Short term memory in the calcium messenger system

Evidence for a sustained activation of protein kinase C in adrenal glomerulosa cells

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If adrenal glomerulosa cells are treated with angiotensin II for a period of 20–30 min, their subsequent response to either a rechallenge with the same concentration of angiotensin II or treatment with BAY K 8644, a calcium channel agonist, differs from the responses of control cells. Perfusion of control cells with 10 nM-angiotensin II leads to an increase in aldosterone secretory rate from 44 ± 7 to 166 ± 9 pg/min per 10^6 cells, but perfusion of cells pretreated for a 20 min period with angiotensin II leads to an increase in secretory rate from 51 ± 9 to 209 ± 18 pg/min per 10^6 cells. Likewise, treatment of control cells with 10 nM-BAY K 8644 leads to no significant increase in aldosterone secretory rate, but treatment of previously exposed cells to angiotensin II leads to an increase in rate from 51 ± 9 to 130 ± 11 pg/min per 10^6 cells. This memory effect is time-dependent in two ways: (a) cells must be exposed to angiotensin II for 20 min or more before it is apparent; (b) the longer the time between removal of angiotensin II and the rechallenge, the less effect these agents have on aldosterone secretory rate. When cells are exposed to angiotensin II for 20 min and then treated with [Sar¹,Ala⁸]angiotensin II, a competitive antagonist of angiotensin II action, the aldosterone secretory rate falls to basal with a half time of 5–7 min. If BAY K 8644 is added simultaneously with [Sar¹,Ala⁸]angiotensin II, the secretory rate falls with a half-time of 35–60 min. BAY K 8644 increases Ca²⁺ influx rate to the same extent in the presence or absence of [Sar¹,Ala⁸]angiotensin II, and does not alter the effect of either angiotensin II or [Sar¹,Ala⁸]angiotensin II on the production of inositol tris-, bis-, or mono-phosphate. In cells treated with 10 nM-angiotensin II for either 20, 30 or 45 min, the extent of phosphorylation of four cellular proteins is increased. If cells treated for 20 min with angiotensin II are then treated with [Sar¹,Ala⁸]angiotensin II, and examined 15 min later (35 min), there is no longer an increase in the extent of phosphorylation of any of the four proteins. If such cells are then treated with 10 nM-BAY K 8644 and re-examined 5 min later (40 min), all four proteins show an increase in the extent of phosphorylation. Treatment of control cells with 10 nM-BAY K 8644 does not alter the extent of phosphorylation of any cellular proteins. These data indicate that inhibition of angiotensin II action leads to a rapid decrease in calcium influx rate, but a slower rate of relaxation of the C-kinase from its Ca²⁺-sensitive form back to its Ca²⁺-insensitive form.

INTRODUCTION

Angiotensin II stimulates aldosterone production from adrenal glomerulosa cells by a Ca²⁺-dependent mechanism [1–10]. The sustained phase of this response appears to depend upon both the amount of C-kinase which is present in its activated form, and the rate of Ca²⁺ influx across the plasma membrane [9,10]. As reported in the preceding paper [2], during the two phases of angiotensin II-mediated aldosterone secretion the patterns of cellular protein phosphorylation differ. In the first phase (1 min), six proteins become phosphorylated, and during the second phase (30 min) only four. Of these four only one is common to both phases. Further, an angiotensin II-induced increase in Ca²⁺ influx rate is required for a sustained secretory response [10] and for the phosphorylation of the four proteins characteristic of this phase of the response [2].

When either angiotensin II is removed from the perfusion medium, or an angiotensin II antagonist, [Sar¹,Ala⁸]angiotensin II, is added to angiotensin II-treated cells, the aldosterone secretory rate declines

rapidly [2] and is back to the basal rate within approximately 10–15 min. Protein phosphorylation also diminishes [2]. The present experiments were undertaken to determine whether this rapid decline in response is due to the rapid decrease in Ca²⁺ influx rate, and/or to the rapid reversion of the C-kinase from its activated to its non-activated form. Our data support the concept that the immediate fall in aldosterone secretory rate seen after cessation of angiotensin II action is due largely to a prompt decrease in plasma membrane Ca²⁺ influx rate [9], and that the C-kinase remains in an activated Ca²⁺-sensitive state for a significant period of time after hormone removal, but relaxes slowly back to its non-activated form with a half-life of approx. 35–60 min.

MATERIALS AND METHODS

Materials

Reagents used in this study were purchased from the following sources: angiotensin II and [Sar¹,Ala⁸]angiotensin II, Sigma; [³²P]P_i (carrier-free),

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; C-kinase, the calcium-activated, phospholipid-dependent, protein kinase; OAG, 1-oleoyl-2-acetyl-glycerol; AIIA, angiotensin II antagonist ([Sar¹,Ala⁸]angiotensin II).

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$^{45}\text{CaCl}_2$ and *myo*-[^3H]inositol, Amersham; BAY K 8644, Miles Institute for Preclinical Pharmacology; Dowex 1-X8, acrylamide, bisacrylamide, SDS, M_r markers and other gel chemicals, Bio-Rad; Ampholines, LKB; Nonidet P-40, Particle Data Laboratories.

Preparation of adrenal glomerulosa cells

Calf adrenal glands were obtained at a local slaughterhouse. Dispersed adrenal glomerulosa cells were prepared by the collagenase digestion of thinly sliced glomerulosa cell layers as previously described [1,8]. Cells were routinely suspended in Krebs–Ringer bicarbonate buffer containing 3.5 mM-potassium, 0.5 mM-calcium and 5.5 mM-glucose (unless otherwise indicated) and equilibrated with O_2/CO_2 (19:1). For measurement of calcium influx rates, ‘purified’ glomerulosa cells were prepared by use of Percoll density gradient centrifugation [11]. Contaminating red cells, fasciculate cells and cellular debris were removed by this method.

Perfusion of glomerulosa cells

Perfusion was performed using a four-channel flow-through chamber in a Perspex block [6]. Cells were perfused for 40 min with the standard aerated buffer at a flow rate of 0.4 ml/min before addition of a stimulator. Angiotensin II (10 nM), [Sar¹,Ala⁸]angiotensin II (1 μM) and/or BAY K 8644 (10 nM) were added to the perfusate at appropriate times. Angiotensin II and [Sar¹,Ala⁸]angiotensin II were dissolved in buffer and BAY K 8644 in ethanol. The final concentration of ethanol in the perfusate was no greater than 0.1%, which was without effect on either aldosterone secretion or calcium metabolism. Samples for aldosterone determination were collected every 4 min. Aldosterone was measured by radioimmunoassay.

Measurement of calcium influx

Calcium influx rate was measured according to the method of Mauger *et al.* [12] as previously described [9,10]. Statistical analysis of influx and aldosterone secretory data were carried out by using student's *t*-test.

Analysis of inositol phosphates

Cells ($5 \times 10^6/\text{ml}$) were incubated in Krebs–Ringer bicarbonate buffer for 120 min with 10 μCi of

[^3H]inositol/ml and 1×10^{-10} M-angiotensin II [1]. The hormone was added to stimulate incorporation of [^3H]inositol into the inositol phospholipids. For the last 10 min of the labelling period, 10^{-8} M-[Sar¹,Ala⁸]angiotensin II was added to the incubation medium to block angiotensin II action. After the period of incubation, cells were washed three times with Krebs–Ringer bicarbonate buffer containing 10 mM-inositol, and then suspended in Krebs–Ringer bicarbonate buffer containing 10 mM-inositol. Cells were then exposed for various periods of time to agonists. The reaction was terminated by addition of 1.5 ml of chloroform/methanol (1:2, v/v). The aqueous phase obtained from this extraction was dried under N_2 [1]. The samples were dissolved in 1 ml of 6.25 mM-disodium tetraborate and applied to 1 ml columns of Dowex 1-X8 (formate form). The column was washed with 7.5 ml of water to remove unincorporated [^3H]inositol. The labelled inositol phosphates were eluted sequentially by using 5 mM-disodium tetraborate/60 mM-sodium formate (for glycerophosphoinositol), 0.1 M-formic acid/0.2 M-ammonium formate (for inositol phosphate), 0.1 M-formic acid/0.4 M-ammonium formate (for inositol bisphosphate) and 0.1 M-formic acid/1.0 M-ammonium formate (for inositol trisphosphate) as described by Berridge *et al.* [13].

Cellular protein phosphorylation

Adrenal glomerulosa cells ($10^7/\text{ml}$) were incubated in a low-phosphate (0.1 mM) Krebs–Ringer bicarbonate buffer containing 250 μCi of [^{32}P]P_i/ml for 45 min [2]. Agonists were added from 1000-fold concentrates. The final agonist concentrations were angiotensin II (10 nM), [Sar¹,Ala⁸]angiotensin II (1 μM) and BAY K 8644 (10 nM). To terminate the incubation, cells were mixed with 500 μl of NaF stop buffer [14], centrifuged in a Microfuge (7 s), surface-washed with an additional 500 μl of stop buffer (10 mM-Hepes, 20 mM-sucrose, 100 mM-NaF, 15 mM-EDTA and 2 mM-EGTA, pH 7.4), centrifuged again, and then immediately 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% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 122 mM-

Table 1. Effect of angiotensin II antagonist with or without BAY K 8644 on angiotensin II-mediated calcium influx

Cells were incubated with various stimulators for the indicated time and calcium influx rate was measured in the presence of the stimulator as described in Methods. All cells, except the control and BAY K 8644-treated cells, were continuously exposed to angiotensin II, and then at appropriate times either AIIA alone, or AIIA followed by BAY K 8644, was added. Results are the mean $\pm$ S.E.M. for three or four experiments. Angiotensin II + AIIA: 10 nM-angiotensin II was added at 0 min and 1 μM -AIIA was added at 20 min; angiotensin II + AIIA + BAY K 8644: 10 nM-angiotensin II was added at 0 min, 1 μM -AIIA II was added at 20 min and 10 nM-BAY K 8644 was added at 35 min. The values marked * were obtained 40 min after angiotensin II, and 20 min after addition of AIIA without or with BAY K 8644 added 15 min after antagonist.

Stimulation	Ca ²⁺ influx (nmol/min per mg of protein) at:	
	5 min	40 min
Control	0.55 $\pm$ 0.08	0.60 $\pm$ 0.10
Angiotensin II (10 nM)	1.21 $\pm$ 0.19	1.28 $\pm$ 0.21
BAY K 8644 (10 nM)	0.98 $\pm$ 0.10	1.05 $\pm$ 0.13
Angiotensin II + AIIA	1.10 $\pm$ 0.14	0.52 $\pm$ 0.12*
Angiotensin II + AIIA + BAY K 8644	1.19 $\pm$ 0.15	1.02 $\pm$ 0.13*

Fig. 1. Effect of changes in the duration of the interval between initial angiotensin II stimulation and addition of agonist on the subsequent aldosterone response to angiotensin II or BAY K 8644

Glomerulosa cells were stimulated by 10 nM-angiotensin II (AII) from time 0 to 30 min. After 15 min (a), 30 min (b) or 45 min (c), cells were again stimulated by either 10 nM-angiotensin II (●) or 10 nM-BAY K 8644 (○). The dotted line indicates the relative response of control cells to 10 nM-BAY K 8644. Values are the means $\pm$ S.E.M. for three to six experiments using three different cell preparations.

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 [15], using bovine serum albumin as a standard, on an aliquot taken prior to dilution of the sample with the isoelectric-focusing buffer. Prior to loading, all samples within an experiment were adjusted to achieve equivalent protein concentrations (1–3 mg/ml); 40 μ g of protein was loaded on each gel.

The homogenate proteins were resolved by two-dimensional electrophoresis by a modification of the method of Garrison & Wagner [14] as described in the preceding paper [2]. Quantification of the autoradiographic exposures was performed as previously described [2]. 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 at each time of incubation.

RESULTS

Effect of angiotensin II and BAY K 8644 on aldosterone secretion

When 10 nM-BAY K 8644 is added to the perifusate of cells that have not been previously exposed to angiotensin II, there is a sustained, nearly 2-fold increase in the rate of Ca²⁺ uptake into adrenal glomerulosa cells (Table 1), but a barely detectable change in aldosterone secretory rate (Fig. 1a, dotted line). However, in cells previously exposed to 10 nM-angiotensin II for a 30 min period, and then washed free of angiotensin II, the addition of 10 nM-BAY K 8644 15 min later causes a significant

stimulation of the aldosterone secretory rate (Fig. 1a). When the time between angiotensin removal and BAY K 8644 addition is 15 min, then BAY K 8644 induces a prompt 2.8-fold increase in aldosterone secretory rate which is a rate approx. 65% of that induced by the initial dose of angiotensin II (10 nM) (Fig. 1a). When 30 min elapses between angiotensin II removal and BAY K 8644 addition, then BAY K 8644 induces a 1.9-fold increase in aldosterone secretory rate which is only 23% of the increment induced by the original addition of 10 nM-angiotensin II (Fig. 1b). If 45 min elapses between angiotensin II removal and BAY K 8644 addition, there is a brief, barely detectable rise in aldosterone secretory rate (Fig. 1c).

Also illustrated in Fig. 1 is the fact that readdition of 10 nM-angiotensin II 15 min later to cells previously exposed for 30 min to this same concentration of angiotensin II leads to a 5-fold increase in aldosterone secretory rate. This represents an increase of approx. 150% of the aldosterone secretory rate induced by the initial 10 nM dose of angiotensin II (Fig. 1a). However, if an interval of 30 or more min elapses between successive doses of 10 nM-angiotensin II, then the second dose of angiotensin II induces an aldosterone secretory response which is similar to that induced by the initial dose (Figs. 1b and 1c).

To be certain that the results are not explained by residual angiotensin II, a slightly different protocol was employed (Fig. 2). In these experiments, adrenal cells were perifused with 10 mM-angiotensin II for either 30 (Fig. 2a), 20 (Fig. 2b) or 10 (Fig. 2c) min, then, with angiotensin II still in the medium, the angiotensin II antagonist [Sar¹,Ala⁸]angiotensin II (1 μ M) was added, and 15 min later 10 nM-BAY K 8644 was added to the perifusate. The results shown in Fig. 2(a) are comparable

Fig. 2. Effect of changes in the duration of the initial period of stimulation by angiotensin II on the subsequent aldosterone secretory response to BAY K 8644

Glomerulosa cells were stimulated by 10 nM-angiotensin II (AII) from time 0. Stimulation was reversed by adding 1 μ M-AIIA at 30 min (a), 20 min (b) or 10 min (c). After a 15 min interval cells were again stimulated by 10 nM-BAY K 8644 (BAY K). Values are the means $\pm$ S.E.M. for three experiments using three different cell preparations.

Fig. 3. Effect of changes in the duration of the initial period of stimulation of angiotensin II on the subsequent aldosterone secretory response to BAY K 8644

Glomerulosa cells were stimulated by 10 nM-angiotensin II (AII) for 30 min (a), 20 min (b) or 10 min (c) from time 0. Cells were then perfused with Krebs-Ringer bicarbonate buffer containing 1 μ M-AIIA in the presence (●) or absence (○) of 10 nM-BAY K 8644 (BAY K). Values are the means $\pm$ S.E.M. for three experiments using three different cell preparations.

with those in Fig. 1(a). When the angiotensin II antagonist is added, the aldosterone secretory rate rapidly declines and in 15 min is close to its basal value. Addition of BAY K 8644 at this point leads to a rapid 3-fold increase in aldosterone secretory rate. Likewise, if the cells are perfused with angiotensin II for only 20 min before the antagonist is added, addition of 10 nM-BAY K 8644 15 min later leads to a 2.8-fold increase in aldosterone secretory rate (Fig. 2b). However, if the cells are exposed to angiotensin II for only 10 min before addition of antagonist, then the subsequent addition of 10 nM-BAY K 8644 leads to a brief and barely detectable rise in aldosterone secretory rate similar to that seen in cells not previously treated with hormone (Fig. 1a).

Using the angiotensin II antagonist, it was possible to add antagonist and BAY K 8644 simultaneously. As shown in Fig. 3, if cells are perfused with angiotensin II for either 30 (Fig. 3a) or 20 (Fig. 3b) min and then exposed to $1 \mu\text{M}$ -[Sar¹,Ala⁸]angiotensin II without or with the simultaneous addition of 10 nM-BAY K 8644, the subsequent aldosterone secretory patterns are quite different. Addition of antagonist alone leads to a prompt fall in the secretory rate to its basal value (Figs. 3a and 3b). The combined addition of antagonist and BAY K 8644 leads in both instances to a very slow decline in aldosterone secretory rate with a half time of approx. 60 min (compared with 7 min when BAY K 8644 is not present) (Figs. 3a and 3b). However, if cells are perfused with angiotensin II for only 10 min before addition of antagonist, then the aldosterone secretory rate declines rapidly upon addition of antagonist whether BAY K 8644 is present or absent.

In previous studies [10,16] we have demonstrated that raising the extracellular K⁺ concentration from 3.5 to 8 mM leads to a 4-fold increase in Ca²⁺ influx rate, and a sustained 3-fold increase in aldosterone secretory rate, but does not increase the turnover of inositol phosphates or phospholipids, and hence does not lead to the release of intracellular Ca²⁺ or the activation of C-kinase. Likewise, when cells are perfused with 8 mM-K⁺ for 30 min, and then switched to perfusate containing 3.5 mM-K⁺, the aldosterone secretory rate falls to basal within 10 min, and addition of 10 nM-BAY K 8644 to the perfusate does not cause a significant increase in aldosterone rate (results not shown). Likewise, addition of 10 nM-angiotensin II to K⁺-pretreated cells does not elicit any greater response than addition of the hormone to control cells.

Effects of BAY K 8644 and [Sar¹,Ala⁸]angiotensin II on calcium influx

Previous work had shown that addition of BAY K 8644 to cells not previously exposed to angiotensin II causes a nearly 2-fold, sustained increase in rate of Ca²⁺ influx [9]. Similar experiments were performed in the present studies in both control and previously treated cells. The effects of BAY K 8644 on the calcium influx rate under a variety of circumstances are summarized in Table 1. This calcium channel agonist has a similar effect on the rate of Ca²⁺ influx whether added to the cells not previously exposed to angiotensin II or to cells previously primed by angiotensin II. Therefore, the BAY K 8644-induced increase in aldosterone secretory rate (Figs. 1–3), cannot be accounted for by a difference in the effect of BAY K 8644 on Ca²⁺ influx rates in cells previously exposed to angiotensin II compared with

Fig. 4. Effect of angiotensin II or BAY K 8644 on the contents of [³H]inositol phosphates

Glomerulosa cells were prelabelled with [³H]inositol as described in the Materials and methods section. Either 10 nM-angiotensin II (●) or 10 nM-BAY K 8644 (○) was added at time 0. Values are the means $\pm$ S.E.M. for four to six experiments using three different cell preparations. (a) Inositol trisphosphate, (b) inositol bisphosphate, (c) inositol monophosphate.

those which have not previously been exposed. The data recorded in Table 1 also show that addition of [Sar¹,Ala⁸]angiotensin II causes a prompt inhibition of angiotensin II-mediated Ca²⁺ influx, but does not inhibit the effect of BAY K 8644 on Ca²⁺ influx rate.

Effects of BAY K 8644 and [Sar¹,Ala⁸]angiotensin II on angiotensin II-mediated increases in the production of inositol phosphate

It has been shown previously that angiotensin II induces a rapid and sustained increase in the concentrations of inositol trisphosphate, inositol bisphosphate, and with a slower time course, inositol phosphate in adrenal glomerulosa cells [1]. To ascertain whether BAY K 8644 has any effect on the production of inositol phosphates, two types of experiments were performed in the absence of lithium chloride: (a) a comparison was made of the effects of angiotensin II and BAY K 8644 on the initial rates of production of these phosphates, and (b) the effect of [Sar¹,Ala⁸]angiotensin II with or without BAY K 8644 on the concentration of inositol phosphates in angiotensin II-treated cells was examined.

As shown in Fig. 4, 10 nM-angiotensin II induces a rapid increase (within seconds) in the concentrations of

Fig. 5. Effect of [Sar¹,Ala⁸]angiotensin II with or without BAY K 8644 on [³H]inositol phosphates in angiotensin II-pretreated cells

Glomerulosa cells were prelabelled with [³H]inositol as described in the Materials and methods section. Cells were stimulated by 10 nM-angiotensin II from time 0 (●—●). In some cells, 1 µM-AIIA (●—●) or 1 µM-AIIA plus 10 nM-BAY K 8644 (○—○) was added at 20 min. Values are the means ± S.E.M. for four experiments using two different cell preparations. (a) Inositol trisphosphate, (b) inositol bisphosphate, (c) inositol monophosphate.

both inositol trisphosphate and bisphosphate, but BAY K 8644 has no such effect. When cells are exposed to angiotensin II for longer periods of time (20 min) in the absence of lithium, the levels of inositol tris- and bis-phosphate rise rapidly to a sustained plateau, and the level of inositol phosphate rises more slowly but also reaches a plateau after a 10 min exposure (Fig. 5). Addition of [Sar¹,Ala⁸]angiotensin II at 20 min leads to a prompt fall in the levels of both the tris- and bis-phosphates back to their control values, and a slower fall in the inositol phosphate level (Fig. 5). Addition of BAY K 8644 along with the angiotensin II antagonist does not alter the action of the antagonist on the metabolism of inositol phosphates (Fig. 5).

Effects of BAY K 8644 on protein phosphorylation

In a previous study we had shown that the patterns of protein phosphorylation seen at 1 and 30 min after angiotensin II differ substantially [2]. The phosphorylation of six proteins is increased at 1 min, and the phosphorylation of four proteins is increased at 30 min. One of these phosphoproteins is the same at 1 and 30 min. Five of the original six are no longer phosphorylated at 30 min, and three new proteins are phosphorylated at 30 min [2]. Based on a variety of data, it was concluded that the proteins phosphorylated at 1 min are likely to be substrates for calmodulin-dependent protein kinase(s), and those at 30 min substrates for protein kinase C. In an effort to define which kinase(s) is activated by the addition of BAY K 8644 under the circumstances illustrated in Figs. 1–3, we determined the change in pattern of protein phosphorylation induced by the addition of [Sar¹,Ala⁸]angiotensin II to cells exposed to angiotensin II for 20 min, and then treated 15 min later with or without BAY K 8644. The results are summarized in Fig. 6 and Table 2. The phosphorylation patterns of control (40 min) cells (Fig. 6a) were compared with those of cells exposed for either 20 (Table 2) or 40 (Fig. 6b) min to 10 nM-angiotensin II alone, with those of cells treated with 10 nM-BAY K 8644 alone

Fig. 6. Effect of BAY K 8644 on protein phosphorylation in control cells and cells exposed sequentially to angiotensin II and then [Sar¹,Ala⁸]angiotensin II

Glomerulosa cell phosphorylation was analysed as previously described [2]. The phosphorylation patterns seen in control cells incubated for 40 min (a) were compared to those seen in cells exposed to 10 nM-angiotensin II for 40 min (b) or in cells treated with 10 nM-angiotensin II for 20 min, then 1 µM-AIIA for 15 min, and 15 min later 10 nM-BAY K 8644 for 5 min (c).

Table 2. Agonist and antagonist-induced changes in protein phosphorylation in adrenal glomerulosa cells

Cells were incubated with [32 P]P_i for 45 min before being exposed to the agonist. Cells were then exposed to either 10 nM-BAY K 8644 at 35 min for a period of 5 min; 10 nM-angiotensin II for 20 or 40 min; angiotensin II for 20 min and then 1 μ M-AIIA for 15 min; or angiotensin II for 20 min, AIIA for 15 min and 10 nM-BAY K 8644 for 5 min. Homogenate proteins were resolved as described in [2]. The M_r of each spot was calculated from a standard curve as described. The effect of agonists on the protein phosphorylation of each phosphoprotein is presented below as fold over the equivalently timed control. The results are the summary of three experiments. *P* values refer to comparison of treated with control by using Student's paired *t*-test; n.s., not significant; *n* = 3 separate experiments.

Spot	$10^{-3} \times M_r$	Changes in phosphorylation (-fold over control) (mean $\pm$ s.e.m., frequency, <i>P</i>)				
		Angiotensin II (20 min)	Angiotensin II + AIIA (35 min)	Angio II + AIIA + BAY (40 min)	BAY (40 min)	Angiotensin II (40 min)
22	26	2.38 $\pm$ 0.16 (3/3) < 0.02	1.00 $\pm$ 0.17 (1/3) n.s.	2.22 $\pm$ 0.26 (3/3) < 0.05	0.65 $\pm$ 0.05 (0/3) < 0.05	3.12 $\pm$ 0.28 (3/3) < 0.02
18	30	2.09 $\pm$ 0.24 (3/3) < 0.05	0.96 $\pm$ 0.16 (1/3) n.s.	0.92 $\pm$ 0.46 (3/3) n.s.	0.77 $\pm$ 0.02 (0/3) < 0.01	1.92 $\pm$ 0.60 (3/3) n.s.
19	30	1.81 $\pm$ 0.08 (3/3) < 0.01	1.21 $\pm$ 0.24 (1/3) n.s.	1.71 $\pm$ 0.27 (3/3) n.s.	0.78 $\pm$ 0.16 (0/3) n.s.	1.71 $\pm$ 0.23 (3/3) n.s.
26	25	2.42 $\pm$ 0.24 (3/3) < 0.05	1.20 $\pm$ 0.43 (1/3) n.s.	1.50 $\pm$ 0.30 (3/3) < 0.02	0.76 $\pm$ 0.06 (0/3) n.s.	2.45 $\pm$ 0.16 (3/3) < 0.02

(Table 2) or with those of cells exposed to angiotensin II for 20 min followed by [Sar¹,Ala⁸]angiotensin II for 15 min and then either examined (Table 2), or exposed to 10 nM-BAY K 8644 for 5 min (Fig. 6c). Addition of BAY K 8644 alone led to no increase in extent of phosphorylation of any proteins (Table 2). The addition of [Sar¹,Ala⁸]angiotensin II to angiotensin II-treated cells led to a decrease in the extent of labelling of all four of the sustained phase proteins (Table 2) (see also [2]), but the addition of BAY K 8644 to pre-exposed cells led to the rephosphorylation of all four proteins (Fig. 6c) normally seen during the late phase of angiotensin II action (Fig. 6b and Table 2). There was no increase in the extent of phosphorylation of any of the five proteins that appear transiently during the initial phase of angiotensin II action [2].

DISCUSSION

The present results indicate that a critical period of prior exposure of adrenal glomerulosa cells to angiotensin II alters their subsequent aldosterone secretory response to a second exposure to angiotensin II (Fig. 1a) or to the calcium channel agonist, BAY K 8644 (Figs. 1–3). This alteration in response is time-dependent in two respects. First, the initial exposure to angiotensin II must be for a period of 20 min or more in order for the subsequent addition of BAY K 8644 to induce an aldosterone secretory response (Figs. 2 and 3). Second, once the first exposure of the cells to angiotensin II is terminated by removing angiotensin II (Fig. 1) or by adding an angiotensin II antagonist (Figs. 2 and 3), the magnitude of the subsequent aldosterone secretory response to a standard dose of BAY K 8644 decreases as a function of time. The half time for the decay of the aldosterone secretory response to angiotensin II upon addition of an angiotensin II antagonist is approx. 7 min in the absence of BAY K 8644 and 60 min in the presence of BAY K 8644 (Fig. 3a).

From previous work we proposed that the sustained phase of the aldosterone secretory response is determined by the C-kinase branch of the calcium messenger system [1,2]. During the sustained phase two factors appear to determine the magnitude of the aldosterone secretory rate [10]: (a) the amount of protein kinase C that is in an activated Ca²⁺-sensitive form, and (b) the rate of plasma membrane Ca²⁺ influx. Both appear to be increased by angiotensin II [9,10]. Thus, there are two possible explanations for the action of BAY K 8644 in angiotensin II-primed cells; either (a) the effect of the calcium channel agonist on calcium metabolism differs in cells not previously exposed to angiotensin II as compared with cells previously exposed to the hormone, or (b) the effect of the drug on calcium metabolism is the same under the two conditions, but some Ca²⁺-sensitive process or enzyme is still present after angiotensin II action ceases.

A variety of data support the concept that it is the latter of these possibilities. First, BAY K 8644 has the same effect on plasma membrane Ca²⁺ influx rate in normal and angiotensin II-primed cells (Table 1). Second, BAY K 8644 has no effect on the metabolism of inositol phosphates either before or after angiotensin II action (Figs. 4 and 5). Third, when BAY K 8644 alone is added to unprimed glomerulosa cells, it has no effect on aldosterone secretory rate or protein phosphorylation

even though it causes a sustained 2-fold increase in Ca^{2+} influx rate (Table 1). Likewise, if OAG is added to unprimed cells for 30 min, it has only a small effect on aldosterone secretory response. However, if BAY K 8644 and OAG are added together [10], then an aldosterone response is observed which is similar to that seen when BAY K 8644 is added to angiotensin II-primed cells (Figs. 1–3). In both circumstances (OAG addition or angiotensin II priming) BAY K 8644 appears to have a similar time course of action. Hence, it is logical to conclude that both have a similar explanation, i.e., the presence of some protein kinase C in its Ca^{2+} -sensitive form allows BAY K 8644 to activate this enzyme and hence aldosterone secretion. This conclusion is supported by the protein phosphorylation patterns seen in the present studies (Fig. 6 and Table 2). Addition of BAY K 8644 to non-primed cells causes no change in phosphorylation pattern (Table 2) just as it causes no change in aldosterone secretory response [9]. On the other hand, if angiotensin II-primed cells are treated with the angiotensin II antagonist, $[\text{Sar}^1, \text{Ala}^8]$ angiotensin II, there is decline in the state of phosphorylation of the four proteins whose phosphorylation is increased by a 20–40 min exposure to hormone. Addition of BAY K 8644 to such angiotensin II-primed, antagonist-treated cells reinitiates an aldosterone secretory response (Figs. 1–3), and re-induces the phosphorylation of the four proteins characteristic of the sustained phase of the angiotensin II-mediated aldosterone secretory response (Fig. 6). These proteins are presumed substrates for the C-kinase [2]. Noteworthy is the fact that the extent of phosphorylation of the unique proteins that appear immediately (1 min) after angiotensin II action [2] does not increase after BAY K 8644 addition to primed cells.

There are several possible explanations for the slow relaxation of the C-kinase enzyme from its Ca^{2+} -sensitive back to its Ca^{2+} -insensitive state. It is possible that the diacylglycerol produced in response to hormone-receptor-mediated hydrolysis of the phosphatidylinositols is slowly removed from the plasma membrane. However, until a more detailed analysis of the actual amount of appropriate species of diacylglycerols in the plasma membrane can be determined, it is difficult to test this possibility. Alternatively, it is possible that the C-kinase undergoes some type of covalent modification which stabilizes it in its Ca^{2+} -sensitive, plasma membrane-associated state. In this regard, it is of particular interest that exposure of adrenal glomerulosa cells to angiotensin II for a period of 20 min or more is required for the development of this memory effect (Figs. 2 and 3).

The present data provide additional support for a model of angiotensin II action in which the flow of information from cell surface to cell interior occurs via two branches of the calcium messenger system: a calmodulin branch largely responsible for the initial phase and a C-kinase branch for the sustained phase of the aldosterone secretory response [1–3]. Further, the data shown in Figs. 2 and 3 indicate that when angiotensin II action is inhibited, the prompt return of the aldosterone secretory response to its basal value is

due to a rapid decrease in hormone-mediated Ca^{2+} influx (see Fig. 7 in [9]), and not to a rapid relaxation of the C-kinase back to its Ca^{2+} -insensitive state. These data re-emphasize the importance of the angiotensin II-mediated increase in Ca^{2+} influx rate during the sustained phase of the aldosterone secretory response [9,10], and provide a further correlation [2] between changes in extent of protein phosphorylation (Fig. 6) and changes in aldosterone secretory rate (Fig. 2a).

The present experiments were undertaken because of observations made in tracheal smooth muscle [17]. This tissue also displays a short term memory to agonist exposure, and suggests that its basis is similar to that operating in adrenal cells. It is likely that this type of memory is a common behavioral feature of the C-kinase branch of the calcium messenger system.

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