as the means  $\pm$  SEM of three duplicate independent experiments normalized to the  
785 control of each group, set to one. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

786 Supplementary Figure 5. Effects of cAMP stimulation on cell-cycle synchronization. The  
787 distribution of the phases resulting from cell-cycle arrest were not affected by stimulation  
788 with the cAMP analogue (dibutyryl adenosine 3'-5' cyclic monophosphate). The  
789 measurement of PKA activity and Star Luc reporter gene activity are not presented because  
790 they are equally fully saturated in all of the synchronized groups.

### Cell cycle synchronization



### Steroids secretion

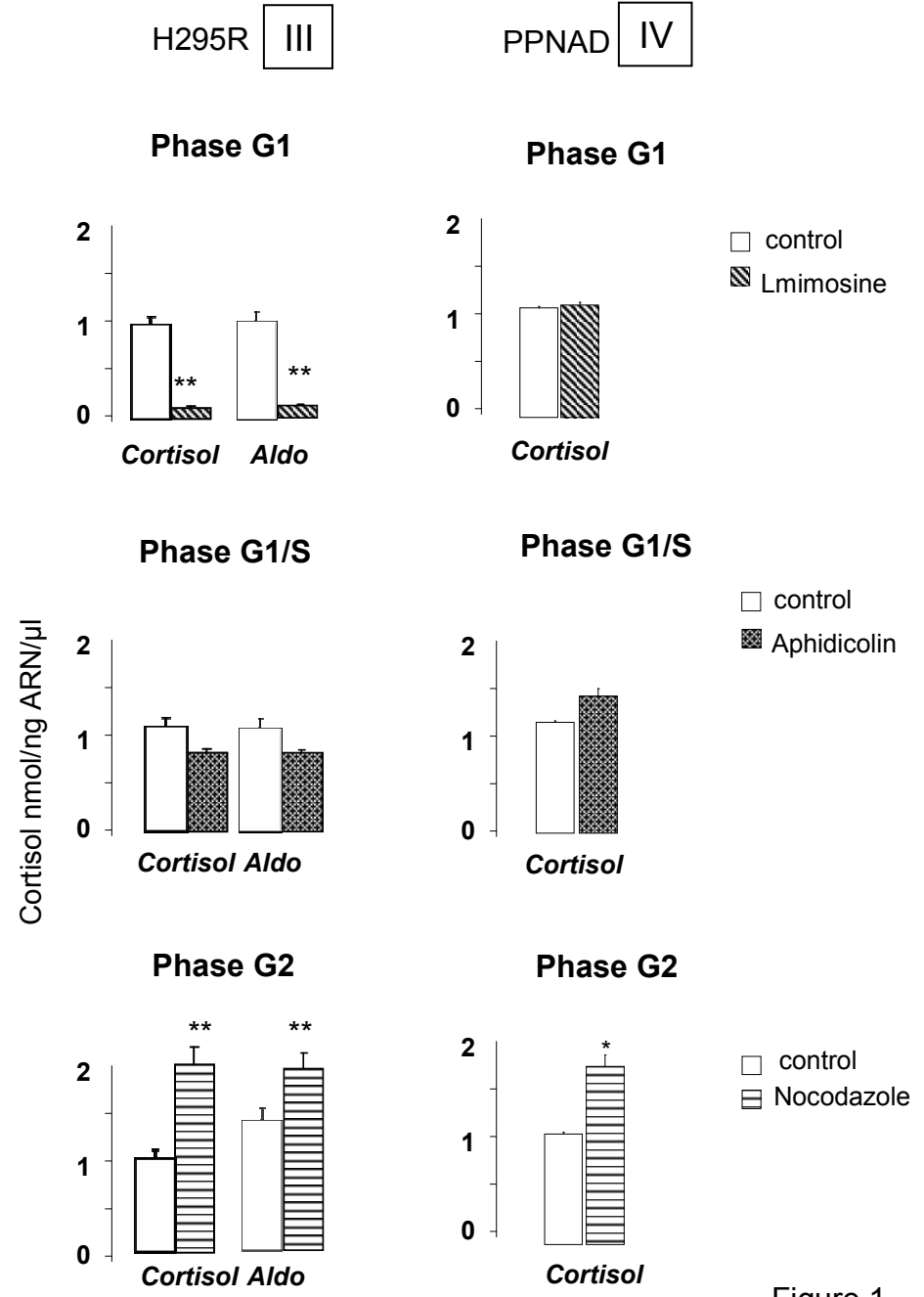


Figure 1

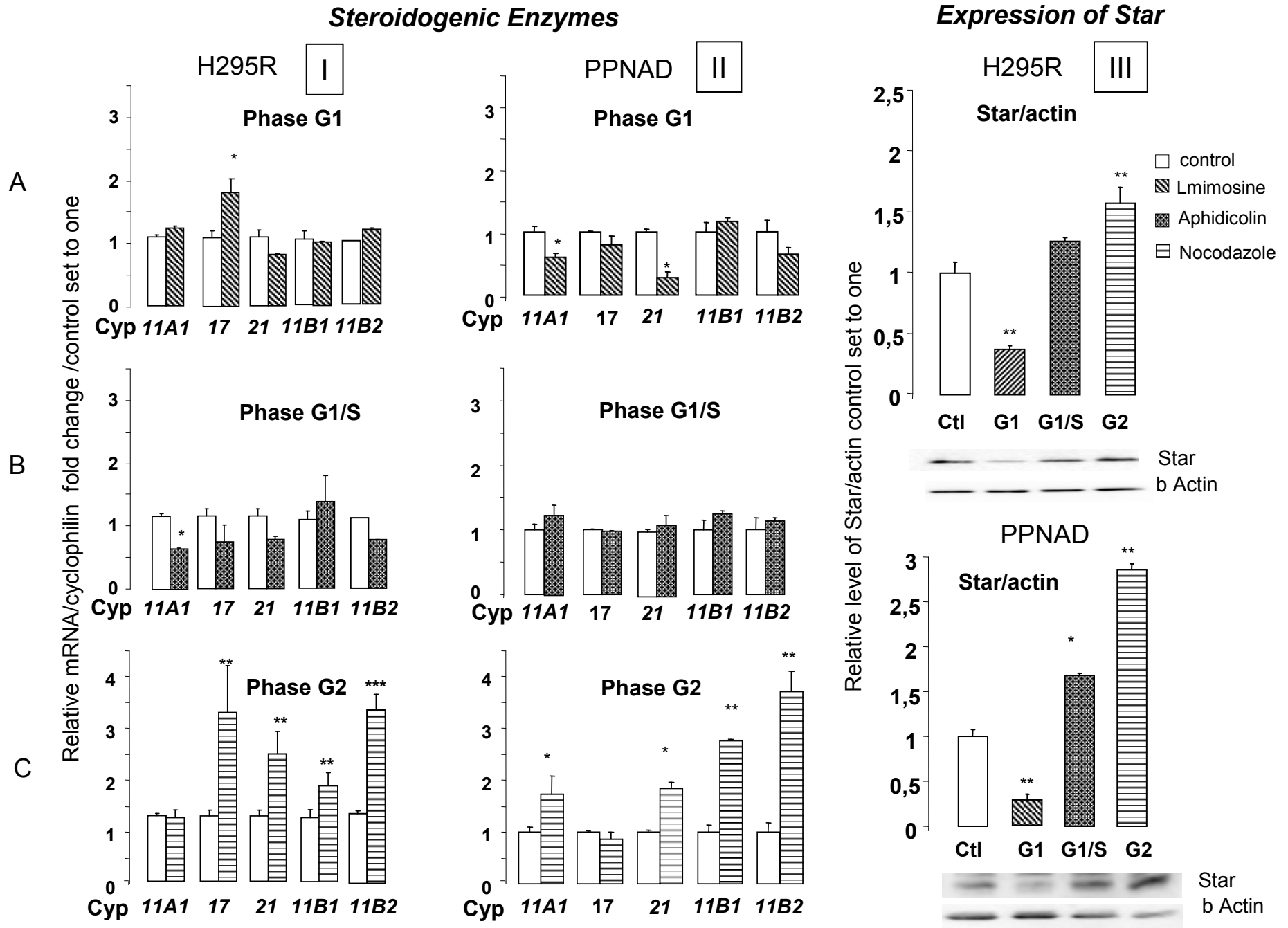


Figure 2



**Release from G1 L -mimosine synchronization**

**Release from G2 nocodazole synchronization**

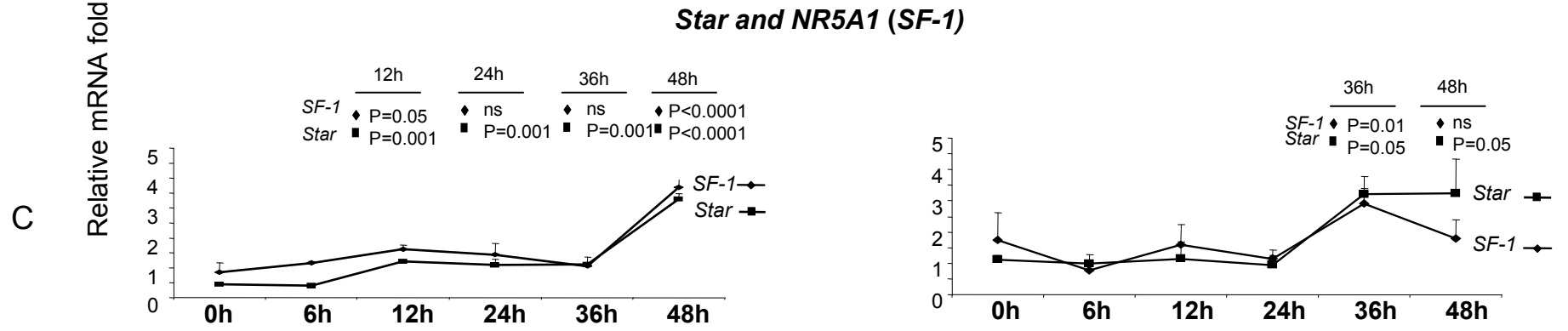
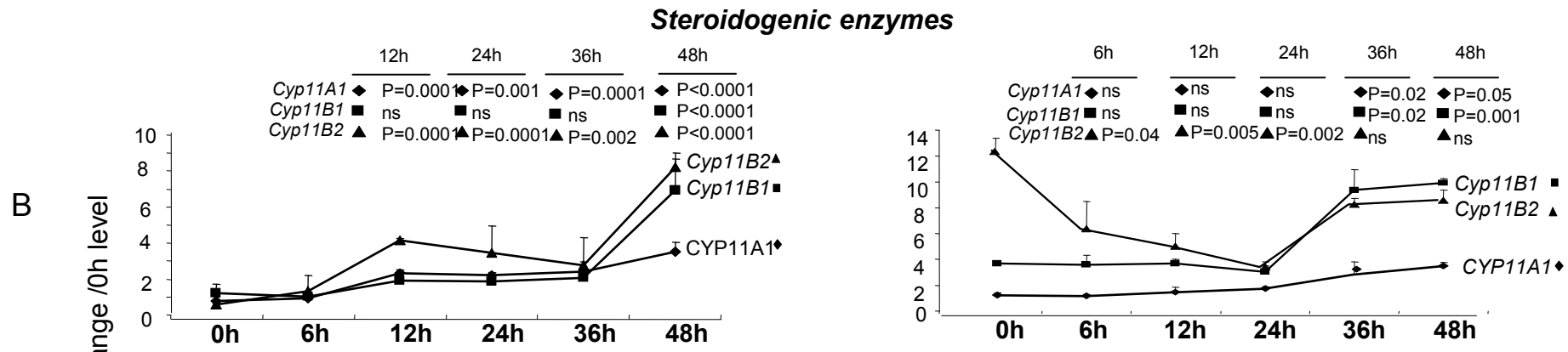
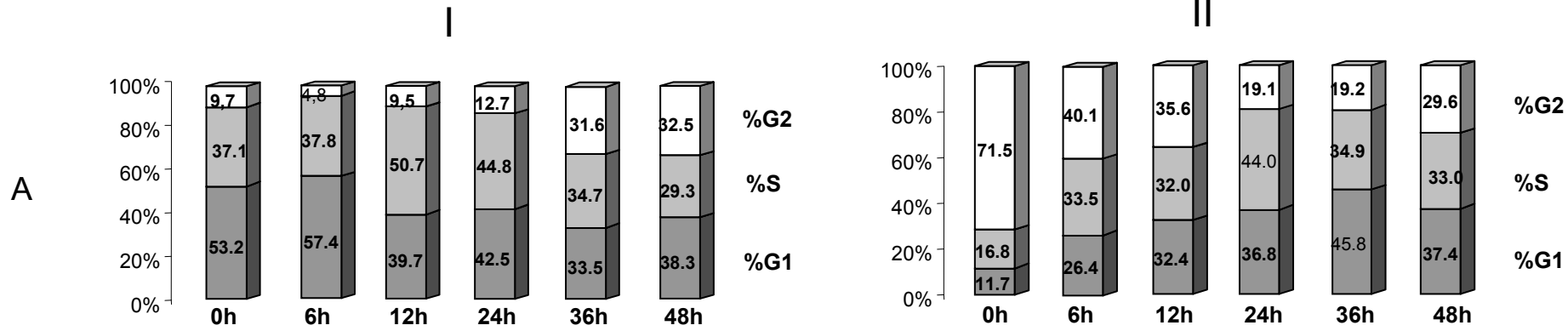


Figure 3

**MAPKinase Cell signaling**

**PKA activity**

**PKA subunits regulation**

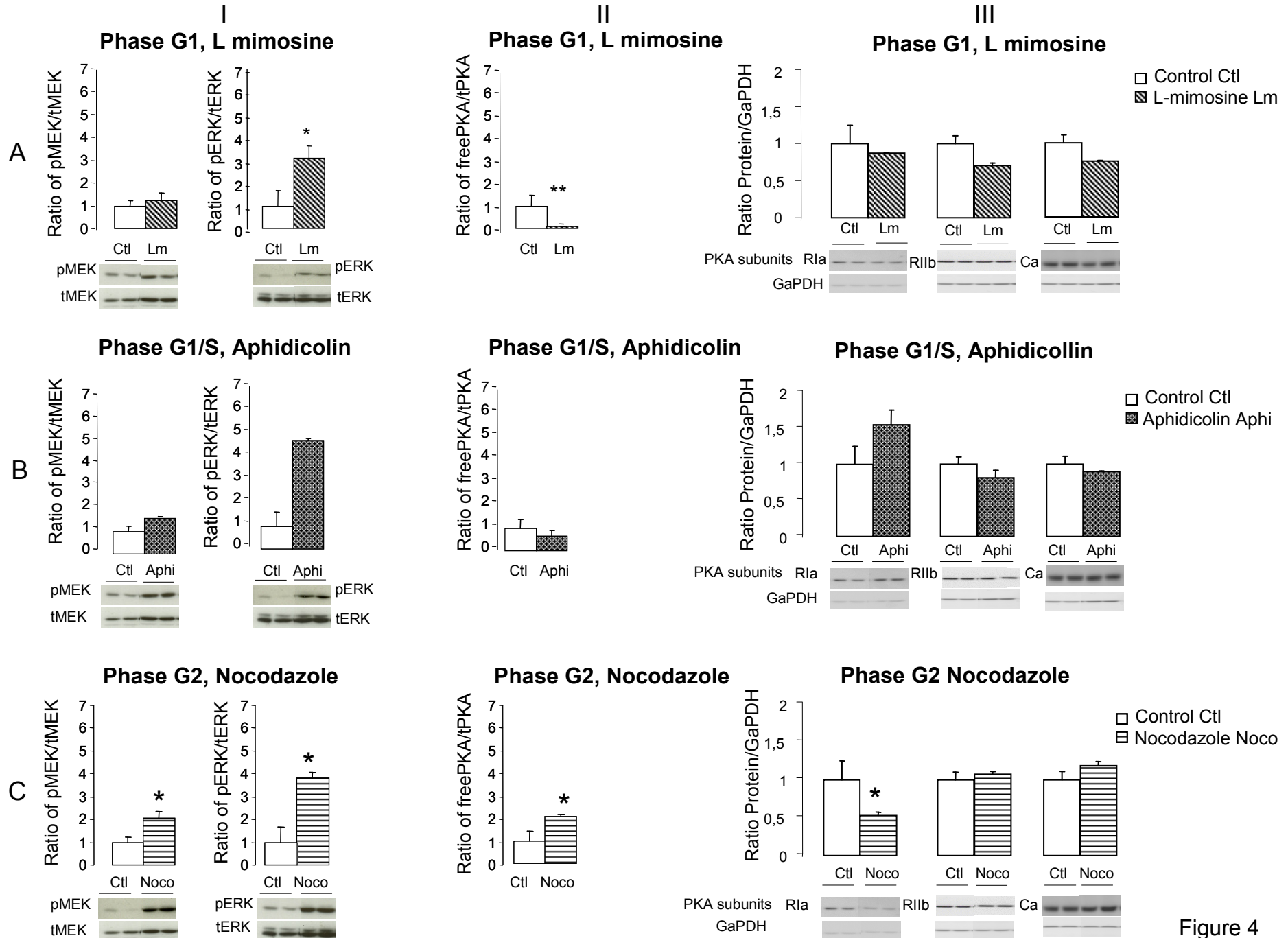


Figure 4

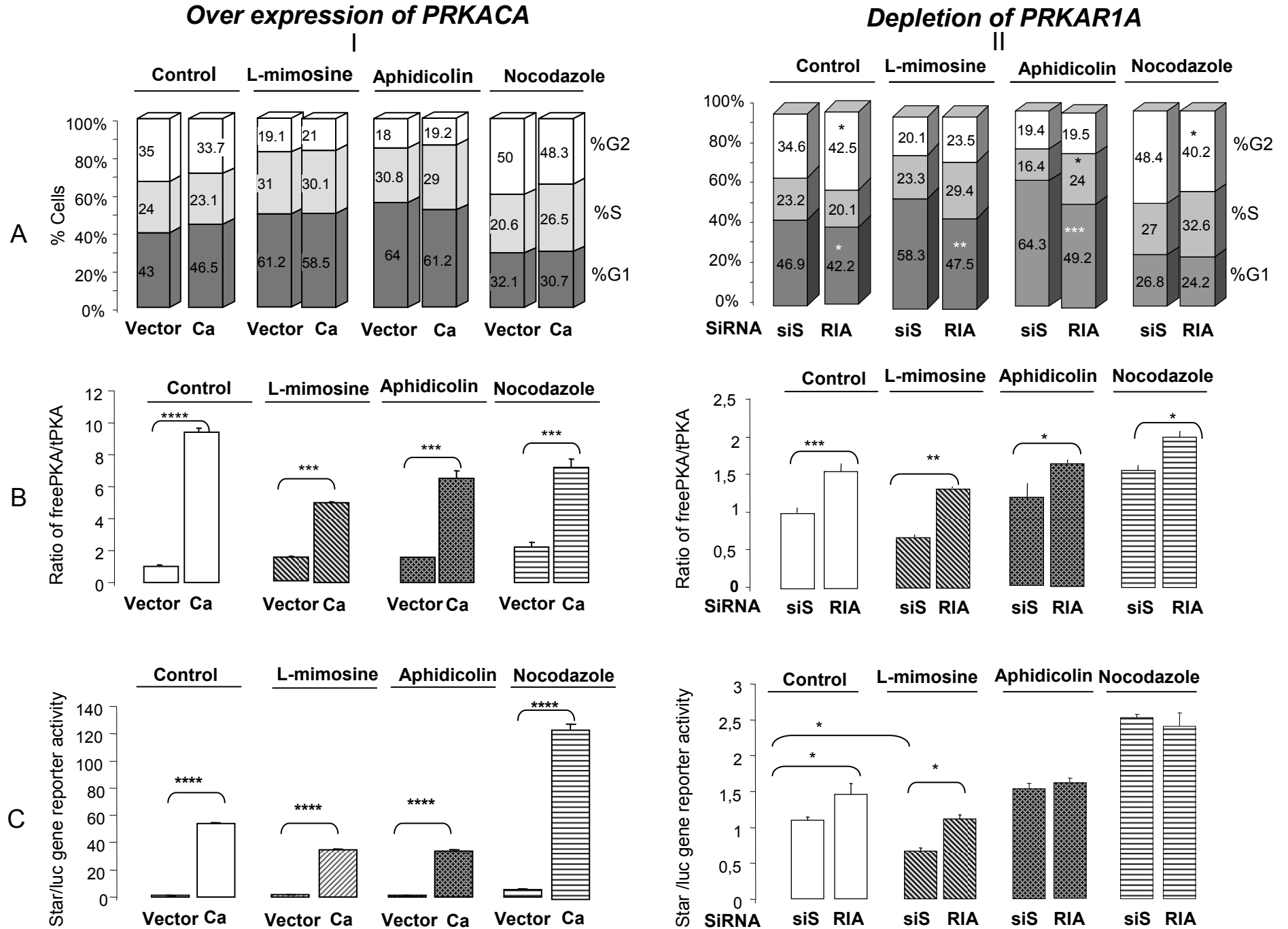


Figure 5

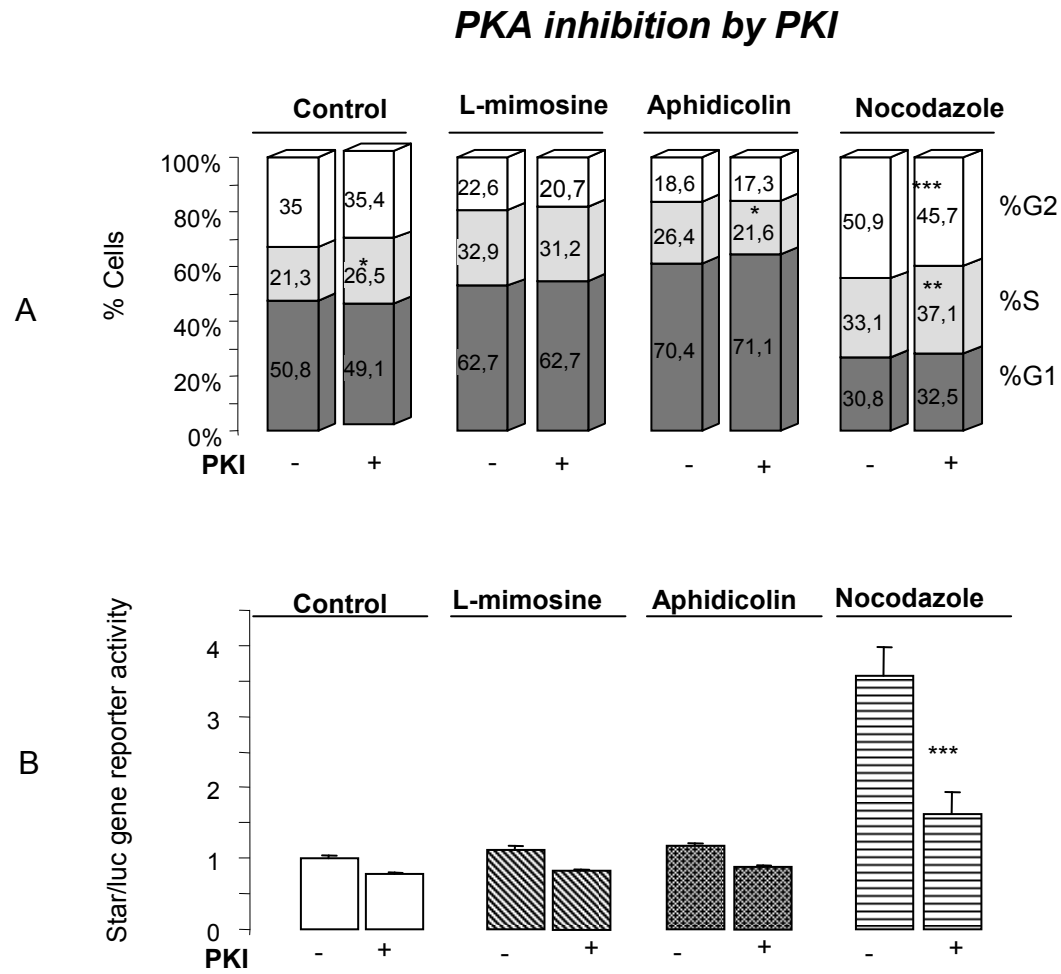
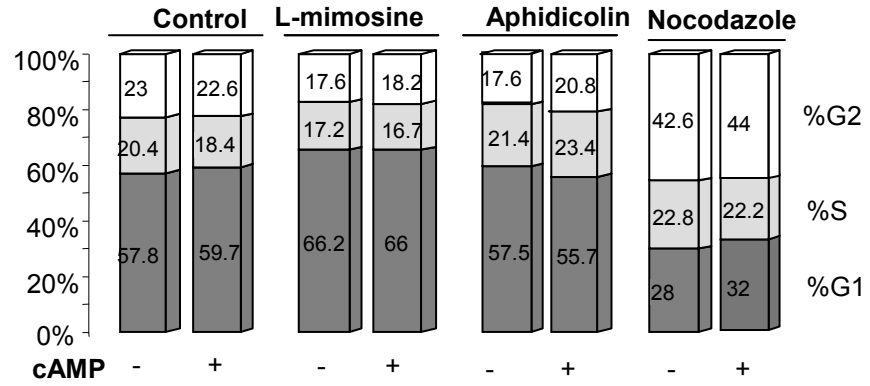
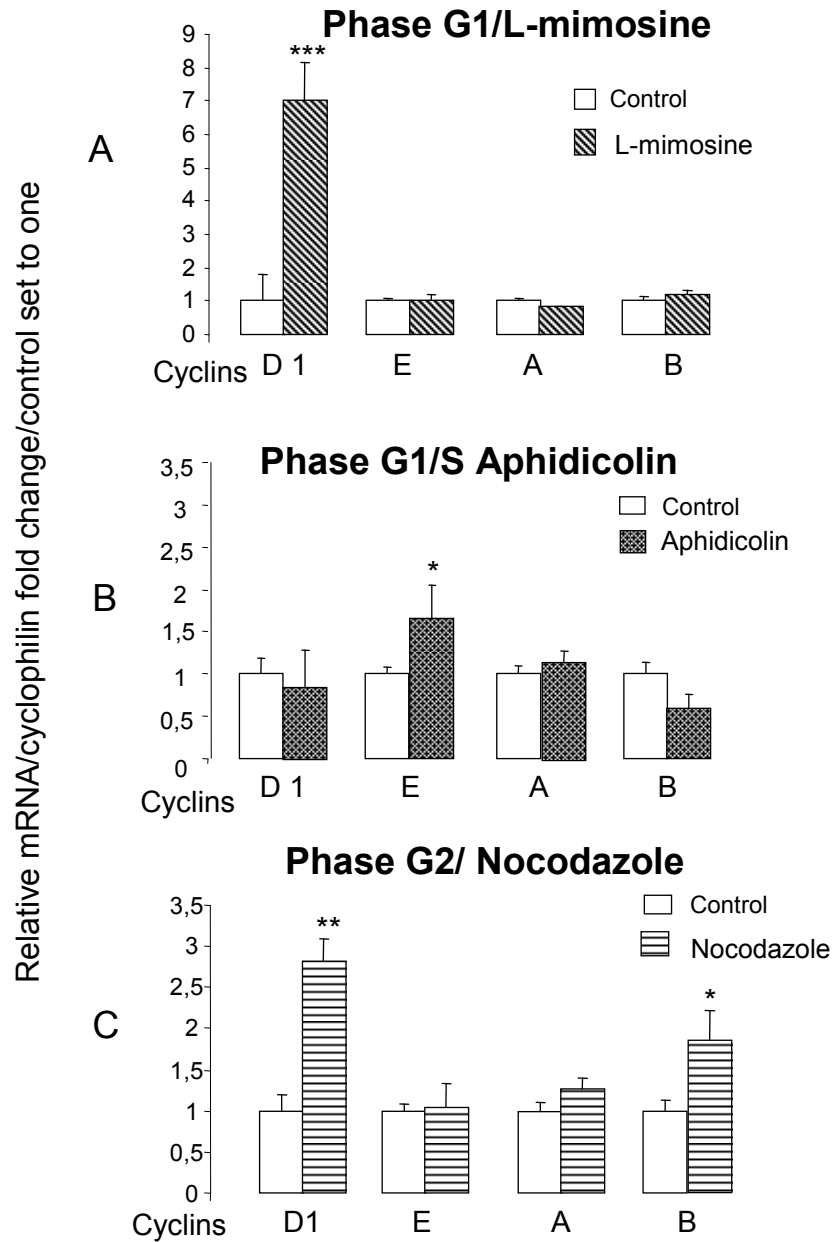


Figure 6

### Activation of PKA by cAMP

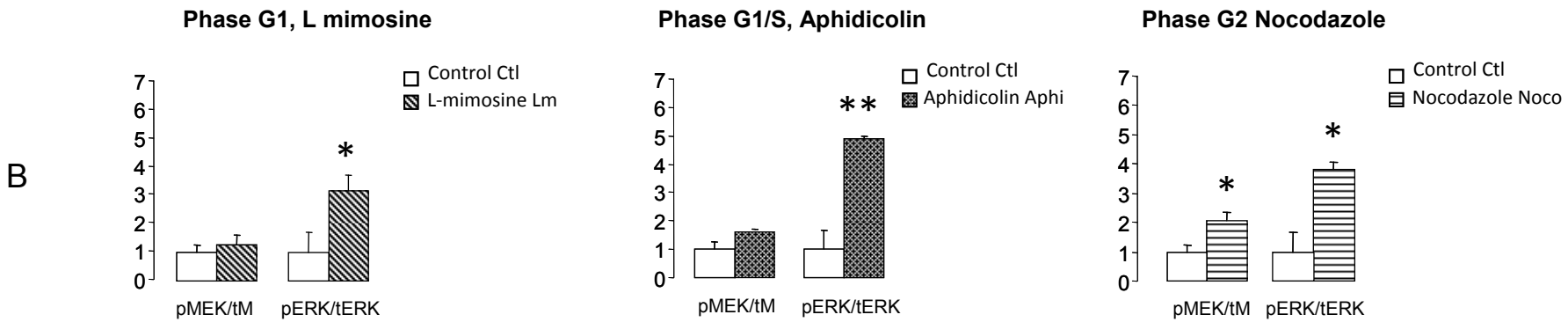
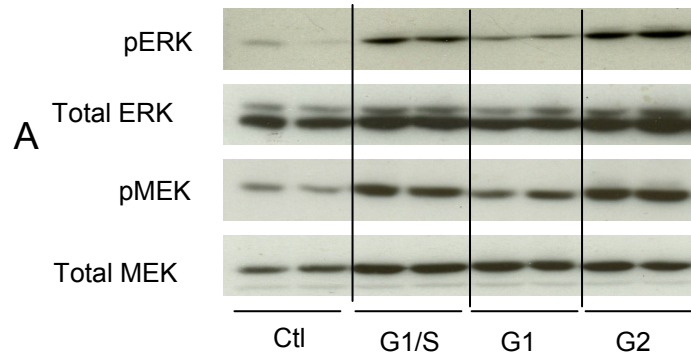


### Cyclins mRNA expression

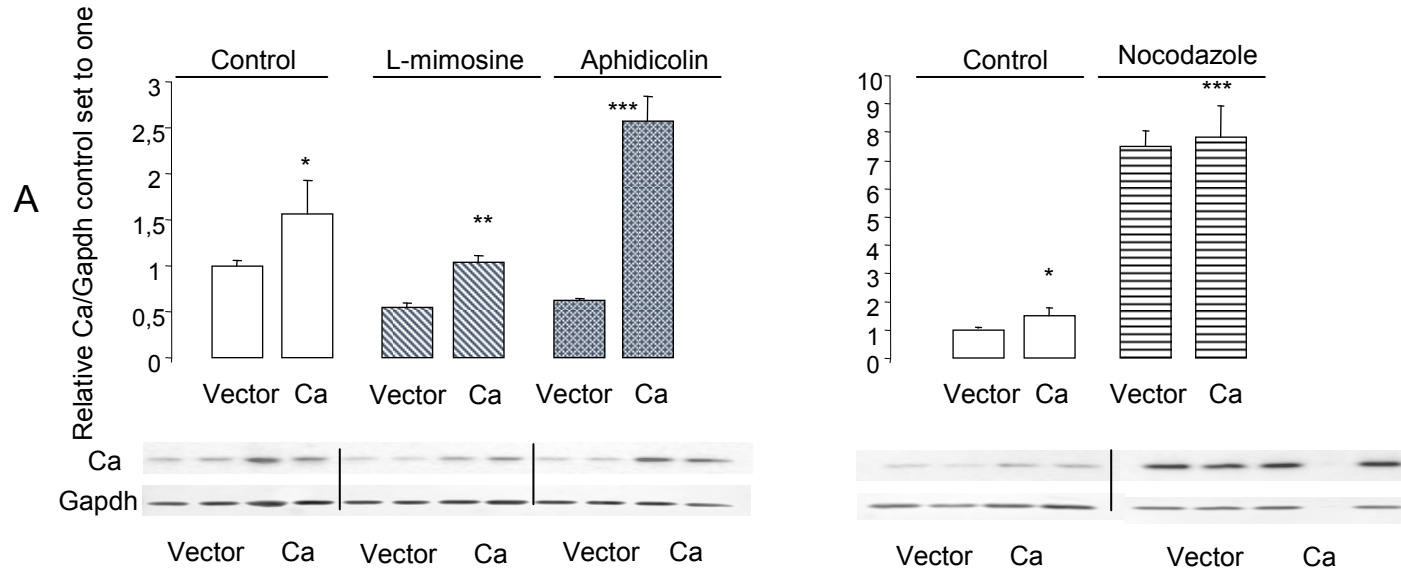


Supplementary Figure 1

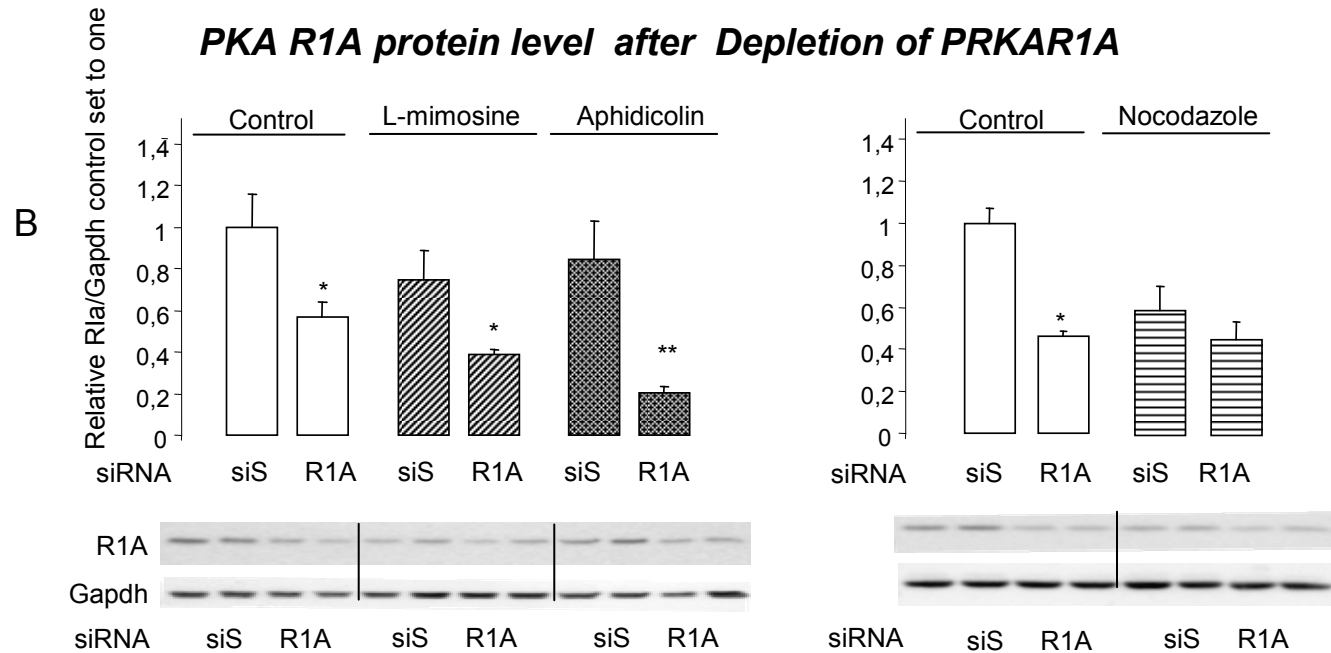
**MAPKinase Cell signaling: full western blot image**



**PKA catalytic protein level after Over expression of PRKACA**

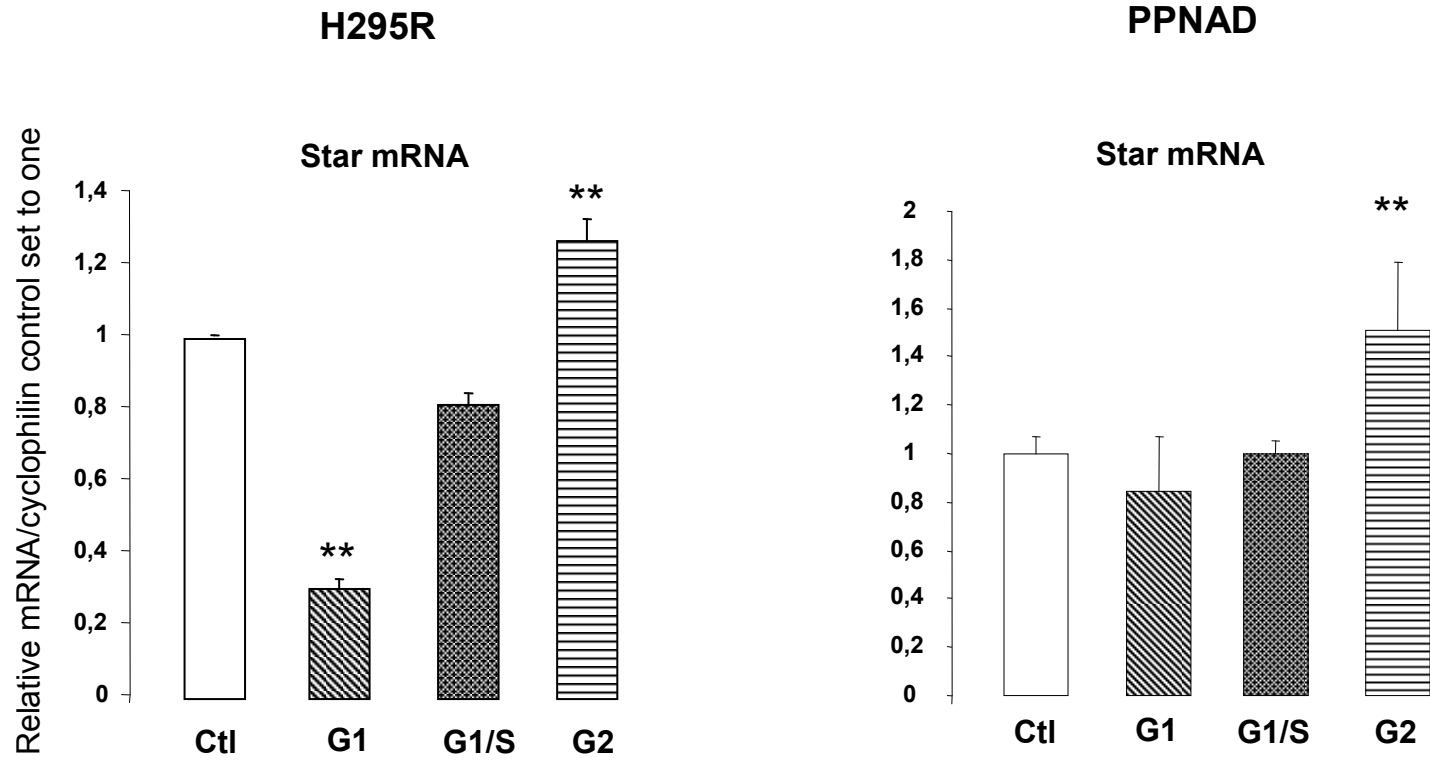


**PKA R1A protein level after Depletion of PRKAR1A**





### Expression of Star mRNA in synchronized cells



Link between steroidogenesis, the cell cycle, and PKA in adrenocortical tumor cells

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**Declaration of interest:** there is no conflict of interest that could be perceived to affect the Impartiality of the research reported. All authors declare that there is no conflict of interest.

## Highlights

Modulation of steroidogenesis by specific cell cycle phases in adrenal cell tumors.

PKA activity varied notably according to the stage of cell cycle in the H295R cells.

Early effect of enhancing PKA activity appears to be critical for steroid excess.

*PRKARIA* mediated the function of PKA activity on both steroidogenesis and cell cycle.