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Temporal patterns of protein phosphorylation after angiotensin II, A23187 and/or 12-*O*-tetradecanoylphorbol 13-acetate in adrenal glomerulosa cells

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The temporal patterns of protein phosphorylation in the adrenal glomerulosa cell were analysed by two-dimensional electrophoresis after stimulation with 10 nM-angiotensin II or various agents [10 nM-12-*O*-tetradecanoylphorbol 13-acetate (TPA), 50 nM-A23187, 1 μM-nitrendipine], administered singly or in combination. These patterns were compared with the temporal patterns of aldosterone secretion induced by the same agonists and antagonists. After 1 and 30 min of stimulation with angiotensin II, different patterns of protein phosphorylation were observed. A comparison of these patterns reveals that: (1) the phosphorylation of only one protein was persistently enhanced during the continuous incubation with angiotensin II; (2) the phosphorylation of five proteins was transiently enhanced (at 1 min but not 30 min); and (3) the phosphorylation of three proteins did not occur at 1 min but was seen at 30 min. Addition of the phorbol ester TPA alone, which at 30 min is without effect in enhancing aldosterone production, has no effect on protein phosphorylation. The combined addition of TPA and the Ca^{2+} ionophore, A23187, which, like angiotensin II, evokes a sustained increase in aldosterone production, reproduced the temporal patterns of protein phosphorylation seen after angiotensin II action. Manipulations (A23187 alone, angiotensin II plus nitrendipine) which evoke only a transient rise in aldosterone production rate induce a transient rise in cellular protein phosphorylation. The 1 min patterns of phosphorylation seen after A23187 or combined angiotensin II and nitrendipine (a Ca^{2+} channel antagonist) are similar to those observed after 1 min of angiotensin II stimulation. These results suggest that, when angiotensin II acts, the initial cellular response is mediated by a different mechanism than that responsible for the sustained response.

INTRODUCTION

Recent studies on the mechanism of action of angiotensin II in hepatocytes [1–8] and adrenal glomerulosa cells [9–23] have shown that the initial events in hormone action are similar. In both cell types, addition of angiotensin II leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate with the production of inositol trisphosphate and diacylglycerol. In both, inositol trisphosphate mobilizes Ca^{2+} from a non-mitochondrial intracellular calcium pool presumed to exist in the endoplasmic reticulum, and by this means causes a transient increase in the free Ca^{2+} concentration in the cell cytosol. Moreover, in each cell type, angiotensin II causes a prompt and sustained increase in the Ca^{2+} influx rate across the plasma membrane [6,23].

In the hepatocyte, work by Garrison and coworkers [8] has also shown that angiotensin II actions leads to an increase in the extent of phosphorylation of at least ten cytosolic proteins only three of which (pyruvate kinase, phosphorylase, and phenylalanine hydroxylase) have been identified. In an effort to identify the protein kinases responsible for the phosphorylation of all of these proteins, Garrison *et al.* [8] compared the patterns of protein phosphorylation seen after the addition of Ca^{2+} ionophore, A23187, or a phorbol ester, TPA, with those induced by angiotensin II. Addition of A23187 induced an increase in the extent of phosphorylation of a subset of seven of the ten proteins affected by angiotensin II while TPA induced an increase in the extent of phosphorylation of the remaining three proteins. From these studies, it was concluded that angiotensin II brought about an increase in the state of phosphorylation of substrate proteins by activating at least three kinases; phosphorylase kinase (a known calmodulin-dependent protein kinase), protein kinase C, and one or more additional calmodulin-dependent kinases. However, while these studies related the activation of phosphorylase kinase to the effects of angiotensin II on glycogenolysis, they did not relate the activation of protein kinase C to the effects of the hormone on hepatocyte function. Equally unresolved was the role played by the angiotensin II-induced sustained increase in Ca^{2+} influx rate in hepatocytes [6].

Recent studies from our own laboratory have begun to link both protein kinase C activation and changes in plasma membrane Ca^{2+} influx rate to the hormone-induced secretion of aldosterone from adrenal glomerulosa cells [17,22,23]. These studies have shown that the combined addition of A23187 and TPA, but neither alone, mimics the effect of angiotensin II on aldosterone secretory rate, that nitrendipine, a calcium channel antagonist, by blocking the hormone-induced increase in Ca^{2+} influx rate, blocks the late but not the early phase

Abbreviation used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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of hormone-induced secretion, and that the combination of an activator of protein kinase C (1-oleoyl-2-acetylglycerol) and a calcium channel agonist (BAY K 8644) can induce a sustained aldosterone secretory response without causing a significant increase in intracellular free Ca^{2+}. Our results suggest that the initial phase of the aldosterone secretory response is mediated by the inositol trisphosphate-induced increase in intracellular free Ca^{2+}, and the sustained phase by the increase in activity of protein kinase C. The activity of this enzyme appears to depend upon two factors: the amount of the enzyme which is available for activation, and the rate of hormone-induced Ca^{2+} influx rate [23].

The purpose of the present study was to examine the changes induced by either angiotensin II, or A23187 and/or TPA, on the phosphorylation of proteins in the adrenal glomerulosa cell. These studies were undertaken in an effort to determine whether there is a correlation between protein kinase activation (as assessed by protein phosphorylation), and the functional response of the cell (aldosterone secretion), and to determine the temporal patterns of phosphorylation as a way of defining the possible protein kinase involved in the early and late phases of hormone action.

MATERIALS AND METHODS

Materials

Reagents used in this study were purchased from the following sources: acrylamide, bisacrylamide, SDS, M_r markers and other gel chemicals, Bio-Rad; Ampholines, LKB; Nonidet P-40, Particle Data Laboratories; NADH, hexokinase, glucose-6-phosphate dehydrogenase, angiotensin II, albumin, TPA, A23187, Sigma; carrier-free $[^{32}P]P_i$, Amersham; nitrendipine was a gift from Dr. A. Scriabine (Miles Laboratories).

Preparation of adrenal cells

Calf adrenal glands were obtained at a local slaughterhouse. Adrenal glomerulosa cells were dispersed by collagenase digestion from thinly sliced glomerulosa layers as previously described [16]. For all phosphorylation experiments, cells were resuspended in low-phosphate (0.1 mM) Krebs–Ringer bicarbonate buffer containing 0.5 mM-Ca^{2+}, 3.5 mM-K^+ and 5.5 mM-glucose which was equilibrated with O_2/CO_2 (19:1). Bovine serum albumin was omitted during the labelling and experimental periods.

Perifusion of adrenal glomerulosa cells

Perifusion was performed using a four channel flow-through chamber in a Perspex block as described previously [17,18]. Cells were perifused with Krebs–Ringer bicarbonate buffer containing 0.5 mM-Ca^{2+} 3.5 mM-K^+, 5.5 mM-glucose and no bovine serum albumin with a flow rate of 0.4 ml/min. Samples for aldosterone were collected every 4 min and aldosterone was measured by radioimmunoassay [12]. Dead space of the perifusion system was 0.8 ml (equivalent to 2 min at this flow rate). A correction for this was made in the aldosterone secretion data plotted in Figs. 1 and 5.

Incubation procedures and sample preparation for phosphorylation

Adrenal cells (10^7/ml) were incubated in low-phosphate (0.1 mM) Krebs–Ringer-bicarbonate buffer containing 250 μCi of $[^{32}P]P_i$/ml for 45 min. Agonists were added to 900 μl aliquots of labelled cells for 1 or 30 min. Appropriate vehicle solutions were added to all controls. Dimethyl sulphoxide never exceeded a final concentration of 0.2%. This concentration does not stimulate phosphatidylinositol turnover nor aldosterone production. To terminate the incubation, cells were mixed with 500 μl of NaF stop buffer (10 mM-Hepes, 20 mM-sucrose, 100 mM-NaF, 15 mM-EDTA and 2 mM-EGTA, pH 7.4), centrifuged (Microfuge) for 7 s, surface washed with an additional 500 μl of the same stop buffer, re-centrifuged, and frozen in a solid CO_2/acetone bath. Upon termination of all incubations, pelleted samples were thawed in ice-cold NaF stop buffer, aspirated 10 times through a 27 gauge needle fitted to 1 ml syringe, mixed with isoelectric focusing sample preparation buffer [2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 122 mM-Tris/HCl (pH 6.8)] in a ratio of 1:2 and vigorously boiled for 5–10 min. The protein content of each sample was determined by the method of Lowry [24] with bovine serum albumin as a standard, on an aliquot taken prior to dilution with isoelectric focusing sample buffer. Prior to loading, all samples within an experiment were adjusted to achieve equivalent protein concentrations (1–3 mg/ml); 70 μg of protein was loaded on each gel.

Two-dimensional gel electrophoresis

Homogenate proteins were resolved by two-dimensional electrophoresis as described by Garrison [7,8] with minor modifications.

In general, 150 μl samples were solubilized with 150 mg of urea and Nonidet P-40 to a final concentration of 3.5% (v/v). Samples were allowed to solubilize at room temperature for more than 2 h. LKB Ampholines, having the following distribution: 1 part of pH 3.5–10, 1.5 parts of pH 5–7 and 2.5 parts of pH 6–8, were added to achieve a final concentration of 2% (v/v). This distribution ratio was also maintained in the isoelectric focusing gel. Prior to loading, each sample was centrifuged at 100000 *g* for 10 min (Beckman Airfuge) to remove gelatinous insoluble material which contained no measurable protein; 70 μg of protein was layered on each isoelectric focusing gel. Each sample was overlayed with 8 M-urea containing 1% Ampholines.

Conditions for gel electrophoresis

Isoelectric focusing gels were prepared in 1.5 mm internal diameter tubes with 3% acrylamide (28.38/1.62) as described by O'Farrell [25] and run for 9050 V·h at 500 V or less and for 1 h at 950 V. Chilled isoelectric focusing gels were removed from tubes by gentle water pressure and equilibrated for exactly 15 min at room temperature in isoelectric focusing sample preparation buffer (see above). Isoelectric gels were layered over a 2.0 cm stacking gel (3%) and secured with 1% agarose. Homogenate proteins were resolved using a gel thickness of 0.75 mm on a 12% (29.2/0.8) separating gel (pH 8.8). Electrophoresis was performed at 20 mA/plate through the stacker and 25 mA/plate through the separating gel.

Gels were stained with Coomassie Brilliant Blue R250, and vacuum-dried on filter paper. Autoradiography was performed using Kodak XAR-5 film with intensifier screens for 24–56 h at −70 °C. The films were developed in an automatic processor and visually analysed. All experiments were repeated on at least three separate cell

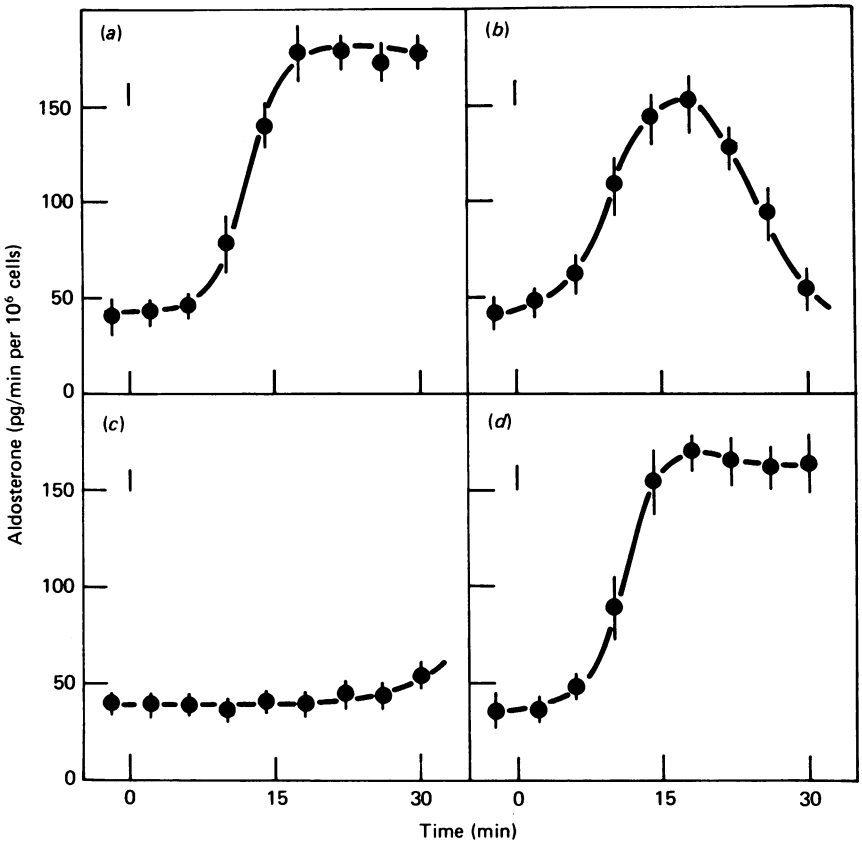

Fig. 1. Time course of effects of angiotensin II, A23187, TPA or combined A23187 and TPA on aldosterone secretion

Cells were stimulated from time 0 to 32 min by either 10 nM-angiotensin II (*a*), 50 nM-A23187 (*b*), 10 nM-TPA (*c*) or combined A23187 and TPA (*d*). Results are the mean ± S.E.M. for three experiments using three different cell preparations.

preparations. All gels within an experimental set were exposed and processed equivalently. Representative autoradiographs are shown. M_r values of the resolved phosphorylated proteins were estimated from M_r markers. Standard proteins used were: phosphorylase *b* (M_r 92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400). The results presented in this paper are restricted to those cellular proteins which can be well resolved by the particular gel system employed. As such, the number of phosphoproteins seen after various treatments must be considered a minimal number.

Quantification of autoradiographs

For each phosphoprotein on the exposed autoradiograph, the integrated density was calculated from the area and intensity of grain exposure. The area was estimated by overlaying the exposed spot with a calibrated stencil which contained 18 circles of known area. The area of the smallest circle which fully exposed each spot was assigned as the spot size. Exposure intensity of each spot was estimated by matching its level of greyness with an X-ray film containing a linear scale of 11 exposure densities ranging from translucent ($A = 0.1$) to opaque ($A > 3.0$). The product of area and intensity estimated in this manner corresponds to the integrated absorbance (units = area × absorbance). The integrated absorbance for each phosphoprotein was determined independently by three observers and a mean value was assigned. The inter-observer variation for a representative group of three phosphoproteins (spots 10, 11 and 12) on four films was 9% ±6% (S.D.). Within each experiment, the integrated absorbance of each phosphoprotein spot seen following hormone or drug treatment is compared as a ratio with that of the same phosphoprotein present in control cells. Separate control cell incubations and autoradiographs were obtained for each experiment. Each experiment was performed with between three and six separate preparations of adrenal cells as indicated in the Results section.

Specific radioactivity of [^{32}P]ATP

An estimation of the specific radioactivity [^{32}P]ATP was obtained by measuring the specific radioactivity of the total [^{32}P]ATP control. Samples were incubated exactly as described for the phosphorylation experiments. At desired times 500 μl of cell suspension was pipetted into ice-cold Microfuge tubes containing 500 μl of 10% $HClO_4$/1 mM-EDTA. After removing the cell protein precipitate, the supernatant was neutralized with ice-cold 1 M-KOH/0.4 M-imidazole/0.4 M-KCl as described by Lowry [26] and recentrifuged. This supernatant was stored at −20 °C until analysis. The content of ATP was determined fluorometrically by measuring the production of NADPH in a hexokinase/glucose-6-phosphate dehydrogenase enzyme system [26]. The radioactivity incorporated into ATP was determined by anion-exchange t.l.c. using poly(ethylene imine)/cellulose F plates (Merck, Darmstadt, Germany) and a formic

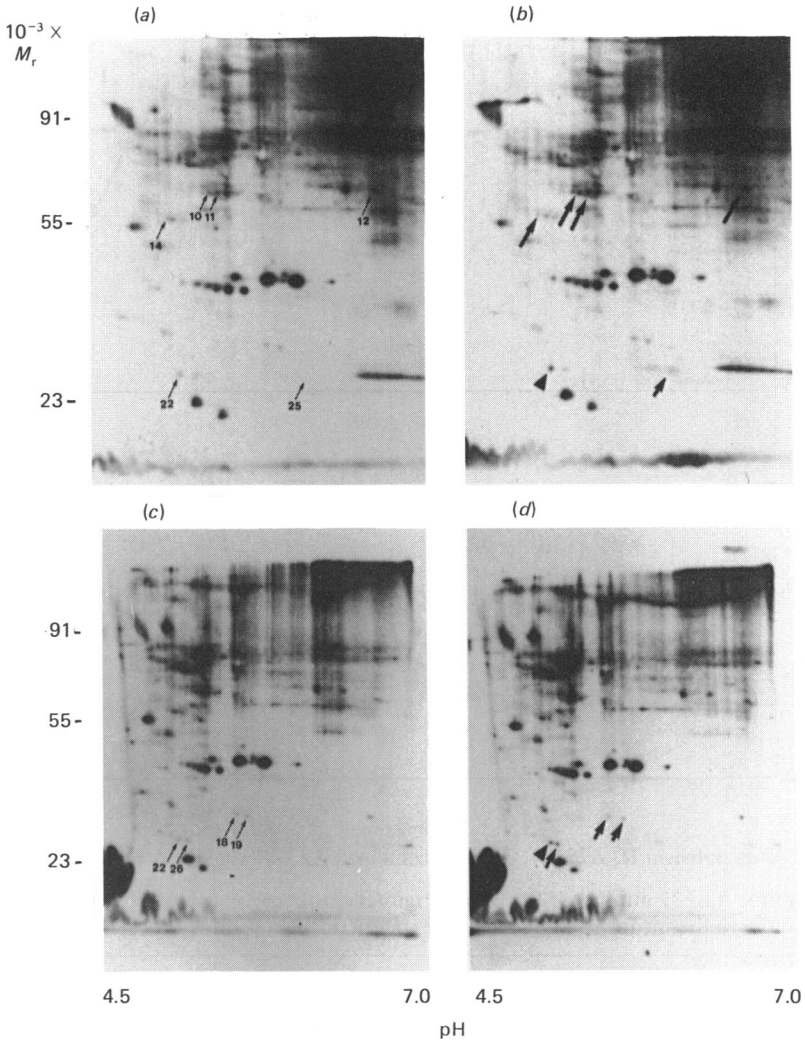

Fig. 2. Autoradiographs showing the effect of angiotensin II on protein phosphorylation in adrenal glomerulosa cells

Cells were incubated with [^{32}P]P_i, treated with 10 nM-angiotensin II for 1–30 min and prepared for two-dimensional gel electrophoresis as described in the Materials and methods section. Numbered arrows in control autoradiograms (*a* and *c*) indicate the position of the phosphoproteins whose phosphorylation state was subsequently enhanced by angiotensin II treatment. (*a*) Control, (*b*) angiotensin II at 1 min, (*c*) control. (*d*) angiotensin II at 30 min. Long arrows identify putative calcium-linked substrates, short arrows identify putative protein kinase C substrates, and solid triangles identify common substrates.

acid/sodium formate buffer system (0.5 M/2.0 M/4.0 M) according to the procedure of Randerath & Randerath [27, 28]. Radioactivity migrating in bands with R_F values equivalent to ATP standards (visualized by u.v. absorbance) was determined by liquid-scintillation counting. The specific radioactivity of the [^{32}P]ATP pool in the cell was analysed at 35 min of incubation and at 46 and 75 min of incubation, i.e. 1 and 30 min after the addition of 10 nM-angiotensin II. The specific radioactivity at 35 min of incubation (321 ± 10 c.p.m./nmol) was not significantly different from the specific radioactivity of the labelled ATP pool at 75 min of incubation in either the absence (293 ± 13 c.p.m./nmol) or the presence (312 ± 11 c.p.m./nmol) of angiotensin II, suggesting that the changes in the patterns of protein phosphorylation induced by time and agonist addition were not due to a change in the specific radioactivity of the [^{32}P]ATP pool.

RESULTS

Activation of aldosterone secretion by angiotensin II, A23187, TPA, or combined A23187 and TPA

Previous work has shown that combined A23187 plus TPA, but neither agent alone, induce a change in aldosterone secretory rate which is qualitatively and quantitatively similar to that induced by angiotensin II [16,17]. In those studies, the dose of A23187 employed was 500 nM. This concentration is sufficient to cause a prompt increase in aldosterone secretory rate, which reaches a peak in approx. 20 min and subsequently declines to a sustained rate which is approx. twice the basal rate of secretion [17]. In an effort to refine the present analysis, the effect of lower concentrations of A23187 alone, and in combination with TPA, on aldosterone secretory rate was explored. As shown in Fig. 1(*b*) as little as 50 nM-A23187 induces a prompt but

Table 1. Agonist-induced changes in protein phosphorylation at 1 min

Glomerulosa cells were incubated with [^{32}P]P_i for 45 min and subsequently stimulated for 1 min. Agonists were added alone or in combination at the indicated concentrations (10 nM-angiotensin II, 50 nM-A23187, 10 nM-TPA). Homogenate proteins were resolved as described. The M_r of each spot was calculated from a standard curve as described. The effect of agonists on the state of protein phosphorylation of each phosphoprotein is presented below as -fold over control. *P* values refer to comparison of treated with control by using Student's *t*-test; n.s., not significant; $n = 3$–6 separate experiments.

		Changes in phosphorylation (-fold over control) (mean ± S.E.M., frequency, *P*)			
Spot	$10^{-3} \times M_r$	Angiotensin II	A23187	A23187 + TPA	TPA
10	64	2.30 ± 0.25 (4/4) < 0.02	2.30 ± 0.28 (4/4) < 0.02	2.67 ± 0.29 (3/3) < 0.05	1.52 ± 0.25 (1/3) n.s.
11	64	2.12 ± 0.21 (5/5) < 0.01	2.28 ± 0.16 (4/4) < 0.01	2.00 ± 0.23 (3/3) < 0.05	0.97 ± 0.10 (0/4) n.s.
12	60	2.15 ± 0.18 (5/5) < 0.01	2.17 ± 0.13 (4/4) < 0.01	2.34 ± 0.43 (3/3) < 0.05	1.47 ± 0.34 (1/4) n.s.
14	56	1.95 ± 0.29 (5/6) < 0.05	2.07 ± 0.48 (4/5) n.s.	2.09 ± 0.48 (3/4) n.s.	1.05 ± 0.10 (0/4) n.s.
22	26	2.38 ± 0.33 (6/6) < 0.01	2.11 ± 0.49 (4/5) n.s.	2.30 ± 0.40 (4/4) n.s.	0.96 ± 0.20 (0/4) n.s.
25	26	137.0 ± 88 (4/4) n.s.	4.8 ± 3.8 (1/3) n.s.	177.0 ± 128 (3/3) n.s.	1.09 ± 0.09 (0/3) n.s.

Table 2. Agonist-induced changes in protein phosphorylation at 30 min

Glomerulosa cells were incubated with [^{32}P]P_i for 45 min and subsequently stimulated for 30 min. Agonists were added alone or in combination at the indicated concentrations (10 nM-angiotensin II, 50 nM-A23187, 10 nM-TPA). Homogenate proteins were resolved as described. The M_r of each spot was calculated from a standard curve as described. The effect of agonists on the state of protein phosphorylation of each phosphoprotein is presented below as -fold over control. *P* values refer to comparison of treated with control by using Student's paired *t*-test; n.s., not significant; $n = 3$ separate experiments.

		Changes in phosphorylation (-fold over control) (mean ± S.E.M., frequency, *P*)			
Spot	$10^{-3} \times M_r$	Angiotensin II	A23187	A23187 + TPA	TPA
10	64	5.20 ± 4.22 (1/3) n.s.	1.21 ± 0.21 (1/3) n.s.	1.04 ± 0.03 (0/3) n.s.	1.00 ± 0.08 (0/3) n.s.
11	64	1.00 ± 0.2 (0/3) n.s.	1.03 ± 0.03 (0/3) n.s.	1.03 ± 0.02 (0/3) n.s.	0.84 ± 0.10 (0/3) n.s.
12	60	1.11 ± 0.10 (0/3) n.s.	1.05 ± 0.05 (0/3) n.s.	1.07 ± 0.08 (1/3) n.s.	0.97 ± 0.12 (0/3) n.s.
14	56	1.23 ± 0.09 (1/3) n.s.	1.16 ± 0.24 (1/3) n.s.	1.34 ± 0.10 (2/3) n.s.	0.86 ± 0.16 (0/3) n.s.
25	26	11.03 ± 10 (1/13) n.s.	0.96 ± 0.05 (0/3) n.s.	9.96 ± 9.03 (1/3) n.s.	0.75 ± 0.24 (0/3) n.s.
22	26	2.28 ± 0.27 (3/3) < 0.05	1.08 ± 0.53 (1/3) n.s.	2.39 ± 0.53 (3/3) n.s.	1.00 ± 0.10 (0/3) n.s.
18	30	1.83 ± 0.09 (3/3) < 0.02	0.97 ± 0.31 (1/3) n.s.	2.05 ± 0.08 (3/3) < 0.01	0.91 ± 0.10 (0/3) n.s.
19	30	1.35 ± 0.06 (3/3) < 0.05	0.95 ± 0.20 (1/3) n.s.	1.46 ± 0.09 (3/3) < 0.05	1.07 ± 0.33 (1/3) n.s.
26	25	3.02 ± 0.35 (3/3) < 0.05	1.37 ± 0.26 (2/3) n.s.	2.73 ± 0.31 (3/3) < 0.05	0.99 ± 0.17 (1/3) n.s.

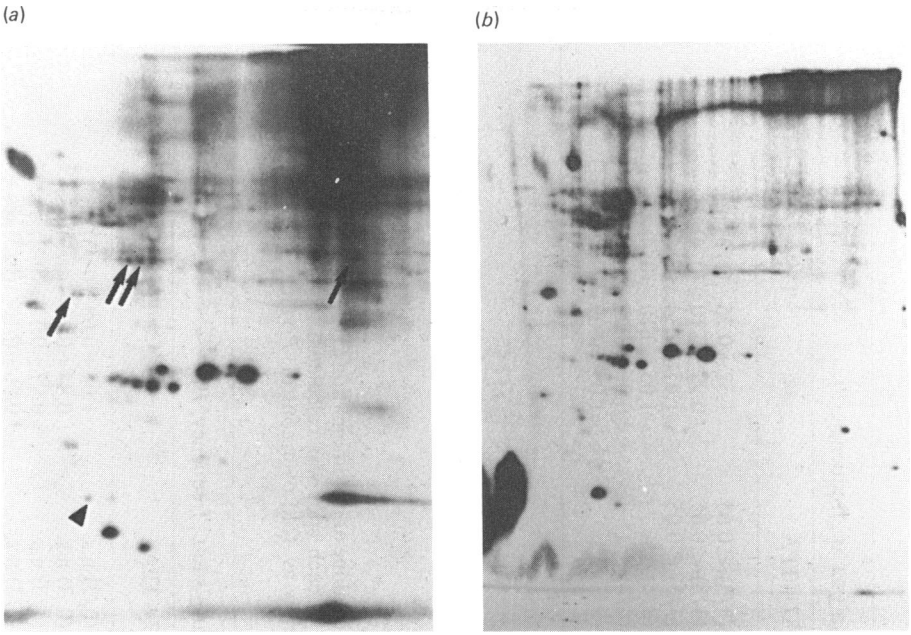

Fig. 3. Autoradiographs showing the effect of A23187 on protein phosphorylation in adrenal glomerulosa cells

Cells were incubated with [^{32}P]P_i, treated with 50 nM-A23187 for 1 or 30 min, and prepared for two-dimensional gel electrophoresis as described in the Materials and methods section. (*a*) A23187 at 1 min, (*b*) A23187 at 30 min. Long arrows identify putative calcium-linked substrates whose phosphorylation was enhanced. For appropriate controls compare with Figs. 2(*a*) and 2(*c*).

transient increase in aldosterone secretory rate which reaches a peak in approx. 17 min, and then declines to its basal value by 30 min. Also illustrated (Fig. 1*c*) is the fact that the addition of 10 nM-TPA alone causes practically no change in aldosterone secretory rate for the first 25 min, and only a minimal rise at 30 min. However, the combination of 10 nM-TPA and 50 nM-A23187 induces a prompt and sustained increase in aldosterone secretory rate (Fig. 1*d*) which is nearly identical with that induced by 10 nM-angiotensin II (Fig. 1*a*).

It is noteworthy that both angiotensin II and A23187 induce a prompt and sustained increase in Ca^{2+} influx rate (control 0.59 ± 0.09, angiotensin II 1.38 ± 0.15 and A23187 2.70 ± 0.26 nmol/min per mg of protein [22,23]). In spite of the fact that the effect of A23187 on Ca^{2+} influx rate is twice as large as the effect of angiotensin II, the change in aldosterone secretory rate induced by A23187 is transient and that induced by angiotensin II is sustained. Further, addition of TPA has no effect on Ca^{2+} influx rate in either control or A23187-treated cells (results not shown). Hence, the synergistic effect of A23187 and TPA on aldosterone secretion (Fig. 1*d*) is not due to their having a synergistic effect on Ca^{2+} influx rate.

Protein phosphorylation: general

Based upon the secretory patterns shown in Fig. 1, and the fact that the peak increase in intracellular free Ca^{2+} concentration occurs within 1 min of angiotensin II addition (W. Apfeldorf & H. Rasmussen, unpublished work), protein phosphorylation was analysed at 1 and 30 min. The 1 min point enabled the detection of early phosphorylation reactions when intracellular calcium was elevated. The 30 min point enabled the detection of late or sustained phosphorylation reactions when intracellular calcium was not elevated. Moreover, at 30 min of incubation, the aldosterone secretory response to either angiotensin II (Fig. 1*a*) or combined A23187 and TPA (Fig. 1*d*) is well-established, but the responses to either A23187 (Fig. 1*b*) alone or TPA (Fig. 1*c*) alone are close to the basal value. In the initial studies to be reported, the decision was made to analyse the phosphoproteins found in total cell extracts rather than to restrict our analysis to those phosphoproteins found in the cytosol, as done by Garrison *et al.* [7,8] in their hepatocyte studies.

The two-dimensional gel system employed in the present study separates approx. 100 Coomassie Blue-stained proteins (not shown) and resolves approx. 40 phosphorylated ones. Only alterations that could be visually detected were scored as a change in phosphorylation state. In general, this confined our analysis to phosphoproteins having M_r values in the range 90000–20000 with isoelectric points below 7.0, because in this region of the autoradiograph alterations in density could be analysed unambiguously. Hence, the phosphoproteins discussed below represent only those cellular proteins that were well resolved by the gel system employed and probably underestimate the total number of phosphoproteins whose phosphorylation state was altered.

Protein phosphorylation: effect of angiotensin II

The patterns of protein phosphorylation seen at 1 and 30 min in the absence of angiotensin II are shown in the representative autoradiographs contained in Fig. 2 (panels *a* and *c*). In these photographs, only those phosphoproteins are numbered whose phosphorylation state was enhanced by agonist or drug addition in subsequent studies. When cells are incubated with

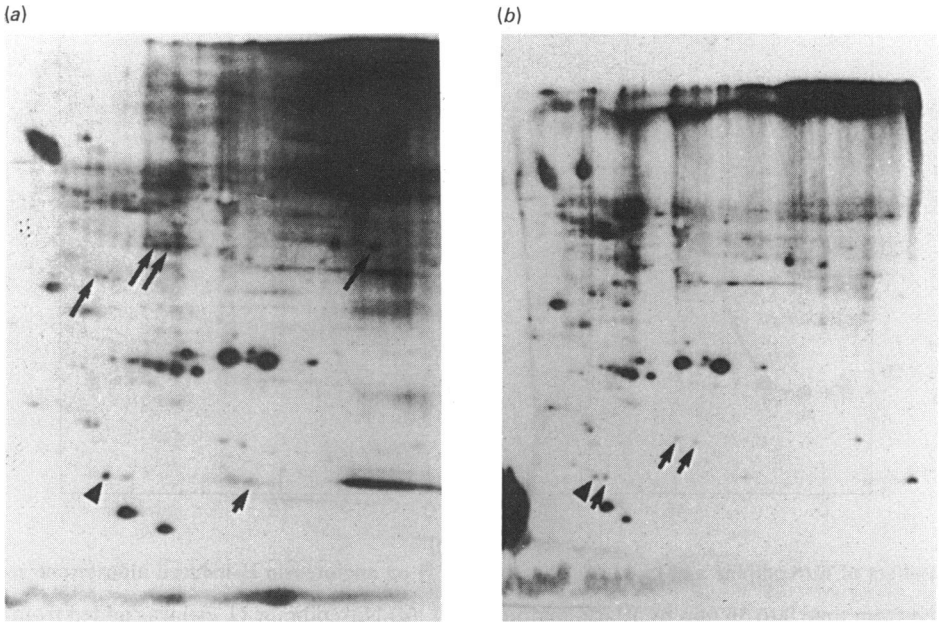

Fig. 4. Autoradiographs showing the combined effect of A23187 and TPA on protein phosphorylation in adrenal glomerulosa cells

Cells were incubated with [^{32}P]P_i, treated with 50 nM-A23187 + 10 nM-TPA for 1 or 30 min and prepared for two-dimensional gel electrophoresis as described in the Materials and methods section. (*a*) A23187 + TPA at 1 min, (*b*) A23187 + TPA at 30 min. Long arrows identify putative calcium-linked substrates, short arrows identify putative protein kinase C substrates, and solid triangles identify common substrates. For appropriate controls compare with Figs. 2(*a*) and 2(*c*).

10 nM-angiotensin II for 1 min before being harvested, the phosphorylation of at least six proteins is increased. A representative autoradiograph is shown in Fig. 2(*b*). This pattern of agonist-induced protein phosphorylation is not static but changes with time. After 30 min of incubation with angiotensin II, the phosphorylation of only four proteins is increased (Fig. 2*d*). Of these, only one is from the original six seen at 1 min. Three additional proteins not seen after 1 min of incubation, spots 18, 19, and 22, are newly phosphorylated, and five proteins of the original six are no longer phosphorylated (Fig. 2*d*). Thus, one can recognize a distinct temporal shift in the phosphorylation pattern during the time course of angiotensin II action.

Because small differences exist among autoradiographs obtained from separate experiments, a summary of this quantified data is given in Tables 1 and 2. Tabulated are the numbered phosphoproteins which showed an increase in the state of phosphorylation, their estimated M_r values and the number of separate experiments performed using different cell isolates. Enhanced protein phosphorylation was expressed as the mean ± S.E.M. of the ratio of the integrated intensity (see the Materials and methods section) of each phosphoprotein spot in autoradiographic exposures of extracts of treated and control cell incubations. The statistical significance of these changes in phosphorylation state is given by the indicated P value. The frequency of occurrence of these changes, however, is also reported because in certain instances (e.g., spot 25), although the enhanced state of phosphorylation was dramatic, significance was not achieved because of the large variation in the absolute magnitude of the enhancement. The results recorded in Tables 1 and 2 emphasize the highly reproducible nature of the results obtained from three to six extracts prepared from separate incubations using different cell preparations.

In separate experiments, it was found that the specific radioactivity of the intracellular ATP pool came into equilibrium within 30–35 min of incubation (see the Materials and methods section). Therefore, addition of hormone (or drugs) was routinely made at 45 min to preclude the possibility that the measured changes in protein phosphorylation were merely a consequence of a more rapid approach to steady state. However, it remained possible that, even though the ATP labelling became constant, the labelling of phosphoproteins with a slow turnover time did not, and that the increase in state of phosphorylation of one or more of the proteins seen 30 min after hormone addition was due to an increased rate rather than extent of labelling. To rule out this possibility, two studies were performed in which cells were prelabelled for 100 min before angiotensin II addition, and the patterns of protein phosphorylation seen 30 min later in control and hormone-treated cells analysed. Hormone addition caused an increase in the extent of labelling of the same four proteins observed in the shorter protocol (results not shown).

Protein phosphorylation: effect of A23187 or TPA alone or in combination

In an attempt to determine the relative contributions of calcium-sensitive calmodulin-dependent protein kinase(s) activity and protein kinase C activity to the phosphorylation patterns obtained at 1 and 30 min of incubation, experiments were performed using either A23187 or TPA alone. Incubation of cells with 50 nM-A23187 for 1 min leads to a change in the state of phosphorylation of five of the six proteins whose phosphorylation is increased in response to angiotensin

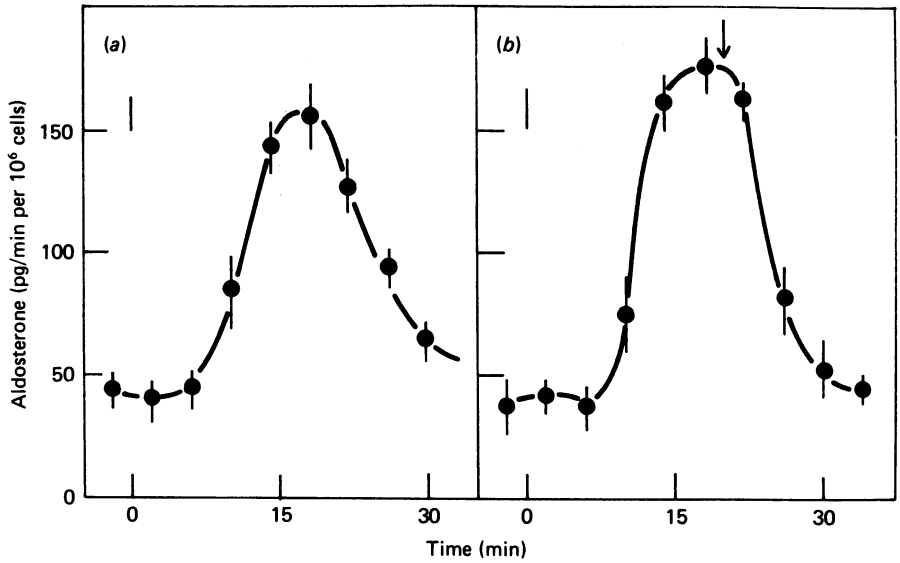

Fig. 5. Time course of effect of nitrendipine and [Sar^1,Ala^8]angiotensin II on angiotensin II-induced aldosterone secretion

Cells were stimulated from time 0 to 36 min by 10 nM-angiotensin II. (*a*) Nitrendipine (1 μM) was added from time 0 to 36 min, (*b*) [Sar^1,Ala^8]angiotensin II (1 μM) was added from time 20 min to 36 min.

II (Fig. 3*a*). Therefore, as in many systems, Ca^{2+}-dependent protein phosphorylation presumably mediated largely by calmodulin-dependent protein kinases, can account for only a subset of the phosphoproteins induced by the natural agonist [7,8,29,30]. Despite the very marked effect of A23187 on the state of protein phosphorylation in the adrenal cell at 1 min of incubation, A23187 is without effect after 30 min of exposure (Fig. 3*b*). Thus, A23187 alone evokes only a transient increase in the aldosterone secretory response (Fig. 1*b*) and in the state of phosphorylation of at least five proteins in the adrenal cell (Fig. 3).

Treatment of cells with 10 nM-TPA for either 1 or 30 min had no significant effect on the phosphorylation of any cellular proteins (Tables 1 and 2). Thus, this dose of TPA is ineffective either in inducing the phosphorylation of proteins (Tables 1 and 2) or in increasing the aldosterone secretory rate (Fig. 1*c*). However, when adrenal glomerulosa cells are exposed to the combination of 10 nM-TPA and 50 nM-A23187, an increase in the state of phosphorylation of six proteins is seen at 1 min (Fig. 4*a*) and of four proteins at 30 min (Fig. 4*b*). At each of these time points, the change in state of phosphorylation of the proteins altered by combined A23187 and TPA are identical with those altered by treatment of the cells with 10 nM-angiotensin II (Tables 1 and 2, and compare Figs. 2 and 4).

Effect of nitrendipine on angiotensin II action

To evaluate the role of Ca^{2+} influx on the patterns of protein phosphorylation seen during the two phases of angiotensin II action, adrenal cells were treated with 1 μM-nitrendipine, a Ca^{2+} channel antagonist, and 10 nM-angiotensin II for either 1 or 30 min of incubation. Nitrendipine at 1 μM causes little change in the basal rate of Ca^{2+} influx but nearly completely inhibits the angiotensin II-mediated increase in Ca^{2+} influx rate [23]. The consequence of this is to alter the time course of the aldosterone secretory response to angiotensin II (compare Fig. 5*a* with Fig. 1*a*). The initial phase of the aldosterone secretory response to angiotensin II is unaltered by the presence of nitrendipine but the sustained phase of aldosterone secretion is greatly attenuated. As illustrated in Fig. 5(*a*), in the presence of nitrendipine, the secretory response to angiotensin II reaches a peak at approx. 17 min, and returns close to its basal value by 30 min (Fig. 5*a*). When cells are simultaneously exposed to nitrendipine and angiotensin II, all six proteins of the first phase are phosphorylated at 1 min (Fig. 6 and Table 3). However, there is no increase in the phosphorylation of any of the four proteins normally seen at 30 min during the second phase of angiotensin II action (Table 3). Thus, when the angiotensin II-mediated increase in Ca^{2+} influx rate is blocked, the late effects of angiotensin II on both aldosterone secretory rate (Fig. 5*a*) and on protein phosphorylation (Fig. 6) are not observed (Table 3).

In previous work [22,23] it was shown that this concentration of nitrendipine, although effective in inhibiting the late phase of angiotensin II-induced aldosterone secretion, had no effect on the late phase of the aldosterone secretory response induced by the combined addition of A23187 and TPA. Likewise, in a series of two experiments, nitrendipine had no effect on the phosphorylation reactions catalysed by the combined addition of A23187 and TPA. At 30 min of incubation, the extent of phosphorylation of all four proteins was equivalently enhanced (results not shown).

Effect of [Sar^1, Ala^8]angiotensin II on secretion and protein phosphorylation

A final feature of the angiotensin II-mediated response which was characterized is the rapidity with which the aldosterone secretory response, and the increased phosphorylation of cellular proteins, is reversed when an angiotensin II antagonist, [Sar^1,Ala^8]angiotensin II [31], is added to cells exposed to angiotensin II. As shown in Fig. 5(*b*), if the antagonist is added 20 min after angiotensin II stimulation, the aldosterone secretory rate falls rapidly, and returns to its basal value by 35 min

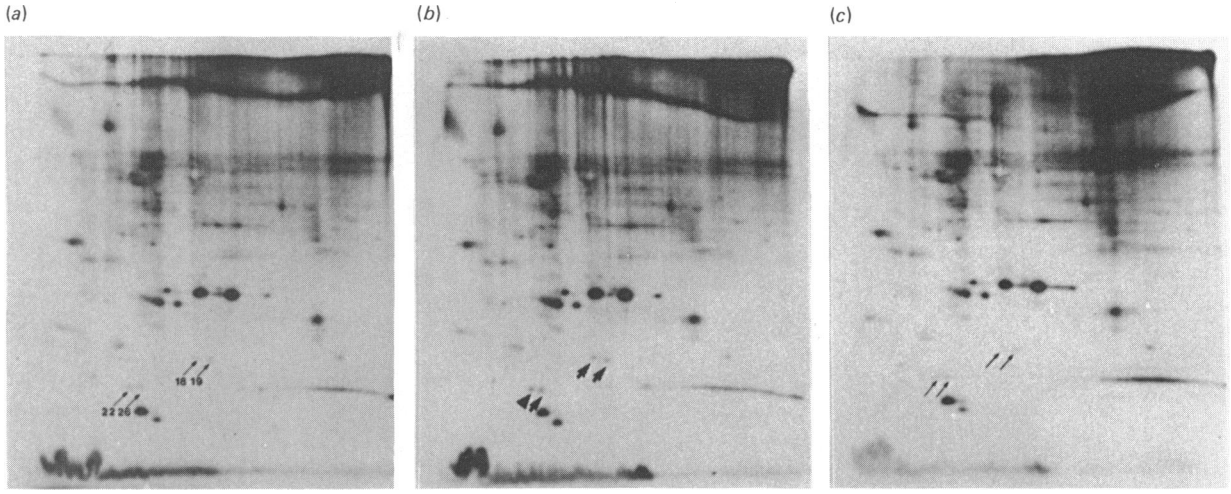

Fig. 6. Autoradiographs showing the effect of nitrendipine on protein phosphorylation in angiotensin II-stimulated adrenal glomerulosa cells

Cells were incubated with [^{32}P]P_i, treated for 30 min with 10 nM-angiotensin II alone or in combination with 1 μM-nitrendipine and prepared for two-dimensional gel electrophoresis as described in the Materials and methods section. (*a*) Control, (*b*) angiotensin II, (*c*) angiotensin II + nitrendipine. Short arrows identify putative protein kinase C substrates, and solid triangles identify common substrates.

Table 3. Effect of nitrendipine on angiotensin II-induced changes in protein phosphorylation

Glomerulosa cells were incubated with [^{32}P]P_i for 45 min and subsequently stimulated for 1 or 30 min. Angiotensin II (10 nM) was added alone or in combination with 1 μM-nitrendipine. Homogenate proteins were resolved as described. The effect of nitrendipine on the state of protein phosphorylation of each phosphoprotein in the angiotensin II-stimulated cell is presented as -fold over angiotensin II. *P* values refer to comparison of angiotensin II-nitrendipine with angiotensin II by using Student's paired *t*-test; n.s., not significant; $n = 3$ separate experiments.

	Nitrendipine-induced changes in phosphorylation (-fold over angiotensin II-stimulated) (mean ± S.E.M., frequency, *P*)			
Spot	1 min		30 min	
10	1.47 ± 0.49	(2/3) n.s.	–	
11	1.20 ± 0.15	(1/3) n.s.	–	
12	0.61 ± 0.13	(0.3) n.s.	–	
14	1.23 ± 0.47	(1/3) n.s.	–	
25	1.56 ± 0.81	(1/3) n.s.	–	
22	1.00 ± 0.14	(1/3) n.s.	0.39 ± 0.02	(0/3) < 0.002
18	–		0.51 ± 0.12	(0/3) < 0.05
19	–		0.67 ± 0.14	(0/3) n.s.
26	–		0.47 ± 0.14	(0/3) n.s.

(15 min after the addition of antagonist). An analysis of the pattern of protein phosphorylation shows that 20 min of stimulation is sufficient to induce the late phase pattern of protein phosphorylation routinely analysed at 30 min of incubation (Fig. 7*b*), and that 15 min after antagonist addition at 35 min of incubation, none of these four proteins (or other proteins) shows an increase in extent of phosphorylation (Fig. 7). Thus, the addition of antagonist provides another circumstance in which protein phosphorylation correlates with aldosterone secretion. Moreover, the rapidity with which protein phosphorylation is reversed in these experiments supports the notion that the enhanced densities measured after 30 min of hormone stimulation are not a consequence of hormone-induced protein synthesis, but are an indication of protein kinase activation.

DISCUSSION

The present results provide additional insights into the mechanism by which angiotensin II regulates cell function. Our data confirm and extend those of Garrison *et al.* [7,8] in hepatocytes. Like these workers we have shown that the combination of A23187 and TPA, but neither agent alone (Fig. 3, Tables 1 and 2), mimics the effect of angiotensin II on the state of phosphorylation of cellular proteins (Figs. 2 and 4). The present results: (1) extend this analysis in the temporal domain and (2) relate the changes in protein phosphorylation to changes in a specific cellular response, aldosterone secretion rate (Figs. 1–7). In particular, the data recorded in Fig. 2 and Tables 1 and 2 demonstrate that when angiotensin II acts, the extent of phosphorylation of a group of cellular proteins increases within 1 min of hormone addition, and that an increased extent of protein phosphorylation is also observed at 30 min. However, the data clearly show that the change in the phosphoprotein pattern seen at 30 min is not identical with that observed at 1 min. Five of the proteins displaying increased phosphorylation at 1 min no longer display this change at 30 min, and three proteins, not phosphorylated at 1 min, are phosphorylated at 30 min. Only one protein that displays an increased extent of phosphorylation at 1 min does so at 30 min. Furthermore, when cells exposed to angiotensin II for 20 min are treated with an angiotensin II antagonist, the aldosterone secretory response declines

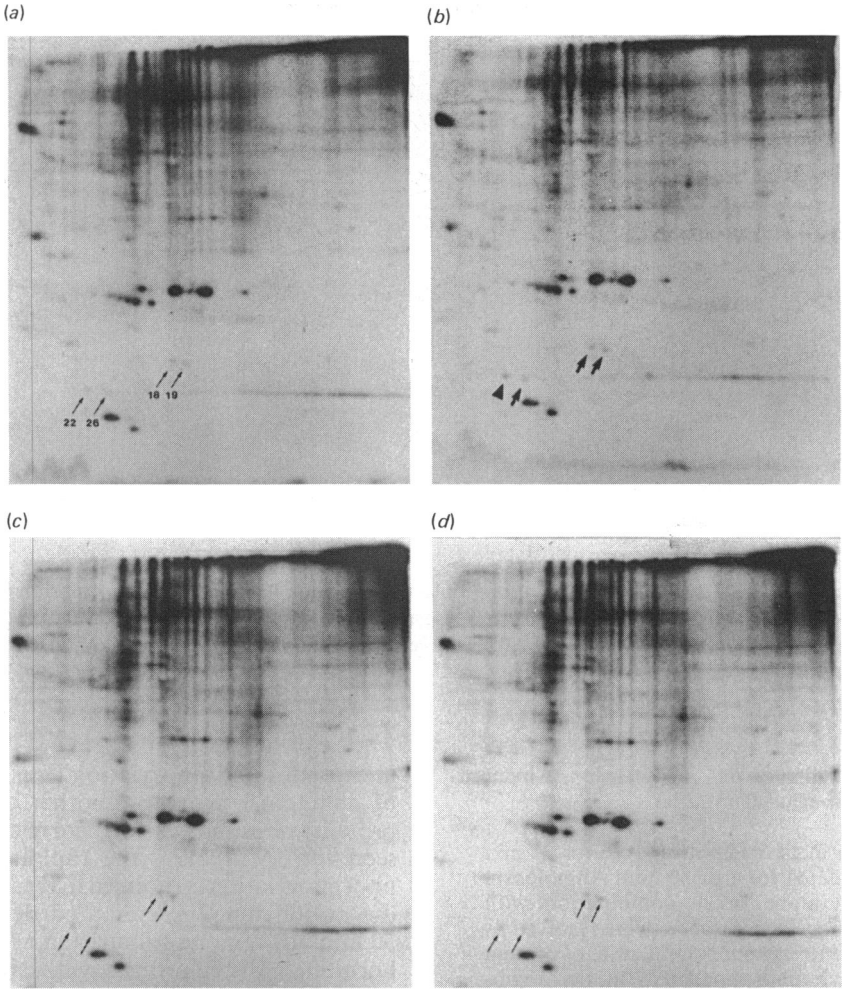

Fig. 7. Autoradiographs showing the effect of [Sar¹,Ala⁸]angiotensin II on angiotensin II-induced protein phosphorylation in adrenal glomerulosa cells

Cells were incubated with [^{32}P]P_i and treated with 10 nM-angiotensin II for 20 min. [Sar¹, Ala⁸]Angiotensin II (1 μM) was subsequently added for an additional 15 min. Cells were prepared for two-dimensional gel electrophoresis as described in the Materials and methods section. (*a*) 20 min control, (*b*) 20 min of angiotensin II, (*c*) 35 min control, (*d*) angiotensin II+[Sar¹,Ala⁸] angiotensin II. Short arrows identify putative kinase C substrates, and solid triangles identify common substrates.

quickly (Fig. 5*b*), and is paralleled by a decrease in extent of phosphorylation of the four late-phase proteins (Fig. 7 and Table 2).

The results shown in Figs. 5(*a*) and 6 and Table 3 are of particular interest. Based on the fact that nitrendipine blocks the angiotensin II-induced sustained increase in Ca^{2+} influx rate [23], and inhibits the sustained phase of the angiotensin II-induced aldosterone secretory response ([23] and Fig. 5*a*), we have concluded that a sustained increase in hormone-mediated calcium influx rate is a critical component of the mechanism by which the sustained phase of the angiotensin II-mediated aldosterone secretory rate is maintained. The data in Fig. 6 and Table 3 support this conclusion by demonstrating that nitrendipine prevents only the late phase of protein phosphorylation induced by angiotensin II.

With the present type of analysis, it is not possible to establish whether the increase in extent of phosphorylation of a specific protein is due to an activation of a specific kinase, and/or an inhibition of a phosphoprotein phosphatase. A resolution of this issue will require extensive further work. However, at the present time little is known about the regulation of phosphatase activity in the adrenal. On the other hand, if one assumes that these increases require at least an activation of a kinase, whether or not an inhibition of a phosphatase also operates, then the data obtained with A23187 and TPA help to define further the mechanism by which Ca^{2+} regulates aldosterone secretion. Based on previous data, we developed a model in which angiotensin II-induced aldosterone secretion is initiated by a transient rise in the intracellular free Ca^{2+} concentration leading to the presumed activation of calmodulin-dependent enzymes, including one or more protein kinases [17,32]. Indirect support for this view is provided by the evidence that A23187 induces a Ca^{2+}-dependent phasic secretory resonse (Fig. 1*b*). The fact that, when A23187 acts, five of the same six proteins whose phosphorylation increases in response to angiotensin II are phosphorylated early but transiently, fully supports this view. Further, we (K. Kojima, I. Kojima & H. Rasmussen, unpublished work) have demonstrated the presence of a multi-

functional calmodulin-dependent protein kinase in this tissue. Likewise, previous data suggested that the late phase of the aldosterone secretory response may be mediated by the Ca^{2+}-dependent regulation of an activated (Ca^{2+}-sensitive) form of protein kinase C: an enzyme also present in this tissue (K. Kojima, I. Kojima, P. Q. Barrett & H. Rasmussen, unpublished work). The fact that the pattern of protein phosphorylation seen 30 min after the combined addition of A23187 and TPA is identical with that seen after angiotensin II action provides support for this view (Fig. 4). In order to validate these concepts more completely, it will be necessary to isolate and more completely characterize both the calmodulin-dependent protein kinase and protein kinase C from this tissue, and in particular to determine their respective substrate specificities.

If one compares our studies in glomerulosa cells with those obtained by other investigators in other tissues, there is one striking difference. In GH_3 cells, hepatocytes and leukocytes, the addition of TPA alone has been reported to cause an immediate and sustained phosphorylation of a number of specific proteins [8,29,30,34,35]. However, in most other studies in which TPA was employed, large concentrations of this drug (160–1600 nM) have been employed [29,30,32,34,35]. In contrast, we carefully defined the minimally effective doses of this drug [16,17] and that of A23187 which would reproduce the pattern of cellular response induced by the natural agonist, angiotensin II (Fig. 1*d*). Our combined results from the present and previous studies [16,17] illustrate that when used in this way these drugs can serve as valuable probes for analysing the temporal sequence of cellular responses in the calcium messenger system.

The demonstration of different patterns of protein phosphorylation during the early and late phase of angiotensin II action provides new insights into the question of how trophic hormones produce not only immediate secretory responses in target cells, but also more long term trophic changes in steroid synthetic capacity and/or cell size and cell number. These data suggest that the flow of information from cell surface to cell interior is different in the early and late phases of hormone action.

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