The phosphatidylinositol–Ca²⁺ hypothesis does not apply to the steroidogenic action of corticotropin

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The hypothesis that ACTH (corticotropin) stimulates steroidogenesis by a mechanism that involves breakdown of polyphosphoinositides and increase in intracellular Ca²⁺ (called here the ‘phosphatidylinositol–Ca²⁺ mechanism’) was tested in Y-1 adrenal-tumour cells and in bovine fasciculata cells, by using incorporation of ³²P and *myo*-[³H]inositol to study phospholipid metabolism, and quin-2 and fura 2 to measure intracellular Ca²⁺. As a positive control, we repeated experiments showing that angiotensin II stimulates glomerulosa cells by way of the phosphatidylinositol–Ca²⁺ mechanism, by using the same methods. With Y-1 and fasciculata cells, no change was observed in the incorporation of either of the labelled precursors into any phosphatidylinositol or into any of three major phosphoinositols, i.e. inositol phosphate, bisphosphate and trisphosphate. Moreover, no change in mass of any of these compounds was seen. No change was observed in the concentration of intracellular Ca²⁺ in Y-1 or fasciculata cells on addition of ACTH, by using either quin-2 or fura 2. By contrast, decreased incorporation of ³²P into phosphatidylinositol bisphosphate and an increase in intracellular Ca²⁺ were seen when glomerulosa cells were treated with angiotensin II. It is concluded that the phosphatidylinositol–Ca²⁺ mechanism is not used by Y-1 adrenal or bovine fasciculata cells in the steroidogenic response to ACTH unless the mechanism is radically different from that seen with all other hormones so far tested in which this mechanism occurs.

INTRODUCTION

It has been known since 1953 that Ca²⁺ is necessary for the response of adrenal cells to ACTH *in vitro* (Birmingham *et al.*, 1953). Since that time, numerous workers have attempted to resolve the question of whether or not Ca²⁺ is required for the intracellular (post-receptor) events that result in increased production of steroids (Kuo *et al.*, 1975; Lefkowitz *et al.*, 1970; Leier & Jungman, 1973; Yanagibashi, 1979; Sibley *et al.*, 1981). In spite of these investigations, the matter remains unresolved, and the findings of different workers have led to different conclusions (Kuo *et al.*, 1975; Lefkowitz *et al.*, 1970; Leier & Jungman, 1973; Yanagibashi, 1979; Sibley *et al.*, 1981). A subsequent contribution suggests that the major effect of Ca²⁺ is exerted on the binding of ACTH to its receptor (Cheitlin *et al.*, 1985).

An important development in understanding the mechanism of action of hormones has come from studies of polyphosphoinositides, which have shown that some hormones produce rapid breakdown of these phospholipids to give inositol trisphosphate and diacylglycerol as possible second messengers, which appear to act, at least in part, by increasing intracellular Ca²⁺ (Berridge, 1984; Berridge & Irvine, 1984; Farese, 1984). We will refer here to these changes as the ‘phosphatidylinositol–Ca²⁺ mechanism’. These findings are particularly important in explaining the actions of such hormones as vasopressin and angiotensin, which do not appear to use cyclic AMP as a second messenger (Berridge, 1984; Berridge & Irvine, 1984; Farese, 1984). In the adrenal cortex this mechanism

is involved in the response of glomerulosa cells to angiotensin II (Williams *et al.*, 1981), and may account for at least part of the response to ACTH as well (Kojima & Rasmussen, 1985). On the other hand, it has been reported that efflux of Ca²⁺ is not affected by ACTH in cells from glomerulosa or fasciculata (Williams *et al.*, 1981). On the contrary, it is well known that cyclic AMP acts as second messenger for ACTH (Haynes, 1958; Haynes *et al.*, 1959). These observations might suggest that ACTH does not employ the polyphosphatidylinositol–Ca²⁺ mechanism. This idea has been challenged by a series of papers from one laboratory that show rapid increase in the synthesis of polyphosphatidylinositol in rat adrenal under the influence of ACTH (Farese *et al.*, 1979, 1980; Farese, 1983). These findings are of great importance in understanding the action of ACTH on the adrenal. However, the response is unusual in at least two respects. Firstly, ACTH was reported to increase the synthesis or mass of polyphosphatidylinositides rather than the breakdown; and secondly, the synthesis of phosphatidylinositides in amounts that can be detected by increase in mass, as opposed to incorporation of labelled precursors, is without precedent in similar responses in other cells (Berridge, 1984; Berridge & Irvine, 1984; Farese, 1984), except for lutropin, which was reported by the same laboratory to produce the same effects as they noticed with ACTH (Farese, 1983).

Clearly, these generalizations cannot be used to dismiss the possibility that ACTH is an exception to our current concepts of the actions of hormones. We have set out to test the hypothesis that ACTH alters the metabolism of

Abbreviations used: ACTH, corticotropin; G-PtdIns, G-PtdIns4P, G-PtdIns(4,5)P₂, the deacylated glycerophosphatidylinositides 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol, 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol 4-phosphate and 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol 4,5-bisphosphate respectively; InsP, InsP₂, InsP₃, inositol phosphate, bisphosphate and trisphosphate respectively; (Bu)₂cAMP, N⁶,O²-dibutyryl adenosine 3',5'-cyclic monophosphate.

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adrenal phospholipids and, since such changes in phospholipid metabolism are known to increase the intracellular concentrations of Ca^{2+} in other cells, we measured the concentration of this cation in adrenal cells incubated with and without ACTH. For these experiments, we used Y-1 adrenal tumour cells, bovine fasciculata cells and, as a positive control, bovine glomerulosa cells. We report here our failure to support either the phosphatidylinoside- Ca^{2+} hypothesis or the occurrence of increased synthesis of phosphatidylinositol in response to ACTH.

METHODS AND MATERIALS

Cultured adrenal cells

Methods for the culture of Y-1 cells (Mrotek & Hall, 1975) and for measuring steroid production by these cells have been given elsewhere (Mrotek & Hall, 1977). Y-1 cells are generally regarded as a model for fasciculata cells. They do not show demonstrable responses to changes in medium $[\text{K}^+]$ or to physiological concentrations of angiotensin II (P. F. Hall, unpublished work). These cells show an EC_{50} (half-maximally effective concentration) for ACTH of 100 pM, with a maximum response to 2 nM; the maximal steroidogenic response involves a 5–10-fold increase above control values. The methods used for preparing and culturing bovine fasciculata-reticularis cells (Gospodarowicz *et al.*, 1977) and glomerulosa cells (Crivello *et al.*, 1982) were those reported by Gill and colleagues. Bovine fasciculata-reticularis cells will be referred to as 'fasciculata cells' for brevity. Cultured bovine fasciculata cells secreted cortisol at rates of 1.88 ± 0.02 , 3.79 ± 0.07 and 4.89 ± 0.07 nmol/8 h per 10^6 cells in response to ACTH at concentrations of 0, 0.01 and 100 nM respectively (the values are means $\pm$ ranges of duplicate determinations). In some studies 20α -dihydroprogesterone was measured by radioimmunoassay (Mrotek & Hall, 1975, 1977) under the same experimental conditions as those used to study lipids and Ca^{2+} . Cortisol was measured by competitive protein-binding assay (Murphy, 1967).

Phospholipid analysis

Two methods were used to examine turnover of phospholipids (including phosphatidylinositides). In the first method incorporation of ^{32}P (50 μCi ; 1.9 pmol/dish) to steady state (2 h) was followed by incubation in fresh medium with and without various agents. After incubation, cells were extracted with chloroform/methanol/2.4 M-HCl (2:2:1, by vol.) (referred to here as acidic chloroform/methanol) and then deacylated by alkaline digestion. The deacylated phospholipids were applied to an h.p.l.c. column which separates the various groups of glycerophosphatidyl lipids. The entire column effluent was collected in 0.78 ml fractions, which were examined for ^{32}P content by liquid-scintillation spectrometry. This method follows the procedure described by Seyfred & Wells (1984). The elution pattern has been standardized with authentic phospholipids and is highly reproducible (Seyfred & Wells, 1984). The incorporation of ^{32}P into glycerophosphatidylinositides and phosphoinositols was also measured by using a Dowex column (X8; formate form) (Berridge, 1983).

The second method used to measure turnover of phospholipids is based on incorporation of *myo*- ^3H inositol. Cells were incubated with this substrate

Fig. 1. Incorporation of ^{32}P into phosphatidylinositides by Y-1 adrenal cells

Y-1 cells were incubated with ^{32}P as described in the Methods and materials section. Cells were incubated with ACTH (100 nM) or saline (controls) for the times shown, and then processed as described in the Methods and materials section. The values are shown as the ratio ACTH/control and represent means $\pm$ S.E.M. for duplicate determinations in each of the three experiments. $\circ$, G-PtdIns; $\triangle$, G-PtdIns4P; $\square$, G-PtdIns(4,5) P_2 .

(5 μCi ; 303 pmol/dish) for 48 h. Incubation medium was changed, and incubation was continued without *myo*- ^3H inositol and with various agents. After incubation, cells were extracted with acidic chloroform/methanol, and extracts were applied to small columns of Dowex-1 by a published procedure (Berridge, 1983). In some studies Li^+ (10 mM) was added to the incubation medium immediately before ACTH. In other studies, the concentrations of unlabelled glycerophosphatidylinositides (mass as opposed to labelling) were measured by extraction into acidic chloroform/methanol, followed by acid hydrolysis (Hess & Derr, 1975). P_i was measured by the method of Hess & Derr (1975).

Measurement of intracellular Ca^{2+}

A modification of a published method (Charest *et al.*, 1983) was used to measure the intracellular concentration of Ca^{2+} by using quin-2. Cells were loaded for 25 min in a shaking water bath at 37 $^{\circ}\text{C}$ with quin-2/AM (50 μM) or vehicle in Krebs buffer containing Hepes (25 mM), glucose (0.2%, w/v), bovine serum albumin (0.1%, w/v) and CaCl_2 (2 mM). After loading, the cells were washed with the same buffer three times and then incubated for 30 min at 37 $^{\circ}\text{C}$ in the same buffer without quin-2. The cells were washed once more and resuspended in 2 ml of the buffer (no quin-2) at a concentration of 2×10^6 – 5×10^6 cells/2 ml. After addition of various agents (see below), mixing was performed in less than 5 s. The cells were examined in a Perkin-Elmer fluorescence spectrophotometer MPF-3 with Hitachi recorder QPD 33 and Xenon power supply (Perkin-Elmer 150). Excitation was at 340 nm with slit width at 4 nm and emission was monitored at 490 nm (slit width 4 nm). Quin-2/AM was effectively de-esterified in the cells, as judged by a shift in the peak emission from 440 to 490 nm. Calculation of internal Ca^{2+} was performed as described by Tsien *et al.* (1982). The intracellular concentration of quin-2 was determined with the aid of $[\text{G-}^3\text{H}]$ quin-2/AM. In some experiments, changes in intracellular Ca^{2+} were measured with the aid of fura 2. The loading procedure for this

Table 1. Incorporation of *myo*-[³H]inositol into glycerophosphatidylinositides and phosphoinositols by Y-1 cells with and without ACTH

Y-1 cells were incubated with *myo*-[³H]inositol as described in the Methods and materials section before addition of ACTH (100 nM) or saline (controls). After incubation for the times shown, cells were processed as described in the Methods and materials section. The values shown represent means $\pm$ S.E.M. of the means for duplicate determinations in each of the three experiments.

Incubation time (min)	Ratio ACTH/control					
	G-PtdIns	G-PTdIns4P	G-PtdIns(4,5)P ₂	InsP	InsP ₂	InsP ₃
0.5	0.94 $\pm$ 0.06	0.92 $\pm$ 0.09	0.91 $\pm$ 0.07	1.02 $\pm$ 0.02	0.88 $\pm$ 0.06	1.01 $\pm$ 0.01
1.0	1.00 $\pm$ 0.02	1.00 $\pm$ 0.02	1.02 $\pm$ 0.02	0.92 $\pm$ 0.09	1.01 $\pm$ 0.01	1.01 $\pm$ 0.01
5.0	1.01 $\pm$ 0.01	1.01 $\pm$ 0.01	1.02 $\pm$ 0.02	1.07 $\pm$ 0.04	1.17 $\pm$ 0.02	1.02 $\pm$ 0.01

indicator was essentially the same as that for quin-2, except that the concentration of fura 2 was lowered to 5 μ M. Excitation was at 340 nm (4 nm slit) and emission at 510 nm (4 nm slit) as described by Tsien *et al.* (1985).

Materials

Collagenase type I and bacterial proteinase XIV were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other compounds were obtained from the following sources: ionomycin (batch no. SQ23, 377), E. B. Squibb and Sons (New York, NY, U.S.A.); quin-2/AM, Calbiochem/Boehringer (San Diego, CA, U.S.A.); [G-³H]quin-2/AM (9.2 mCi/mmol; Batch 5), Amersham (Arlington Heights, IL, U.S.A.); quartz cuvettes (3.0 ml), Fisher Scientific (Pittsburg, PA, U.S.A.); fura 2/AM, Molecular Probes Inc. (Junction City, OR, U.S.A.); [³²P]phosphoric acid, *myo*-[³H]inositol (lot no. 1873-283) and [³H]cortisol (lot no. 2191-035; 83.5 Ci/mmol), New England Nuclear Research Products (Boston, MA, U.S.A.).

RESULTS

Incorporation of radioactively labelled precursors into glycerophosphatidylinositides and phosphoinositols by adrenal cells

Y-1 cells. (a) ³²P. Fig. 1 shows values for the incorporation of [³²P]P_i into glycerophosphatidylinositides by Y-1 cells incubated with and without ACTH. In preliminary studies (results not shown), it was found that the specific radioactivity of [³²P]ATP in Y-1 cells incubated under the conditions shown in Fig. 1 reached steady state within 90 min. In this and all other studies with ³²P, preincubation was continued for 120 min to ensure isotopic equilibration. After addition of 0.9% NaCl or ACTH, incubation was continued for the times shown. Values shown are means ($\pm$ S.E.M. for three separate experiments) of the ratios of incorporation with ACTH to that without ACTH. This ratio does not change with time for 5 min. Although the values for incorporation of ³²P vary from experiment to experiment, especially with changes in the specific radioactivity of ³²P, the ratio of ACTH/control is consistent and clearly shows no change with time. An indication of variation between experiments can be seen in mean values $\pm$ S.E.M. for the unstimulated cells ($n = 24$, i.e. three experiments, four time points, duplicate dishes), which were as follows: G-PtdIns 869 $\pm$ 60, G-PtdIns4P 887 $\pm$ 71 and G-PtdIns(4,5)P₂ 4719 $\pm$ 339 (c.p.m./10⁶ cells). The zero-time value

represents the mean of duplicate determinations of the ratio ACTH/control, at zero time, in the three experiments; this value is taken as 1.0. Studies at longer times of incubation (up to 30 min) revealed no effect of ACTH in similar studies (not shown). (b) *myo*-[³H]inositol. Table 1 shows that ACTH did not alter incorporation of *myo*-[³H]inositol into glycerophosphatidylinositides or phosphoinositols in three experiments with Y-1 cells during short incubations (0.5–5.0 min), and that the ratio of ACTH/control does not change. To indicate variation between experiments, the mean values for unstimulated cells ($n = 18$, three times and three experiments) are given: G-PtdIns 13 500 $\pm$ 290, G-PtdIns4P 107 $\pm$ 27, G-PtdIns(4,5)P₂ 122 $\pm$ 23, InsP 207 $\pm$ 23, InsP₂ 263 $\pm$ 38, InsP₃ 77 $\pm$ 4 (c.p.m./10⁶ cells).

Bovine fasciculata cells. (a) ³²P. Fig. 2 shows values for the ratio ACTH/control for incorporation of ³²P into glycerophosphatidylinositides (Fig. 2a) and phosphoinositols (Fig. 2b) by bovine fasciculata cells during incubation for times from 0.5 to 5.0 min. The ratio shows no consistent change with time for the glycerophosphatidylinositides. With phosphoinositols (Fig. 2b), some fall is seen in InsP₂ and InsP₃ at 5 min. For InsP₃ the fall is 25%. Means $\pm$ S.E.M. for unstimulated cells ($n = 24$, three experiments, four time points) were as follows: G-PtdIns 13 405 $\pm$ 1058, G-PtdIns4P 3055 $\pm$ 77, G-PtdIns(4,5)P₂ 6344 $\pm$ 476, InsP 504880 $\pm$ 20078, InsP₂ 11 269 $\pm$ 780, InsP₃ 6537 $\pm$ 581 (c.p.m./10⁶ cells). Similar studies showed that ACTH (10 pM) did not alter incorporation of ³²P into glycerophosphatidylinositides or into phosphoinositols in bovine fasciculata cells at 0.5, 1.0

Table 2. Incorporation of ³²P into glycerophosphatidylinositol bisphosphate by glomerulosa cells from bovine adrenal

Glomerulosa cells were incubated with [³²P]P_i (see the Methods and materials section) before addition of saline or angiotensin II (100 nM). After incubation for 30 s, cells were processed as described in the Methods and materials section. Values are means $\pm$ ranges for duplicate determinations.

Addition	Incorporation into G-PtdIns(4,5)P ₂	
	(c.p.m./10 ⁶ cells)	(%)
Saline	4213 $\pm$ 163	100
Angiotensin II	1528 $\pm$ 178	36

Fig. 2. Incorporation of ^{32}P into phosphatidylinositides and inositol phosphates by bovine fasciculata cells

Fasciculata cells were incubated with ^{32}P as described in the Methods and materials section. After incubation with ACTH (100 nM) or saline (controls) for the times shown, cells were processed as described in the Methods and materials section. The values are shown as the ratio ACTH/control and represent means $\pm$ S.E.M. for duplicate determinations in each of three experiments. (a) Glycerophosphatidylinositides: $\circ$, G-PtdIns; $\triangle$, G-PtdIns4P; $\square$, G-PtdIns(4,5) P_2 . (b) Inositol phosphates: $\bullet$, InsP; $\blacktriangle$, InsP $_2$; $\blacksquare$, InsP $_3$.

and 5.0 min treatment (results not shown). (b) *myo*- ^{3}H]inositol. With *myo*- ^{3}H]inositol, no changes in its incorporation into any precursor were observed after stimulation with ACTH (10 pM and 100 nM) at times up to 5 min (results not shown).

Table 4. Glycerophosphatidylinositide concentrations in Y-1 adrenal cells incubated without and with ACTH

Y-1 cells were incubated for the times shown with saline or ACTH. Cells were processed as described in the Methods and materials section. Values are means $\pm$ ranges of duplicate determinations.

ACTH (nM)	Time (min)	Concn. (nmol/mg of protein)		
		G-PtdIns	G-PtdIns4P	G-PtdIns(4,5) P_2
0	0.0	8.3 $\pm$ 0.3	1.4 $\pm$ 0.2	0.6 $\pm$ 0.1
0	0.5	8.6 $\pm$ 0.1	1.5 $\pm$ 0.1	0.6 $\pm$ 0.1
100	0.5	8.6 $\pm$ 0.1	1.5 $\pm$ 0.1	0.6 $\pm$ 0.1
0	1.0	8.4 $\pm$ 0.2	1.3 $\pm$ 0.0	0.6 $\pm$ 0.1
100	1.0	8.6 $\pm$ 0.6	1.3 $\pm$ 0.2	0.5 $\pm$ 0.2
0	5.0	8.4 $\pm$ 1.1	1.5 $\pm$ 0.2	0.7 $\pm$ 0.2
100	5.0	7.8 $\pm$ 0.8	1.3 $\pm$ 0.0	0.6 $\pm$ 0.1
0	15.0	7.7 $\pm$ 0.3	1.3 $\pm$ 0.0	0.7 $\pm$ 0.1
100	15.0	7.6 $\pm$ 1.5	1.3 $\pm$ 0.1	0.7 $\pm$ 0.3

Bovine glomerulosa cells. (a) ^{32}P . Incorporation of ^{32}P into G-PtdIns(4,5) P_2 by bovine glomerulosa cells was decreased by angiotensin II (Table 2). A smaller decrease was observed with G-PtdIns4P, to 80% of the control value (not shown). (b) *myo*- ^{3}H]inositol. Table 3 shows that angiotensin II (100 nM) caused a rapid decrease in the incorporation of *myo*- ^{3}H]inositol into G-PtdIns(4,5) P_2 at 0.5 min, with a rapid return to previous values. Smaller changes were seen in G-PtdIns4P, and no change was seen in G-PtdIns. All three phosphoinositols showed an increased incorporation of ^3H after 0.5 min (Table 3), suggesting breakdown of G-PtdIns(4,5) P_2 .

Glycerophosphatidylinositide concentrations in Y-1 adrenal cells incubated without and with ACTH

Table 4 shows values for G-PtdIns, G-PtdIns4P and G-PtdIns(4,5) P_2 in extracts of Y-1 cells incubated without and with ACTH for short times. No differences were seen between values with and without ACTH at the times studied.

Table 3. Incorporation of *myo*- ^{3}H]inositol into glycerophosphatidylinositides and phosphoinositols by glomerulosa cells from bovine adrenal

Glomerulosa cells were incubated with *myo*- ^{3}H]inositol as described in the Methods and materials section before addition of angiotensin II (100 nM) or saline (controls). After incubation for the times shown, cells were processed as described in the Methods and materials section. The values shown represent means $\pm$ ranges for duplicate determinations, with values as percentages of controls in parentheses.

Angiotensin II (nM)	Time (min)	Incorporation (c.p.m./10 ⁶ cells)					
		G-PtdIns	G-PtdIns4P	G-PtdIns(4,5) P_2	InsP	InsP $_2$	InsP $_3$
0	0.0	8750 $\pm$ 50	170 $\pm$ 10	195 $\pm$ 13	313 $\pm$ 6	261 $\pm$ 18	87 $\pm$ 12
0	0.5	9500 $\pm$ 300 (100)	185 $\pm$ 13 (100)	194 $\pm$ 2 (100)	356 $\pm$ 4 (100)	259 $\pm$ 26 (100)	79 $\pm$ 4 (100)
100	0.5	9200 $\pm$ 600 (97)	134 $\pm$ 6 (72)	107 $\pm$ 3 (55)	474 $\pm$ 14 (133)	343 $\pm$ 8 (132)	116 $\pm$ 4 (147)
0	1.0	11200 $\pm$ 200 (100)	165 $\pm$ 15 (100)	196 $\pm$ 4 (100)	325 $\pm$ 16 (100)	244 $\pm$ 56 (100)	84 $\pm$ 2 (100)
100	1.0	11100 $\pm$ 350 (99)	136 $\pm$ 6 (82)	159 $\pm$ 3 (81)	413 $\pm$ 11 (127)	279 $\pm$ 72 (114)	101 $\pm$ 11 (120)
0	5.0	12100 $\pm$ 400 (100)	200 $\pm$ 20 (100)	178 $\pm$ 36 (100)	324 $\pm$ 7 (100)	270 $\pm$ 9 (100)	76 $\pm$ 4 (100)
100	5.0	9800 $\pm$ 200 (81)	182 $\pm$ 18 (91)	160 $\pm$ 26 (90)	315 $\pm$ 5 (97)	231 $\pm$ 3 (96)	90 $\pm$ 18 (118)

Fig. 3. Changes in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in adrenal cells

(a) Y-1 cells were labelled with quin-2/AM, as described in the Methods and materials section. Excitation was at 340 nm and emission was recorded at 490 nm (4 nm slit width). The results are representative of six similar experiments. Additions were made at the times indicated by the arrows; fluorescence has been corrected for the subsequent changes in volume. Autofluorescence was typically about 15% of the resting value in quin-2-loaded cells, and has not been subtracted. (b) Bovine fasciculata cells were prepared and loaded with quin-2/AM, as described in the Methods and materials section. Additions were made at the times indicated by the arrows. (c) Bovine glomerulosa cells were prepared and loaded with quin-2/AM, as described in the Methods and materials section. Angiotensin II (AII) or KCl (50 mM) was added at the indicated times. (d) Y-1 cells were loaded with fura 2/AM, as described in the Methods and materials section. The tracing was obtained with excitation at 340 nm (4 nm slit) and emission recorded at 510 nm (4 nm slit). In a separate experiment, excitation was at 380 nm, with similar results except that the curve after addition of ionomycin was directed downwards, as expected at this wavelength.

Concentration of intracellular Ca^{2+}

Quin-2. (a) Y-1 cells. Y-1 cells presented no special problems with the quin-2 method. Using a value for intracellular volume of $0.67 \mu\text{l}/10^6$ cells (determined in preliminary studies), the cells were found to load readily with quin-2/AM to a concentration of approx. 0.90 nM, and the ester was rapidly and completely hydrolysed. Although Y-1 cells are not usually studied in suspension, they can survive for days when continually stirred, and in the cuvettes they synthesized 20α -dihydroprogesterone. Under these conditions, addition of ACTH caused an increase in the production of this steroid by a factor of 2.5–3.0 (three determinations), and ACTH and quin-2 increased production by a factor of 1.7–2.0 (three determinations).

Fig. 3(a) shows a stable baseline of fluorescence which could be maintained for more than 5 min. The concentration of Ca^{2+} in the cells shown in Fig. 3(a) was 131 nM. The value for intracellular Ca^{2+} in Y-1 cells was 123 ± 9 nM (mean $\pm$ S.E.M., $N = 6$). Addition of ACTH (final concn. 0.02–20 nM) had no effect on the fluorescence signal (Fig. 3a). Fluorescence was monitored for 5 min, and no change was seen. Dibutyl cyclic AMP (2 mM) was also without effect (Fig. 3a). Addition of ionomycin (200 nM) caused an immediate and sustained increase in fluorescence (Fig. 3a).

(b) Bovine fasciculata cells. Fig. 3(b) shows that neither ACTH nor dibutyl cyclic AMP altered the fluorescent signal from these cells. The concentration of Ca^{2+} in fasciculata cells was 38–43 nM (two determinations).

(c) Bovine glomerulosa cells. The intracellular concentration of Ca^{2+} in these cells was 79 ± 1 nM (two determinations). Addition of angiotensin II (10 nM) caused a rapid increase in the fluorescence signal to a value that corresponds to 112 nM (Fig. 3c). Further addition of angiotensin II caused further increases to intracellular Ca^{2+} , as follows: 100 nM-angiotensin raised $[\text{Ca}^{2+}]$ to 117 nM, and 1000 nM-angiotensin produced an intracellular $[\text{Ca}^{2+}]$ of 133 nM. The increase in fluorescence produced by the hormone persisted unchanged for more than 5 min. If KCl (50 mM) was added after angiotensin II, the fluorescence signal indicated a further increase in the concentration of intracellular Ca^{2+} to 141 nM (Fig. 3c). In a second experiment, similar results were obtained. An increase in the fluorescence signal occurred at concentrations of angiotensin II of 0.2 nM and higher. The value for intracellular Ca^{2+} rose from 78 nM to 93 nM at 0.2 nM-angiotensin II.

Fura 2. Y-1 cells. When Y-1 cells were loaded with the fluorescent indicator fura 2, a stable baseline fluorescence was obtained. On addition of ACTH (0.02–20 nM), no change in the fluorescence signal was observed. On the other hand, addition of ionomycin (300 nM) to the Y-1 cells caused a rapid increase in the fluorescence signal, indicating a rise in intracellular $[\text{Ca}^{2+}]$, followed by a return towards baseline after 1 min (Fig. 3d).

DISCUSSION

The experiments reported here should be considered in relation to two questions concerning phospholipid metabolism in adrenal cells, namely: (1) is the phosphatidylinositol- Ca^{2+} mechanism involved in the responses to ACTH? and (2) does ACTH increase the

synthesis of polyphosphatidylinositol in adrenal cells? This distinction is important, since increase in the synthesis or mass of polyphosphatidylinositol is not part of the phosphatidylinositol- Ca^{2+} mechanism as it occurs in other cells (e.g. Berridge, 1983; Berridge & Irvine, 1984).

When a cell is labelled at steady state with an isotopically labelled intermediate in a metabolic cycle, it is expected that, when a stimulus accelerates a step in this pathway, the total radioactivity per mg of protein or the specific radioactivity (or both) of the intermediate that is the substrate in this step will decrease. At times between 0.5 and 15 min after addition of ACTH, neither total radioactivity nor specific radioactivity of phosphatidylinositol and polyphosphoinositides of adrenal cells changed. This was true for both $[\text{P}^{32}]\text{P}_i$ and *myo*- $[\text{H}^3]$ inositol, which enter the pathway to polyphosphatidylinositides at different points and pass through very different metabolic pathways. Moreover, no change was observed in the incorporation of *myo*- $[\text{H}^3]$ inositol into phosphatidylinositides or in the mass of any polyphosphatidylinositides or phosphoinositol. Farese (1982) and Whitley *et al.* (1984) reported that, at low concentrations, ACTH increased incorporation of $[\text{P}^{32}]\text{P}_i$ into PtdIns in rat fasciculata and glomerulosa cells, respectively. However, in our experiments with Y-1 or bovine fasciculata cells, ACTH was without effect even at a concentration of 10 μM . By contrast, an unequivocal decrease in the total radioactivity in polyphosphatidylinositides was seen with angiotensin II. From the work of other investigators with other stimuli and other cells, the conditions that we have used would have detected a clear response in such diverse situations as blowfly salivary gland (Fain *et al.*, 1983), rat mammatropic pituitary cells (Rebecchi & Gershengorn, 1983) and rat liver (Williamson, 1985).

In considering the second aspect of the phosphatidylinositol- Ca^{2+} mechanism, we have seen no change in the concentration of intracellular Ca^{2+} , using two different methods in two different cell types, both of which showed good steroidogenic responses to ACTH. Again, glomerulosa cells showed a clear increase in the concentration of intracellular Ca^{2+} on addition of angiotensin II. This response, although small when compared with responses observed in other cell types (Thomas *et al.*, 1984; Albert & Tashjian, 1984; Gershengorn & Thaw, 1985), is similar to that observed by other workers using bovine or rat glomerulosa cells (Capponi *et al.*, 1984; Braley *et al.*, 1984). Both of these groups found that angiotensin II, at concentrations similar to those used in the present study, produced a maximal increase in cytosolic Ca^{2+} of less than 2-fold (Capponi *et al.*, 1984; Braley *et al.*, 1984). On the basis of these results, we conclude that the phosphatidylinositol- Ca^{2+} mechanism, as it occurs in other cells, is not involved in the responses of Y-1 or bovine fasciculata cells to ACTH. This mechanism cannot therefore be an essential part of the steroidogenic response to ACTH. However, these negative observations cannot exclude some modified form of the mechanism with changes in phospholipid and Ca^{2+} metabolism that are too small or too fleeting to be detected by the methods that we have used. In this case, the response must be radically different from that described with other hormones (Thomas *et al.*, 1984; Albert & Tashjian, 1984; Gershengorn & Thaw, 1985). Until methods are developed to detect such hypothetical changes, it is not constructive to consider the phospho-

tidylinosotide-Ca²⁺ mechanism as a necessary part of the response to ACTH.

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