Angiotensin II (AII) regulates the secretion of aldosterone from adrenal glomerulosa cells by a calcium-dependent mechanism which involves both the uptake of calcium from the extracellular pool, and the release of calcium from a dantrolene-sensitive intracellular pool. In the present study, it was shown that AII induces the rapid (10 s) hydrolysis of phosphatidylinositol 4-phosphate and -4,5-bisphosphate, leading to the sustained production of inositol bis- and trisphosphate (Ins-P_2, PtdIns-4-P, and diacylglycerol rich in arachidonic acid. Saponin-permeabilized glomerulosa cells accumulate calcium into a nonmitochondrial pool by an ATP-dependent manner. Ins-P_2 (0.5–5 μM) induces a release of Ca^{2+} from this pool. This release was blocked by dantrolene (10 μM). Adrenal glomerulosa cells were shown to contain the calcium-activated, phospholipid-dependent protein kinase (C-kinase). Perfusion of glomerulosa cells with combined 12-O-tetradecanoyl phorbol 13-acetate and A23187 induced an immediately developing, sustained, maximal secretory response similar to that induced by AII. These data are interpreted in terms of a model in which, after AII addition, there is a flow of information through two separate branches of the calcium messenger system, each with its unique temporal role: a calmodulin branch activated by the transient rise in the [Ca^{2+}] in the cell cytosol, which is largely responsible for the initial transient cellular response; and a C-kinase branch activated by the increase in both cytosolic [Ca^{2+}] and the diacylglycerol content of the plasma membrane, which is largely responsible for the sustained phase of cellular response. The temporal integration of these two phases underlies the observed pattern of cellular response.

AII is a major regulator of aldosterone secretion from adrenal glomerulosa cells (1). When AII acts, there is a prompt and sustained increase in aldosterone secretion which is maintained as long as AII is present (1). In eliciting this response, AII appears to act via the calcium messenger system: this hormone is effective only if extracellular calcium is present (2, 3); its effect is blocked by calcium channel blockers such as verapamil, D-600, La^{3+}, and dihydropyridines (3–5); AII stimulates both the efflux of calcium from a dantrolene-sensitive intracellular pool (6–8) and an uptake of calcium into the cell across the plasma membrane (3); and finally, addition of the AII-receptor agonist, A23187, can partially mimic the action of AII (3).

One of the most interesting results in other systems which employ Ca^{2+} as a messenger and display sustained responses to the sustained presence of an extracellular messenger, is that the rise in the concentration of free Ca^{2+} in the cell cytosol, [Ca^{2+}], is transient even though cellular response is sustained (9–17). Specifically, Morgan and Morgan (10) have reported that in another AII-responsive tissue, vascular smooth muscle, addition of hormone causes only a transient rise in intracellular free Ca^{2+} even though it induces a sustained contractile response.

We reported previously that calcium ionophore, A23187, and phorbol ester, TPA, together, but neither alone, induce aldosterone secretion qualitatively and quantitatively similar to that induced by maximally stimulatory concentrations of AII (15). Similar results were obtained for insulin secretion from perfused rat islets of Langerhans (19), and prolactin secretion from perfused clonal pituitary cells, GH_{3}, (2). Likewise, in vascular smooth muscle, TPA has been shown to induce a calcium-dependent sustained contraction which is reversed by addition of forskolin (20). From these data a model has been developed to account for the regulation of a sustained cellular response by the calcium messenger system (21). In this model, it is proposed that the flow of information from the cell surface to the cell interior takes place via two branches: a calmodulin branch that is activated by the transient rise in the Ca^{2+} concentration in the cell cytosol, [Ca^{2+}], and is largely responsible for the initial phase of cellular response; and a C-kinase branch that is regulated both by the transient rise in [Ca^{2+}], and an increase in the diacylglycerol content of the plasma membrane and is largely responsible for the sustained phase of cellular response. It is postulated that the C-kinase branch provides a type of gain control in the calcium messenger system (22). Recent studies of the early events in the calcium messenger system have provided evidence as to the nature of the events in signal transduction in the plasma membrane (23–30, 32, 33). Binding of extracellular messenger to its receptor is

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The abbreviations used are: AII, angiotensin II; [Ca^{2+}], calcium concentration in cytosol; PtdIns-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns-4-P, phosphatidylinositol 4-phosphate; Ins-P_2, myo-inositol 1,4,5-trisphosphate; Ins-P_1, myo-inositol 1,4-bisphosphate; Ins-P, myo-inositol 1-monophosphate; TPA, 12-O-tetradecanoyl phorbol 13-acetate; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Me_2SO, dimethyl sulfoxide; ATP, adenosine 5'-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, N-2-hydroxyethylpipеразine-N'-еthaneNsulfonic acid.

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1 The abbreviations used are: AII, angiotensin II; [Ca^{2+}], calcium concentration in cytosol; PtdIns-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns-4-P, phosphatidylinositol 4-phosphate; Ins-P_2, myo-inositol 1,4,5-trisphosphate; Ins-P_1, myo-inositol 1,4-bisphosphate; Ins-P, myo-inositol 1-monophosphate; TPA, 12-O-tetradecanoyl phorbol 13-acetate; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Me_2SO, dimethyl sulfoxide; ATP, adenosine 5'-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, N-2-hydroxyethylpipеразine-N'-еthaneNsulfonic acid.

2 D. Delbeke, I. Kojima, P. Dannies, and H. Rasmussen, manuscript submitted for publication.
thought to lead to the activation of a phospholipase C specific for PtdIns-4,5-P$_2$ and possibly PtdIns-4-P (23, 28–30, 32). Activation of this enzyme leads to the release of two or three products, diacylglycerol (24), and Ins-P$_3$ (32), and possibly Ins-P$_4$. Diacylglycerol is a known activator of C-kinase (24).

It increases the sensitivity of the enzyme to Ca$^{2+}$ such that it is active at Ca$^{2+}$ concentrations found in the resting cytosol. Recent studies in the pancreatic acinar cells (26) and isolated hepatocytes (27, 29, 33) show that Ins-P$_3$ is capable of mobilizing calcium from a nonmitochondrial intracellular pool. Thus, agonist-induced breakdown of PtdIns-4,5-P$_2$ by phospholipase C generates two intracellular messengers which activate the two branches of the calcium messenger system: one (Ins-P$_3$) by causing a rise in [Ca$^{2+}$], and the other, diacylglycerol, by altering the sensitivity of the C-kinase to calcium (22, 24).

In the present study we examined the early events in AII action in bovine adrenal glomerulosa cells. Answers to the following specific questions were sought: first, do A23187 and TPA mimic AII action on aldosterone secretion in bovine as they do in porcine cells; second, can oleoylacetoylglycerol substitute for TPA in activating aldosterone secretion; third, does A23187 cause Ins-P$_3$-independent hydrolysis of PtdIns-4,5-P$_2$ and/or PtdIns-4-P with a resultant generation of diacylglycerol and Ins-P$_3$ and Ins-P$_4$; fourth, does C-kinase exist in adrenal glomerulosa cells; and fifth, does Ins-P$_3$ induce release of calcium from a dantroline-sensitive intracellular pool.

Bovine rather than porcine cells were employed because previous studies had shown that bovine cells give a more consistent response of greater magnitude to AII addition than that seen with porcine cells (7, 8).

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Effect of A23187 and TPA on Aldosterone Secretion—As shown in Fig. 1A, perfusion of bovine adrenal glomerulosa cells with 1 X 10$^{-8}$ M AII leads to a sustained 5-fold increase in the rate of aldosterone secretion. The effect begins within 8 min (column dead space time, 2 min) and rises monotonically to a plateau at 20 min and remains at this rate for at least the next 40 min. If the cells are perfused with 0.5 $\mu$M A23187, the rate of aldosterone secretion increases within 8 min, rises to a peak at 20 min, which is 70% of the peak increment in the rate seen after AII action, but then declines so that the increment in rate seen after 60 min is only 35% of that seen when AII acts (Fig. 1B). When the cells are perfused with 10 $\mu$M TPA (Fig. 1C), there is no immediate increase in aldosterone secretory rate, but after 20 min, there is a very slow increase to a value at 60 min which represents an increment in rate of only 20% of that induced by AII. Perfusion with combined A23187 and TPA (Fig. 1D) leads to a stimulation in aldosterone secretory rate which is qualitatively and quantitatively similar to that produced by perfusion with AII (compare Fig. 1D with A). The only difference is that the peak response is achieved approximately 5 min later when A23187 and TPA were used more than additive.

These agents acted synergistically. These results are similar to those previously reported in glomerulosa cells from porcine gland (18). However, in the bovine glands, the magnitude of the responses to AII, A23187, and combined A23187 and TPA, are all greater than those seen in cells from porcine glands.

Effect of Oleoylacetoylglycerol and A23187—To further evaluate the role of C-kinase in aldosterone secretory response to AII, 1-oleoyl-2-acetoylglycerol was employed as a stimulator of C-kinase (39). When cells are perfused with 60 $\mu$M 1-oleoyl-2-acetoylglycerol, aldosterone production rate increases gradually after a 20-min lag (Fig. 2A). The increment in hormone production rate at 60 min is only 15% of that seen after AII addition (Fig. 1A). The stimulatory effect of this concentration of 1-oleoyl-2-acetoylglycerol is slightly smaller than that of 10 $\mu$M TPA (compare Figs. 1C and 2A). Perfusion with combined 1-oleoyl-2-acetoylglycerol and A23187 (0.5 $\mu$M) causes a rapid and sustained increase in aldosterone secretion (Fig. 2B) which is similar to that produced by AII. Thus, 1-oleoyl-2-acetoylglycerol and A23187 act synergistically, just as do A23187 and TPA.

Existence of Protein Kinase C in Cytosol of Adrenal Glomerulosa Tissue—To obtain further support for the notion that TPA, 1-oleoyl-2-acetoylglycerol and AII are activating C-kinase, the cytosolic content of this enzyme was determined. Adrenal glomerulosa slices produce aldosterone in response to AII while cortisol production is negligible (data not shown). Protein kinase activity in the cytosol was assayed by measuring $^{32}$P incorporation into histone HIIIS (Table 1). In the presence of no calcium and 1 mM EGTA, protein kinase activity was 73.4 $\pm$ 1.5 pmol/min/mg of protein. The presence of 4.8 X 10$^{-6}$ M calcium, protein kinase activity was 129.0 $\pm$ 9.2 pmol/min/mg of protein. Addition of phosphatidylserine (2.5 $\mu$g) in the presence of 4.8 X 10$^{-6}$ M calcium caused a 2-fold increase in kinase activity. Addition of diolein (0.2 $\mu$g) increased the activity further to 385.8 $\pm$ 4.3 pmol/min/mg of protein.

Effect of AII on the Turnover of $^{32}$P-labeled Phosphoinositides—The results shown in Figs. 1 and 2 and Table 1 lend further support to the concept that control of aldosterone secretion occurs via two branches of the calcium messenger system. A prediction of our model is that AII should stimulate...
the hydrolysis of polyphosphoinositides in this tissue. The effect of AII on the turnover of these compounds in adrenal cells was studied. After a 75-min period of incubation with [32P]PO4, the ratios of radioactivity incorporated into PtdIns-4,5-P2, PtdIns-4-P, and PtdIns were 6:5:5. Addition of 1 x 10^-9 M AII causes a rapid decrease in radioactivity from both PtdIns-4,5-P2 and PtdIns-4-P (Fig. 3). A loss of 45% of the [32P] radioactivity from PtdIns-4,5-P2 is detected at 10 s and the loss of radioactivity is maximal after 20 s of stimulation (55% of control). The initial loss of PtdIns-4,5-P2 is associated with a significant and equally rapid loss of PtdIns-4-P. The amount of radioactive phosphate in the PtdIns-4,5-P2 and PtdIns-4-P pools remains reduced for at least 120 s. In contrast, the radioactivity in the PtdIns pool falls only slightly, but not significantly, within this time frame.

To determine the dependency of AII-mediated breakdown of PtdIns-4,5-P2 and PtdIns-4-P on extracellular calcium, 32P-labeled cells were incubated in Kreb-Ringer bicarbonate buffer containing no calcium and 0.5 mM EGTA to that observed in the presence of extracellular calcium. A decrease in radioactivity from PtdIns-4,5-P2 labeled cells was studied. After a 75-min period of incubation with 32P as described under "Experimental Procedures." Results are expressed as per cent of unstimulated control values. At the start of the incubation, the radioactivity present in each phospholipid was PtdIns-4,5-P2, 4647 ± 424; PtdIns-4-P, 4513 ± 246; and PtdIns-4,5-P2, 5280 ± 524 cpm.

**Fig. 3.** The time course for angiotensin II-stimulated changes in [32P]-labeled phosphatidylinositol, phosphatidylly
ositol 4-phosphate, and phosphatidylglycerol in vitro with [32P] as described under "Experimental Procedures." A cell suspension containing 2 x 10^6 cells was incubated with 1 x 10^-8 M AII for each time point. Samples were taken at the indicated and radioactivity in each lipid fraction was measured as described under "Experimental Procedures." Results are the mean ± S.E. for six determinations from three different cell preparations. Values are expressed as per cent of unstimulated control values. At the start of the incubation, the radioactivity present in each phospholipid was PtdIns-4,5-P2, 4647 ± 424; PtdIns-4-P, 4513 ± 246; and PtdIns-4,5-P2, 5280 ± 524 cpm.

**Effect of AII on the Turnover of [3H]InsP-labeled Phosphoinositides—**Similar experiments were carried out in cells in which the various phosphoinositides were labeled with [3H]inositol rather than [32P]phosphate. When cells are incubated with [3H]inositol in the presence of 1 x 10^-9 M AII followed by receptor blockade with [Sar^1,Ala^8]AII, approximately twice as much radioactivity is incorporated into the phosphoinositides as compared to incubation with [3H]inositol alone. However, the proportionality between radioactive inositol phosphates and phosphoinositides is not affected (data not shown). The ratio of radioactivity incorporated into PtdIns-4,5-P2, PtdIns-4-P, and PtdIns after [3H]inositol labeling is 1:4:95. When [3H]inositol-labeled cells are stimulated with 1 x 10^-9 M AII in the presence of 50 mM LiCl, radioactivity in PtdIns-4,5-P2 decreases rapidly (Fig. 5). There is approximately a 30% decrease at 10 s and a 50% decrease at 20 s. This decrease in radioactivity from PtdIns-4,5-P2 is accompanied by a decrease in label in the PtdIns-4-P fraction with a small delay in time course. At 10 s, the decrease in PtdIns-4-P labeling is not significant, but by 20 s the radioactivity in this pool is only 50% of the control value. Radioactivity from both PtdIns-4,5-P2 and PtdIns-4-P remains decreased at 60 s. Label in PtdIns does not change significantly throughout the entire 60 s.

**Effect of AII on Inositol Phosphates Production—**In addition to measuring the rates of hydrolysis of phosphoinositides, the rate of appearance of the water-soluble products, inositol bisphosphate (Ins-P2) and inositol trisphosphate (Ins-P3), were determined. Production of water-soluble inositol phosphates was evaluated in [3H]inositol-labeled adrenal glomerulosa cells in the presence of 50 mM LiCl. In unstimulated cells the ratio of Ins-P2, Ins-P3, and Ins-P1 is 20:1:2. AII causes a rapid increase in [3H]Ins-P2 (Fig. 6). Radioactivity in Ins-P increases 2-fold at 10 s and 5-fold by 60 s. Ins-P2 continues to increase for at least 30 min. At 30 min, Ins-P showed a 7-fold increase. The increase in [3H]Ins-P3 is accompanied by an increase in [3H]Ins-P2. Radioactivity in Ins-P increases 3-fold at 10 s and 6-fold by 60 s. In contrast, Ins-P does not increase significantly beyond 10 min. Ins-P increases gradually. At 30 min Ins-P is 130% of control.

**Effect of AII on the Turnover of [3H]Arachidonic Acid-labeled Phosphoinositides and Diacylglycerol—**When cells are incubated with [3H]arachidonic acid for 2 h, the ratios of radioactivity incorporated into PtdIns-4,5-P2, PtdIns-4-P, and PtdIns are 1:4:95. When [3H]arachidonic acid-labeled cells are stimulated with 1 x 10^-9 M AII, radioactivity in the PtdIns-4,5-P2 pool decreases rapidly (Table 3). There is approximately a 30% decrease at 10 s and 40% at 20 s. At 60 s radioactivity in PtdIns-4,5-P2 is 80% of the control value. Label in the PtdIns-4-P pool also decreases rapidly. It is approximately 80% of control at 10 s and 70% at 20 s. It then recovers to 90% of control by 60 s. In contrast to [32P] and [3H]inositol labeling, the radioactive [3H]arachidonic in
FIG. 6. The time course of angiotensin II-stimulated changes in inositol monophosphate, inositol bisphosphate, and inositol trisphosphate. Calf adrenal glomerulosa cells were labeled in vitro with \(^{3}H\)inositol as described under "Experimental Procedures." A cell suspension containing \(4 \times 10^6\) cells was incubated with \(1 \times 10^{-5} \text{M AI1}\) in the presence of \(50 \text{mM LiCl}\) for each time point. Inositol phosphates were separated as described under "Experimental Procedures." The separate curves represent the time courses of change in Ins-P \(_2\) ( ), Ins-P \(_3\) ( ) and Ins-P \(_1\) ( ). Results are the mean \(\pm\) S.E. for four to six determinations from three separate cell preparations.

FIG. 7. The time course of angiotensin II-stimulated changes in \(^{3}H\)arachidonic acid-labeled diacylglycerol. Adrenal glomerulosa cells were labeled for 120 min with \(^{3}H\)arachidonic acid as described under "Experimental Procedures." A cell suspension containing \(2 \times 10^6\) cells was incubated with \(1 \times 10^{-5} \text{M AI1}\) for each time point ( ) and \(1 \times 10^{-5} \text{M [Sar\',Ala\']AI1}\) was added 6 min after AI1 to some incubation tubes ( ). Results are the mean \(\pm\) S.E. for four to six determinations from five different cell preparations. Values are expressed as counts/min/\(10^6\) cells.

\(\text{PtdIns}\) also decreases slightly but significantly \((p < 0.05)\) by 60 s, at which time it is 80.4\% of the control value.

The other product of polyphosphoinositide hydrolysis is diacylglycerol. Measurement of its production was made in cells prelabeled with \(^{3}H\)arachidonic acid. Glomerulosa cells were incubated with \(^{3}H\)arachidonic acid for 2 h. Treatment of \(^{3}H\)arachidonic acid-labeled cells with AI1 leads to a rapid increase in the amount of radioactively labeled diacylglycerol. As shown in Fig. 7, AI1 causes a biphasic change in \(^{3}H\)-labeled diacylglycerol. A peak is observed at 20 s, which is 3.5-fold greater than control. The amount of \(^{3}H\)diacylglycerol then decreases to a value which is 2-fold the basal value and stays there for at least 10 min. When AI1 action is blocked after 5 min by adding a competitive inhibitor, [Sar\',Ala\']AI1, the amount of \(^{3}H\)diacylglycerol decreases rapidly to a value at 10 min which is less than 50\% of the original control value, and less than 25\% of that seen in cells treated only with AI1.

Effect of Inositol Trisphosphate on Nonmitochondrial Calcium Pool—Saponin-permeabilized cells were incubated in cytosol-like medium containing 270 nM free calcium, oligomycin, rotenone, and an ATP-regenerating system. When ATP is added to the medium, saponin-premobilized cells accumulate radioactivity rapidly (Fig. 8) and radioactivity reaches a steady state within 5 min. Since the uptake is observed in the presence of oligomycin and rotenone, the ATP-dependent uptake of calcium is into a nonmitochondrial pool, presumably the endoplasmic reticulum. As shown in Fig. 8, addition of 5 \(\mu\)M Ins-P \(_2\) causes a rapid net release of radioactivity from this pool within 1 min. This effect of Ins-P \(_2\) on calcium release is dose-dependent (Fig. 9). The maximal effect is obtained with 2.5 \(\mu\)M Ins-P \(_2\). Addition of myo-inositol-2-monophosphate has no effect on calcium release from this pool (data not shown). When saponin-treated cells are incubated in a cytosol-like medium containing 5 \(\mu\)M dantrolene, the cells accumulate more radioactivity in response to ATP (Fig. 10B) than do control cells (Fig. 10A). In the presence of 5 \(\mu\)M dantrolene, however, the release of Ca in response to Ins-P \(_2\) is significantly smaller (Fig. 10B) than in control cells (Fig. 10A). In the presence of 10 \(\mu\)M dantrolene, the ATP-dependent uptake of calcium into saponin-treated cells is identical to that in control cells (Fig. 10, A and B). Under these conditions, the Ins-P \(_2\)-induced release of calcium is nearly totally inhibited (Fig. 10B). When saponin-treated cells are incubated with 25 \(\mu\)M dantrolene, the ATP-dependent uptake of calcium is less than that in control cells (Fig. 10, A and B) and Ins-P \(_2\) induced calcium release in a dose-dependent manner and at higher dose dantrolene appears to affect ATP-dependent calcium uptake into the pool.

**DISCUSSION**

Our working model of how the present and past data can be explained is depicted schematically in Fig. 11 (also see Ref.
21). When the natural agonist, AII, acts there is an increase in the production of both Ins-P3 and diacylglycerol. The rise in Ins-P3 is responsible for inducing the release of calcium from an intracellular pool (probably in the endoplasmic reticulum), which in addition to an AII-mediated increase in Ca\(^{2+}\) influx across the plasma membrane (by an undefined mechanism), causes a rise in [Ca\(^{2+}\)]. This leads to the activation of a number of calmodulin-dependent enzymes including one or more calmodulin-dependent protein kinases. As a consequence, cellular response (aldosterone secretion) is initiated. However, because calcium release is transient and because calmodulin activates the calcium pump in the plasma membrane (21), the [Ca\(^{2+}\)], falls (predicted, not yet measured) so that calmodulin-regulated events are not sustained. The rise in diacylglycerol along with the rise in [Ca\(^{2+}\)], leads to the activation of C-kinase. This enzyme in its activated state is considerably more sensitive to activation by Ca\(^{2+}\), so that when the [Ca\(^{2+}\)], falls back to near its original value, this enzyme remains activated. In this activated state, it is largely responsible for mediating the sustained phase of cellular response. One can bypass the receptor-mediated events by using a calcium ionophore A23187 to induce a rise in [Ca\(^{2+}\)], and a phorbol ester TPA (or synthetic diacylglycerol) (39, 40) to activate the C-kinase (Figs. 1 and 2). If these agents (A23187 and TPA or A23187 and 1-oleoyl-2-acetoylglycerol) are applied simultaneously to the tissue, a cellular response is induced which is similar in time course and magnitude to that induced by the natural agonist, AII.

The data shown in Figs. 3 and 5 and Table 3 indicate that whether the pools of phosphoinositides are prelabeled with \(^{32}P\)phosphate, \(^{3}H\)labeled inositol, or \(^{3}H\)-labeled arachidonic acid, addition of AII causes a rapid and marked fall in the contents of both PtdIns-4,5-P\(_2\) and PtdIns-4-P without causing any significant change in the PtdIns pool. The time course of change in both PtdIns-4,5-P\(_2\) and PtdIns-4-P are sufficiently similar that it is not possible to determine whether PtdIns-4,5-P\(_2\) hydrolysis is the primary event with PtdIns-4-P serving as a precursor pool to supply further PtdIns-4,5-P\(_2\), or whether the hydrolysis of both PtdIns-4,5-P\(_2\) and PtdIns-4-P is a primary consequence of hormone action.

Our results confirm and extend the recently reported data of Farese et al. (41) in rat adrenal glomerulosa cells. These workers reported that AII induces a decrease in the size of the PtdIns-4,5-P\(_2\) and PtdIns-4-P pools in rat adrenal capsules. However, the responses observed in the present studies at physiological relevant concentrations of AII are greater than those reported by Farese et al. (41) even though these workers employed higher doses of AII. Furthermore, these workers have stressed that AII increases the total "mass" of the various phosphoinositides (42). However, these are late effects of AII detected at 60 min which have little or no role in the acute action of AII, since AII-mediated calcium efflux is detected within 1 min after hormone addition (8).

One of the major questions in all studies of this type is whether isotopic equilibrium has been reached before hormone or agonist is added. The accumulated results reported from similar experiments in other tissues and cells are summarized in Table 4 and compared to the present results. As can be seen, the results obtained with \(^{32}P\)phosphate labeling and those with \(^{3}H\)inositol labeling differ considerably in the same tissue as they do in adrenal glomerulosa cells. Nonetheless, the relative distributions of either label in the various pools in adrenal glomerulosa cells fall in the same ranges as those reported for other cells. The most striking difference between the present data (Figs. 3 and 5) and those reported for other tissues (Table 4) is the fact that in adrenal glomerulosa cells AII causes a proportionally greater decrease in the size of both the PtdIns-4,5-P\(_2\) and PtdIns-4-P pools than has generally been reported for other hormonally activated systems.

The data shown in Fig. 4 and Table 2 indicate that the effect of AII on polyphosphoinositide breakdown is not dependent on extracellular Ca\(^{2+}\), and that simply raising intracellular [Ca\(^{2+}\)] with the ionophore A23187 is not sufficient to activate breakdown. These results are similar to those seen in a variety of other, but not all other, systems in which phosphoinositide turnover or breakdown has been linked to early events in the calcium messenger system (23). Results shown in Fig. 5 and Table 3 indicate that \(^{[3]H}\) arachidonic acid-labeled PtdIns disappears more rapidly than \(^{[3]H}\)inositol-labeled PtdIns. As shown in platelet (43); PtdIns-containing arachidonic acid may turnover more quickly. Alternatively, AII may cause the activation of phospholipase A\(_2\), which would lead to the direct release of arachidonic acid.

The data shown in Fig. 6 indicate that simultaneously with the breakdown of PtdIns-4,5-P\(_2\) and PtdIns-4-P, there is the appearance of the water-soluble inositol phosphates, Ins-P\(_2\), and Ins-P\(_3\). The rates of appearance of the water-soluble inositol phosphates are very rapid and nearly simultaneous, so it is not possible to determine whether Ins-P\(_3\) production leads secondarily to Ins-P\(_2\) production or whether these compounds result from the simultaneous hydrolysis of, respectively, PtdIns-4,5-P\(_2\) and PtdIns-4-P. In the presence of 50 mM Li\(^{+}\), the increases in Ins-P\(_3\) and Ins-P\(_2\) are not accompanied by an increase in Ins-P suggesting that 50 mM Li\(^{+}\) blocks phosphoinositid 4-monophosphatase and possibly 5-monophosphatase as well as phosphoinositol 1-monophospha-
In recent work both in isolated permeabilized pancreatic acinar cells and permeabilized hepatocytes, it has been reported that Ins-P$_3$, but not Ins-P$_2$, induces the release of Ca$^{2+}$ from an intracellular pool other than the mitochondria (26, 27, 29, 33). The suggestion has been made that this is the same pool that the natural agonists, acetylcholine and epinephrine, respectively, mobilize during their actions. It is known that when AII acts on adrenal glomerulosa cells, it induces a mobilization of Ca$^{2+}$ from a dantrolene-sensitive intracellular pool (8). Mobilization is detected within 1 min and is maximal 2–3 min after AII addition. This intracellular pool of calcium remains depleted as long as AII exists suggesting that the intracellular messenger which causes the release of calcium remains elevated. The results shown in Fig. 6 indicate that the production of Ins-P$_3$ after AII action is rapid (10–60 s) and persists for at least 30 min. The data shown in Figs. 8 and 9 indicate that Ins-P$_3$ mobilizes calcium from a nonmitochondrial pool, presumably the endoplasmic reticulum. The effect of Ins-P$_3$ is dose-dependent and E$_{D50}$ is 0.88 μM which is in good agreement with values reported in pancreatic acinar cells and hepatocytes (26, 27, 29, 33). As shown in Fig. 10, the Ins-P$_3$-induced release of calcium is inhibited by pretreatment with dantrolene, a muscle relaxant that inhibits calcium release from sarcoplasmic reticulum (46, 47). Since the AII-mediated mobilization of calcium from an intracellular pool is blocked by pretreatment with dantrolene in intact cells (8), the present data provide the first direct evidence that Ins-P$_3$ and a natural agonist, such as AII, are inducing the mobilization of calcium from the same intracellular pool.

The data shown in Fig. 7 indicate that diacylglycerol-containing arachidonic acid, a known activator of C-kinase (24), is also produced in response to AII addition in adrenal glomerulosa cells. The results shown in Table 1 indicate that calcium-activated, phospholipid-dependent protein kinase, C-kinase, exists in adrenal glomerulosa cytosol. The activity detected in glomerulosa cytosol is much higher than that found in whole adrenal tissue (48) or in fasiculata cytosol (49), but is about half of that found in spleen (48), one of the tissues richest in C-kinase.

One of the unresolved issues concerning the Ca$^{2+}$ messenger system is how a sustained response is achieved. It appears that [Ca$^{2+}$], rises only transiently in most cells that employ the calcium messenger system to couple stimulus to response and display a sustained response to the sustained presence of agonist. Once agonist is removed, the response decays rapidly. It is our postulate that the major intracellular pathway involved in the sustained phase of the response is the C-kinase pathway, and that the sensitivity of the C-kinase to activation by Ca$^{2+}$ has been enhanced sufficiently so that this enzyme operates efficiently at [Ca$^{2+}$] in the range of 0.1–0.3 μM. If this model is correct, it follows that hormone-receptor interaction provides some type of continuing message other than Ca$^{2+}$, to keep the C-kinase in its altered state of sensitivity. One possibility is that the diacylglycerol content remains high as long as the hormonal receptor is occupied. Another is that the diacylglycerol serves as a source for arachidonic acid, and a product of arachidonic acid metabolism provides a positive feedback effect. The data shown in Fig. 7 indicate that when AII acts, the amount of labeled diacylglycerol remains high for at least 10 min, and that it declines promptly when an AII antagonist is added to the tissue. Such data might be used to support the concept that continued high diacylglycerol concentrations is the means of maintaining the C-kinase in its Ca$^{2+}$-sensitive state. However, the data in Fig. 7 provide a measure of labeled diacylglycerol only, and do not provide a means of determining the actual diacylglycerol concentration in these cells. Until it is possible to measure actual amounts of the total diacylglycerol as a function of time after AII action, it is not possible to determine the messenger responsible for keeping the C-kinase in its sensitive state during the sustained phase of cellular response.

The model developed to account for results of the present studies is an extension of our previous model (21), and is similar in many respects to that recently presented by Niizukza (62). He has emphasized that calcium mobilization and C-kinase activation appear to act synergistically in the control of platelet and neutrophil function, but has not explicitly considered the importance of the temporal roles of these two inputs. In our model, a major emphasis is placed on the temporal roles of the two branches of the calcium messenger system in cells displaying sustained responses. Our present data plus that previously published (5, 8, 18–20) and discussed (21, 22) lead us to conclude that calcium mobilization and the subsequent activation of calmodulin-dependent cellular responses is a brief initial event that is responsible for the initiation of an integrated cellular response, and that C-kinase activation and the resulting phosphorylation of a specific subset of cellular proteins is responsible for modulating the sustained phase of cellular response. The activated C-kinase operates effectively at a [Ca$^{2+}$] similar to, or only slightly greater than, the [Ca$^{2+}$] found in the unstimulated cells. Hence, the C-kinase branch provides a type of gain control in the calcium messenger system.

It is our belief that the model developed to account for the manner in which AII regulates aldosterone secretion is not unique to this hormone or this tissue but, may be a general mechanism by which sustained cellular responses are achieved in cells which employ the calcium messenger system to couple stimulus to response and display a sustained response to the sustained presence of an extracellular messenger (18–23).

Acknowledgments—We thank Dr. R. Irvine of the ARC Institute of Animal Physiology, of Cambridge, United Kingdom for his generosity in supplying us with the inositol 1,4,5-trisphosphate used in the present studies, and Dr. Frank Ebetino of the Norwich Eaton Pharmaceutical of Norwich, NY for the dantrolene. We are grateful to Nancy Canetti and Ann Levine for their expert editorial and secretarial assistance.

REFERENCES
Experimental Procedures

Angiotensin II, 1-12, 1-8, aldosterone, 200 pmole/ml, and L-[3H]inositol (15.6 Ci/mole) were purchased from Amersham. [3H]GTP was a gift from Dr. F. E. Ebeling of Novichh London Pharmaceuticals, Inc., Norwich, VT.

Preparation of Adrenal Glomerulosa Cells

Calf adrenal glands were obtained at a local slaughterhouse. Thin slices containing glomerulosa layer were prepared as reported previously (35). Calf adrenal glomerulosa cells were prepared by collagenase digestion (36). Cells were suspended in Krebs-Ringer bicarbonate buffer containing 0.5% glucose (KRBG buffer) incubated with USP 25,000 IU/ml. bovine serum albumin was omitted except when cells were incubated with [3H]-inositol.

Labeling of Adrenal Glomerulosa Cells

Perfusion was performed as described previously (36) using four-channel flow-through chamber in a lactic block. Cells were perfused with KRBG buffer at 0.4 ml/min and 25°C. Adrenal in the perfusate was measured by radioimmunoassay using anti-adrenosterone antibody (antiserum was purchased as part of [125I]-collagen/cells [19,1g]), [3H]inositol and 2-(methyl) glycerol were dissolved in MeOH. [3H]inositol was less than 0.2%, and has no effect on aldosterone production.

Measurement of Protein Kinase C Activity in Cytosol

Adrenal glomerulosa slices were homogenized in 25 ml tri-chloroacetic acid (TCA) buffer (pH 7.5) containing 2.5 ml GTA buffer (2.5 M sucrose, 5 M morpholinoethanesulfonic acid) using glass tissue homogenizer. The homogenate was centrifuged at 30,000 rpm for 10 min. The supernatant was collected at 100,000 x g for 5 min in an air fuse (Beckman) and supernatant was designated cytosol. The cytosol fraction was assigned for protein kinase C activity by measuring the transfer of 32P from [32P]ATP to histone. All reactions were carried out in plastic tubes. The standard reaction mixture (32) contained 10 pmole of [32P]ATP (5 x 106 cpm), 1 pmole of acetyl-CoA, 40 pmole of histidine, 200 pmole of BSA, 2 mmole of MgCl2, and cytosol proteins. 

Concentration of calcium in the reaction mixture was adjusted by using calcium/EDTA buffer (32). Lipids were dissolved in 200 pmole of HEPES (0.75) by sonication for 5 min at 4°C. The reaction was initiated by adding 1.0-2.0 pmole of CaCl2 and terminated by adding 0.5 ml TCA. Acid precipitable material was collected on pellets of silica gel dried under a flow of nitrogen gas. After washing with six ml portions of TCA, the filters were dried and counted for radioactivity. Protein was measured by the method of Lowry et al. (19). 

Extraction of Lipids and Thin Layer Chromatography

For phosphoinositide measurement, incubation was done with stimulators as described in the figure legends. The reaction was terminated by adding 1 ml of chloroform/methanol (2:1, v/v). Lipids were extracted (35). The organic phase was dried under a flow of nitrogen gas. Phosphoinositides were separated by preparative thin layer electrophoresis using chloroform/methanol/acetone/acidic acid/water (40:15:13:12:8 v/v) as solvent (35). In this solvent system, the following phosphoinositides were observed: 0.25 (PS), 0.19 (PtdIns), 0.26 (PtdInsP2), 0.45 (PtdInsP3), 0.32 (PtdInsP4), and 0.65 (PtdInsP5) as radioactive phospholipids were visualized by autoradiography. When phosphoinositides were labeled with [3H]-inositol or [3H]-choline, phosphoinositides authentic (50 pmole), were added to the sample before applying to the thin layer plate and TCA precipitable was studied by autoradiography. The concentration of calcium in the reaction mixture was adjusted by using calcium/EDTA buffer (32). Lipids were dissolved in 200 pmole of HEPES (0.75) by sonication for 5 min at 4°C. After washing with six ml portions of TCA, the filters were dried and counted for radioactivity. Protein was measured by the method of Lowry et al. (19).

Reduction of Calcium Release from Saponin-Permeabilized Cells

A single experiment was done in triplicate. Incubation was done with stimulators as described in the figure legends. The reaction was terminated by adding 1 ml of chloroform/methanol (2:1, v/v). Lipids were extracted (35). The organic phase was dried under a flow of nitrogen gas. Phosphoinositides were separated by preparative thin layer electrophoresis using chloroform/methanol/acetone/acidic acid/water (40:15:13:12:8 v/v) as solvent (35). In this solvent system, the following phosphoinositides were observed: 0.25 (PS), 0.19 (PtdIns), 0.26 (PtdInsP2), 0.45 (PtdInsP3), 0.32 (PtdInsP4), and 0.65 (PtdInsP5). After washing with six ml portions of TCA, the filters were dried and counted for radioactivity. Protein was measured by the method of Lowry et al. (19).

Adrenal glomerulosa cells were labeled with 75 ml of [3H]inositol and 50% as described in Methods. Then cells were resuspended in KRBG buffer containing no calcium and 0.5 mM GTP for 5 min. Cell suspension containing 2 x 106 cells was incubated with 1 x 10-7 M A1 for indicated times. Results are the mean ± S.E. of four determinations from two separate cell preparations. Values are percent of stimulated control. At the start of the incubation, the radioactivity present in each phosphoinositide was 4.18±0.96 (PtdInsP5), 15.43±1.08 (PtdInsP4), 30.34±0.85 (PtdInsP3), 86.1±0.10 (PtdInsP2) and 99.8±0.64 (PtdInsP1) pmole/mg protein.

Table 1

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Adrenal glomerulosa cells were labeled for 60 min in KRBG buffer containing 50% [3H]inositol. A single experiment was done in triplicate. Incubation was done with stimulators as described in the figure legends. The reaction was terminated by adding 1 ml of chloroform/methanol (2:1, v/v). Lipids were extracted (35). The organic phase was dried under a flow of nitrogen gas. Phosphoinositides were separated by preparative thin layer electrophoresis using chloroform/methanol/acetone/acidic acid/water (40:15:13:12:8 v/v) as solvent (35). In this solvent system, the following phosphoinositides were observed: 0.25 (PS), 0.19 (PtdIns), 0.26 (PtdInsP2), 0.45 (PtdInsP3), 0.32 (PtdInsP4), and 0.65 (PtdInsP5). After washing with six ml portions of TCA, the filters were dried and counted for radioactivity. Protein was measured by the method of Lowry et al. (19).

Table 2

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Table 4

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Control of Aldosterone Secretion

Figure 4. The time course of A23187-stimulated changes in 32P-labeled phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate.

Cell adrenal glomerulosa cells were labeled for 75 min in vitro with 32P as described in Methods. A cell suspension containing 2 x 10^6 cells was incubated with 5 x 10^{-6} M A23187 for each time point. Results are the mean ± S.E. for six determinations from three different cell preparations. Values are expressed as percent of unstimulated control. At the start of incubation, the radioactivity in each phospholipid was PtdIns, 1309±242; PtdIns4P, 1620±248; and PtdIns4,5P2, 587±501 cpm.

Figure 5. The time course of angiotensin II-stimulated changes in 3H-labeled phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate.

Cell adrenal glomerulosa cells were labeled in vitro for 120 min with 3H-inositol as described in Methods. A cell suspension containing 4 x 10^6 cells was incubated with 3 x 10^{-7} M AII in the presence of 50 mM LiCl for each time point. Results are the mean ± S.E. for six determinations from three different cell preparations. Values are expressed as percent of unstimulated control. At the start of incubation, the radioactivity in each phospholipid was PtdIns, 1244±98; PtdIns4P, 579±28; and PtdIns4,5P2, 331±4 cpm.

Figure 3. Effect of 1-oleoyl-2-acetoyl-glycerol and A23187 on the rate of aldosterone secretion from bovine adrenal glomerulosa cells. 1-oleoyl-2-acetoyl-glycerol (A) or 1-oleoyl-2-acetoyl-glycerol plus 0.5 pg A23187 (B). Each value is the mean ± S.E. of three different experiments.
Control of Aldosterone Secretion

Figure 8: Effect of inositol 1,4,5-trisphosphate on the \(^{45}\text{Ca}\) content of saponin-permeabilized glomerulosa cells.

Saponin-treated adrenal glomerulosa cells were incubated at 37°C in cytosol-like medium containing 270 nM free calcium, oligomycin, rotenone, ATP regenerating system and \(^{45}\text{Ca}\) as described in Methods. The reaction was started by adding 2 mM ATP at time 0; 5 μM Ins-P\(_3\) was added at 20 min. Cells were incubated for the indicated time, and their \(^{45}\text{Ca}\) content was measured by a filtration method. Each value is the mean ± S.E. of four experiments.

Figure 9: The relationship between the amount of \(^{45}\text{Ca}\) released and the concentration of added inositol 1,4,5-trisphosphate.

The amount of released \(^{45}\text{Ca}\) in response to various doses of Ins-P\(_3\) was determined from experiments similar to that shown in Figure 8. Each value is the mean ± S.E. of 3 to 6 experiments from three different cell preparations.