Evidence for the Role of Phosphorylase Kinase, Protein Kinase C, and Other Ca^{2+}-sensitive Protein Kinases in the Response of Hepatocytes to Angiotensin II and Vasopressin*

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Angiotensin II, catecholamines, and vasopressin can stimulate the phosphorylation of 10 hepatic cytosolic proteins via a Ca^{2+}-linked, cyclic AMP-independent mechanism. To explore the role of known Ca^{2+}-sensitive protein kinases in this response, [³²P]P0₄³⁻-labeled hepatocytes were stimulated with various agonists, the cytoplasmic proteins were separated on two-dimensional gels, and the resulting autoradiographs were computer analyzed. The role of phosphorylase kinase was examined using hepatocytes from gsd/gsd rats which are deficient in this enzyme. The phosphorylation state of phosphorylase kinase was not increased by glucagon, angiotensin II, or vasopressin in hepatocytes from the gsd/gsd animals. The phosphorylation state of all other substrates was chromatography or the Ca^{2+}-linked hormones to the same extent in gsd/gsd hepatocytes as in normal Wistar controls, suggesting that phosphorylase kinase plays a restricted role in the hormone response. The role of the Ca^{2+}- and phospholipid-sensitive protein kinase (protein kinase C) was examined by stimulating hepatocytes with phorbol esters which are thought to activate protein kinase C by substituting for diacylglycerol. Phorbol esters increased the phosphorylation state of 3 of the 10 substrates affected by angiotensin II or vasopressin, but did not stimulate Ca^{2+} fluxes in hepatocytes. Treatment of hepatocytes with the Ca^{2+} ionophore A23187 mimicked the effect of the Ca^{2+}-linked hormones on the phosphorylation of the other 7 substrates. The results demonstrate that at least three Ca^{2+}-sensitive protein kinases are involved in the response of hepatocytes to Ca^{2+}-linked hormones. Since these kinases can be activated independently by phorbol esters or A23187, the results imply that hormones such as vasopressin generate two intracellular messengers, diacylglycerol and Ca^{2+} ion.

Recent studies have shown that glucose-mobilizing hormones can use two distinct pathways to activate carbohydrate metabolism in hepatocytes. One mechanism is used by glucagon which acts via adenylate cyclase to generate its intracellular messenger, cyclic AMP (1-3). In contrast, angiotensin II, vasopressin, and α₁-adrenergic agonists stimulate different membrane events including phosphatidylinositol turnover (4-9) and Ca^{2+} fluxes (1-3, 10, 11). Although these two types of hormones generate very different biochemical messengers, their net effects on carbohydrate metabolism are similar, with both stimuli leading to increased glycolysis (1-3) and gluconeogenesis (12-14). Moreover, both types of stimuli appear to control the activity of the important regulatory enzymes of carbohydrate metabolism by changing their phosphorylation state (15-17). These observations suggest that hepatocytes contain protein kinases responsive to a variety of intracellular messengers but that the substrate specificity of these enzymes overlaps to a significant extent (17, 18).

Evidence from a number of experimental approaches suggests that the effect of glucagon on the phosphorylation state of hepatic enzymes is mediated by the cyclic AMP-dependent protein kinase (16, 19-21). In contrast, the nature and role of the protein kinases that respond to a Ca^{2+}-linked hormone such as vasopressin are unknown. Although a number of possibilities exist, the relevant kinases have not been identified. Based on the literature, the candidates must include the Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) (22, 23) and Ca^{2+}-calmodulin-requiring kinases such as phosphorylase kinase (24) and glycogen synthase kinase (25, 26). Other enzymes, such as Ca^{2+}-regulated phosphatases or unknown Ca^{2+}-sensitive kinases, may also participate.

The purpose of the present study was to define the role of some of these enzymes in the response of hepatocytes to Ca^{2+}-linked hormones by studying protein phosphorylation in the intact cell. The role of phosphorylase kinase was examined using hepatocytes from gsd/gsd animals that are deficient in this enzyme (27). The role of protein kinase C was explored by stimulating cells with phorbol esters which have been shown to activate this kinase in intact platelets (28, 29). The role of other Ca^{2+}-sensitive phosphorylation events was examined by treating cells with the Ca^{2+} ionophore A23187. The results indicate that the full response to a Ca^{2+}-linked hormone requires the participation of phosphorylase kinase, protein kinase C, and at least one other Ca^{2+}-sensitive enzyme.

MATERIALS AND METHODS

Preparation of Hepatocytes, Incubation Procedures, and Sample Preparation—Isolated liver cells were prepared from 200-300-g, fasted male Wistar or gsd/gsd rats by published methods (30). For protein phosphorylation experiments, the hepatocytes were suspended in a low phosphate (0.1 mM) Krebs-Ringer bicarbonate buffer to a final concentration of about 60-80 mg of protein/ml and gassed with 95% O₂, 5% CO₂. The effects of hormones on protein phosphorylation in the intact cells were monitored by published methods (15-17). Five hundred ml of cell suspension were incubated with 24 mM L-lactate, 6 mM pyruvate, and about 0.4 ml of [³²P]PO₄ for 45 min and stimulated with hormones, phorbol esters, or A23187 for 1-4 min. Except for the dose response and time course experiments, all doses

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and stimulation times were chosen to give maximal responses (16). Glucagon was dissolved in 0.01 N NaOH containing 1 mg/ml of crystalline bovine serum albumin, phorbol esters and A23187 were dissolved in 100% dimethyl sulfoxide, and angiotensin II and vasopressin in 154 mM NaCl. All agents were added to the cells as 100- fold concentrated stocks. Incubations were terminated by lysing the cells with digitonin (11). Gel filtration of cytoplasmic proteins was resolved by two-dimensional gel electrophoresis as described (17). For the assay of Ca\textsuperscript{2+} -fluxes or phosphorylase activity, they were prepared as described above but reduced and separated at a concentration of 10-20 mg of protein/ml in a regular phosphate Krebs-Ringer bicarbonate buffer. The concentrations of \(l\)-lactate and pyruvate were reduced to 16 mM and 4 mM, respectively, in these experiments.

Two-dimensional Gel Electrophoresis, Autoradiography, and Computer Analysis—Cytoplasmic proteins were resolved by two-dimensional gel electrophoresis exactly as described (17). Gels were stained with Coomassie brilliant blue and dried on filter paper. Autoradiography was performed with Kodak X-Omat K (XK-1) film for 10-14 days. This double emulsion film proved superior to others (e.g. DuPont X-Omat) because it combines fine grain, 14 optical background (about 0.2 A at 550 nm) and a moderately fast response to \(^{32}P\). The use of slightly more \([^{32}P]\)PO\textsubscript{4} in the incubation mixtures raised the background of the gels and limited the resolution, but did not completely prevent the detection of low background profiles. The film was exposed to the gel for 1-3 days. When cells were used for the assay of Ca\textsuperscript{2+} -fluxes or phosphorylase activity, they were prepared as described above but reduced and separated at a concentration of 10-20 mg of protein/ml in a regular phosphate Krebs-Ringer bicarbonate buffer. The concentrations of \(l\)-lactate and pyruvate were reduced to 16 mM and 4 mM, respectively, in these experiments.

- **Assay of Calcium Fluxes**
  - Calcium fluxes were measured using \(^{45}Ca\) as a tracer by an adaptation of the procedures described by Blackmore et al. (10) and Barritt et al. (11). Freshly prepared hepatocytes were washed 3 times in 10 volumes of Ca\textsuperscript{2+}-free Krebs-Ringer bicarbonate buffer containing 1 mg/ml EGTA. Cells were resuspended at a concentration of 0.2-1 \(\times\) 10\textsuperscript{6} cells/ml and preincubated for 30-60 min with 1 \(\mu\)Ci/ml of \(^{45}Ca\), 16 mM \(l\)-lactate, and 4 mM pyruvate in a shaking water bath at 37°C in stopped tubes which were held at 95% \(O_2, CO_2\) in a humid environment. After preincubation, the cells were centrifuged at 50 \(\times\) g and resuspended in Krebs-Ringer bicarbonate buffer containing 2.5 mM \(Ca\textsuperscript{2+}\). Two hundred \(\mu\)l of the cell suspension were added to 5-m1 plastic tubes containing vehicle or varying concentrations of hormone. Cells were incubated at 37°C for 6-10 min under 95% \(O_2, 5% CO_2\), and the incubations were terminated by the addition of 5 ml of an ice-cold wash solution containing 154 mM NaCl, 2.3 mM LaCl\textsubscript{3}, 1% bovine serum albumin (Fraction V). The medium Ca\textsuperscript{2+} was separated from that in the cells by one of two methods. In some experiments, the cells were removed from the medium by centrifugation (13,000 \(\times\) g for 1 min). Equilibrium was reached by a layer of bromodecane (\(p = 1.04\)) such as described by Barritt et al. (11). The Ca\textsuperscript{2+} in the medium and in the cell pellet was counted in a liquid scintillation counter with an efficiency of about 35%. In other experiments, the contents of the tubes were immediately poured over a Whatman GF/A filter which had been prewashed in the wash solution containing NaCl, LaCl\textsubscript{3}, and albumin. Tubes were rinsed with an additional 5 ml of wash solution and the rinse was filtered. \(^{45}Ca\) on the filters was measured in a liquid scintillation counter with an efficiency of 30-35%. Each hormone concentration was paired with a separate control.

1 The abbreviations used are: Mes, 2-(N-morpholino)ethanesulfonic acid; 
2-N-morpholinoethanesulfonic acid; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether) \(\cdot\) \(N\text{, N'\text{, N''\text{, N''''}}\text{)}\) tetraacetic acid; pl, isoelectric point; PMA, 4\(\beta\)-phorbol-12-myristate-13-acetate.  

The two methods of separating the cells from the medium were carefully compared using different hormones and found to give identical results.

2 The filter method was used in most experiments because samples could be processed much more rapidly. Regardless of the method used to separate cells from medium, the Ca\textsuperscript{2+} flux results are expressed as the per cent of Ca\textsuperscript{2+} efflux caused by a given agonist as compared to the matched control.

3 Suppliers—The reagents used in this study were obtained from the following sources. Carrier-free \([^{32}P]\)PO\textsubscript{4} from \(^{32}P\) Nuclear (New England Nuclear); sodium dodecyl sulfate, acrylamide, bisacrylamide, Coomassie brilliant blue, and other gel chemicals, Bio-Rad; crystalline bovine albumin, Miles Laboratories (Elkhart, IN); bromodecane, Aldrich; albumsin (fraction V), collagenase (Lot 1029-6829), EDTA, \(l\)-lactate, EGTA, bromodecane, glucagon, arginine-vasopressin, phorbol esters, dimethyl sulfoxide, digitonin, caffeine, Sigma; Tc, Mes, pyruvate, A23187, Calbiochem; angiotensin II, Vega (Phoenix, AZ); Nonidet P-40, Partical Data Laboratories (Elmhurst, IL); amphotericin, LKB (Rockville, MD). All other chemicals were reagent grade.

**Results**

Expansion of the Autoradiograph Numbering System

The phosphorylated proteins in the cytosolic fraction of the hepatocytes were resolved on two-dimensional polyacrylamide gels and displayed on autoradiographs. In a previous report, the 37 darkest spots on the autoradiograph were assigned numbers and 6 of these proteins were identified as important regulatory enzymes in intermediary metabolism (see Fig. 4 and Table I of Ref. 17). Because of the technical improvements described under "Materials and Methods," two more spots were visualized on the autoradiographs. Moreover, the phosphorylation of each of these proteins is increased by Ca\textsuperscript{2+}-linked hormones such as vasopressin or angiotensin but not by glucagon (see Table I, below). In order to keep the original numbering system intact, these new spots were designated "a" and "b." The alphabetical designation of these and future spots will allow expansion of the spot list without confusion. The positions of spots a, b, and the 21 other spots analyzed in the present experiments are indicated in Fig. 2. The molecular weights and isoelectric points of the phosphorylated forms of a and b are: 1, 70,000, pl = 5.8; 2, 56,000, pl = 5.7.

**Role of Phosphorylase Kinase**

Hepatocytes Deficient in Phosphorylase Kinase—Previous work has shown that phosphorylase kinase in the gsd/gsd rat is inactive in a number of tissues, including the liver (27). As a result, neither glucagon nor the Ca\textsuperscript{2+}-linked hormones are able to stimulate phosphorylase activity in hepatocytes from these animals (27, 36). To ensure that the gsd/gsd animals used in this study were of the proper phenotype, phosphorylase activity was measured before and after treatment with glucagon or vasopressin. Basal phosphorylase activity in the gsd/gsd hepatocyte was 0.14 \(\mu\)mol of glucose/mg of protein/15 min, about 50% lower than basal activity in the Wistar rat. Treatment of the cells with 10\textsuperscript{-6} M glucagon or 24 nM vasopressin for 2 min yielded an activity of 0.16 \(\mu\)mol of glucose/mg of protein/15 min, an 18% increase in activity. Phosphorylase activity in hepatocytes from normal Wistar rats would increase 3-5-fold under these conditions (Table IV and 1,3, 16). These results agree with those of other inves-

C P. Campanile and J.C. Garrison, manuscript in preparation.
tigators (27, 36) and confirm that the animals used were of the proper phenotype.

**Molecular Basis of the Lesion**—A molecular explanation for the inability of hormones to activate phosphorylase in the gsd/gsd rat is shown in Fig. 1. Hepatocytes from Wistar or gsd/gsd animals were labeled to equal specific activities and treated with 10−7 M glucagon and equal amounts of cytoplasmic proteins loaded on two-dimensional gels. The position of phosphorylase in the gel system has been determined (17) and the figure shows the region of the gel containing the molecule. Phosphorylase is identified by the downward pointing arrow in the left-hand panels of the figure representing the stained proteins. The molecule has a Mr of 93,000 and focuses as a string of 5 spots over the pH range of 6.5–6.7. The reason for the multiple isoelectric points is unknown; however, crystalline muscle phosphorylase also has multiple isoelectric forms. The multiple isoelectric forms do not appear to be due to multiple phosphorylation of phosphorylase from either tissue (37). The right-hand side of Fig. 1 presents the corresponding autoradiographs. The phosphorylated forms of phosphorylase also focus as multiple isoelectric species. The protein (spot 4) is identified by the arrows pointing upward toward one major isoelectric form in each panel. One of the benchmark proteins, spot 10, whose phosphorylation state does not change with hormone treatment is also shown. These proteins are used to verify that the 32P specific activities, the amount of protein loaded on the gel, and the autoradiographic exposures are constant within an experiment (17).

Although approximately equal amounts of protein are visible in the stained gels from Wistar or gsd/gsd hepatocytes, there is a large difference in the amount of phosphate incorporated into phosphorylase in the two types of cells. Even in the basal state, the phosphorylase in the Wistar hepatocyte contains measurable amounts of phosphate and this is markedly increased by treatment of the cells with glucagon. In contrast, the phosphorylase molecule in the gsd/gsd hepatocyte contains very little phosphate in the basal state and its content increases only slightly (an average of 1.8-fold—see Table I, below) following treatment of the cells with glucagon. Moreover, neither angiotensin II nor vasopressin is able to stimulate the phosphorylation of phosphorylase in the gsd/gsd hepatocyte (see Table I, below). These results document that phosphorylase kinase does not respond to hormones in the gsd/gsd hepatocyte. Moreover, the observation that phosphorylase in the liver of gsd/gsd animals contains very little phosphate in both the basal and stimulated states provides a molecular explanation for the lack of activity observed in the standard phosphorylase assay (above text and Refs. 27 and 36).

**Role of Phosphorylase Kinase in the Response to Hormones**—Previous work has demonstrated that stimulation of hepatocytes with glucagon or a hormone such as vasopressin stimulates the phosphorylation of separate but overlapping sets of substrates (17, 18). In order to examine the role of phosphorylase kinase in the overall phosphorylation response, [32P]P04−-labeled hepatocytes from gsd/gsd animals were stimulated with glucagon, angiotensin II, or vasopressin and the cytoplasmic proteins were separated on two-dimensional polyacrylamide gels. The pattern of phosphorylated proteins observed on the autoradiographs did not differ from those of normal Wistar hepatocytes (data not shown). The integrated density information on the autoradiographs is presented in Table I along with matched controls performed with hepatocytes from Wistar rats.

Four major points are evident from the data. First, in agreement with previous results, the quantitative effects of angiotensin II and vasopressin were very similar, therefore the data were combined and presented in one column (17). Second, as noted in Fig. 1, none of the hormones significantly increased the phosphorylation of phosphorylase (spot 4) in hepatocytes prepared from gsd/gsd animals. Third, the phosphorylation state of three substrates unique to the Ca2+-linked hormones (a, b, and 29) is still increased in hepatocytes from the gsd/gsd rat. Finally, excluding phosphorylase, there is no statistical difference between the effects of glucagon or the Ca2+-linked hormones on the phosphorylation of the respective substrates in the hepatocytes from the two types of animals.

These results suggest that phosphorylase kinase plays a restricted role in the phosphorylation of hepatic proteins in response to glucagon or either of the Ca2+-linked hormones.

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**Fig. 1. A comparison of the phosphorylation state of phosphorylase in hepatocytes from Wistar and gsd/gsd rats.** A, cells were prepared from Wistar rats, labeled with [32P]P04−, and treated with vehicle (top) or 100 nM glucagon (bottom) for 4 min and the cytoplasmic proteins were resolved on two-dimensional gels. The left half of the figure presents a selected section of the Coomassie blue-stained gel and the right half the corresponding autoradiograph. In each section, the arrows point to the position of phosphorylase (see text). Spot 10 on the autoradiographs is one of the benchmark proteins (see Tables I and II). About 35 µg of protein was loaded on the gels. B, identical with A except that the hepatocytes were prepared from gsd/gsd rats. About 35 µg of protein was loaded on the gels.
Phosphorylase is clearly a substrate for the kinase but apparently none of the other phosphoproteins resolved in the gel system are affected by this enzyme. Therefore, other Ca²⁺-sensitive protein kinases must be involved in the hormone response.

Role of Protein Kinase C

Phorbol Esters and Protein Kinase C—In 1982, Castagna et al. (28) demonstrated that phorbol esters, such as PMA could directly activate protein kinase C purified from brain. The kinetic effects of phorbol esters on the enzyme appeared to be identical with those of a series of diacylglycerol compounds (38) and it was proposed that phorbol esters activated the kinase by replacing the requirement for diolein (28). In addition, recent work has shown that a putative phorbol ester-receptor competence for protein kinase C through two column steps (39). Most importantly, using platelets as an experimental system, it has been demonstrated by a number of criteria that phorbol esters cells activate protein kinase C in the intact cell (28, 29).

In light of these observations, phorbol esters were used to examine the role of protein kinase C in the response of hepatocytes to the Ca²⁺-linked hormones. Hepatocytes were labeled with [³²P]PO₄, stimulated with vasopressin or PMA and the cytoplasmic proteins were resolved on two-dimensional gels. Fig. 2 presents autoradiographs made from such an experiment. As noted in Table I, vasopressin changes the phosphorylation state of 10 proteins as compared to the control. The top right panel indicates the positions of these 10 proteins (labeled with arrows and arrowheads). The bottom left panel shows that addition of PMA to the cells stimulates the phosphorylation of only three of the proteins affected by vasopressin (a, b, and c, labeled with arrows) and does not affect the other seven proteins. Vasopressin’s effects on the phosphorylation state of these seven substrates can be mimicked by adding the Ca²⁺ ionophore A23187 to the cells (see below).

Dose Response and Time Course of the PMA Effects—Dose response and time course experiments were performed to establish the conditions required for maximal effects of phorbol esters. Initially, 1 μg/ml of PMA was added to the hepatocytes and cytoplasmic proteins were prepared after 0.5, 1, 2, 3, and 10 min of stimulation. Two-dimensional autoradiographs were computer-analyzed and the time course of the phosphorylation of spots a, b, and c was measured. Four benchmark proteins were also analyzed and did not change.

The time course of the phosphorylation of all three proteins was very similar. An observable increase (about 40% of maximal) was apparent after 30 s of stimulation and maximal effects occurred between 2.0 and 3.0 min of treatment. This time course is very similar to that seen in platelets (28). The phosphorylation of all three proteins was maintained through-

Ca²⁺-sensitive Protein Kinases in Hepatocytes

Table I

A comparison of the effect of glucagon and the Ca²⁺-linked hormones on the phosphorylation state of cytoplasmic proteins from Wistar and gsd/gsd rats

| Spot No. | Mr  | Identity               | Glucagon | Vasopressin and angiotensin combined |
|----------|-----|------------------------|----------|-------------------------------------|
| A. Phosphorylation state increased |
| 4        | 93,000 | Phosphorylase | 11.5 ± 3.4  | 1.8 ± 0.8 | 6.5 ± 1.0  | 1.1 ± 0.1 |
| 7        | 80,000 | Phosphofructokinase | 11.9 ± 1.1  | 6.0 ± 1.5 | —         | —         |
| a        | 70,600 | —                | 4.7 ± 0.5  | 5.3 ± 0.6 | —         | —         |
| 13       | 61,000 | Pyruvate kinase | 7.4 ± 0.7  | 6.9 ± 2.3 | 2.0 ± 0.1  | 2.6 ± 0.4 |
| b        | 56,000 | —                | 5.5 ± 0.9  | 5.5 ± 0.8 | —         | —         |
| 14       | 55,000 | Fructose-6-phosphate-2-kinase | 5.2 ± 1.0  | 18.0 ± 5.3 | —         | —         |
| 17       | 52,000 | Phenylalanine hydroxylase | 5.6 ± 0.9  | 5.4 ± 0.5 | 1.8 ± 0.1  | 1.6 ± 0.1 |
| 20       | 45,000 | —                | 1.6 ± 0.1  | 2.3 ± 0.3 | 1.7 ± 0.1  | 2.6 ± 0.3 |
| 21       | 45,000 | —                | 5.5 ± 0.3  | 4.1 ± 0.4 | —         | —         |
| 22       | 43,000 | —                | 11.3 ± 1.8 | 11.9 ± 5.3 | —         | —         |
| 23       | 49,000 | —                | 2.8 ± 0.6  | 3.0 ± 0.3 | —         | —         |
| 25       | 42,000 | Fructose-1,6-biphosphatase | 1.0 ± 0.1  | 2.0 ± 0.1 | —         | —         |
| 29       | 35,000 | —                | 0.4 ± 0.04 | 0.04 ± 0.06 | —         | —         |
| B. Benchmark proteins |
| 10       | 67,000 | —                | 1.06 ± 0.02 | 0.82 ± 0.12 | 0.99 ± 0.03 | 1.1 ± 0.04 |
| 24       | 42,000 | —                | 1.04 ± 0.03 | 0.98 ± 0.06 | 1.07 ± 0.02 | 0.93 ± 0.06 |
| 27       | 28,000 | —                | 0.99 ± 0.02 | 0.96 ± 0.07 | 0.98 ± 0.09 | 1.04 ± 0.05 |
| 30       | 29,000 | —                | 1.00 ± 0.05 | 0.97 ± 0.03 | 1.04 ± 0.05 | 1.01 ± 0.03 |
| 33       | 25,000 | —                | 1.00 ± 0.04 | 1.03 ± 0.03 | 1.00 ± 0.04 | 0.94 ± 0.06 |

* Not different from control (p > 0.2).

* Blanks in this column indicate that the identity of the protein is unknown.
Ca\(^{2+}\)-sensitive Protein Kinases in Hepatocytes

CONTROL

MW
93K
50K
18K

VASOPRESSIN

MW
93K
50K
18K

PMA

MW
93K
50K
18K

A23187

MW
93K
50K
18K

FIG. 2. Autoradiographs comparing the effects of vasopressin, PMA, and A23187 on the phosphorylation state of hepatic cytoplasmic proteins. Hepatocytes were labeled with \(^{32}P\)PO\(_4\) and stimulated with 70 nM vasopressin, 1 \(\mu\)g/ml of PMA, or 30 \(\mu\)M A23187 for 2-3 min, cytoplasmic proteins were prepared, and about 45 \(\mu\)g of protein was resolved on two-dimensional gels. The numbers and letters in the control and vasopressin panels identify the positions of the proteins selected for computer analysis (see Tables I–III). The numbers are to the upper right of the spot in each case. In some cases (spots 7, 14, 20–22, and 34), there is very little phosphorylated form of the protein in unstimulated cells and the spot is not easily visible. The proteins whose phosphorylation state is altered by vasopressin treatment are identified by arrows and arrowheads. The arrows represent the subset of proteins in which the vasopressin effects can be mimicked by PMA (lower left quadrant). The arrowheads represent the subset of proteins in which the vasopressin effects can be mimicked by A23187 (lower right quadrant).

Role of Kinases Sensitive to Ca\(^{2+}\) Influx

Effects of Ca\(^{2+}\) Ionophore—Previous results using one-dimensional gels have demonstrated that A23187 could stimulate the phosphorylation of a few proteins in hepatocytes (15). Moreover, known calmodulin-requiring protein kinases such as phosphorylase kinase (24) and myosin light chain kinase (43–45) are activated by treatments which elevate cytosolic Ca\(^{2+}\) levels (46–50). Therefore, in an attempt to investigate the role of putative calmodulin-requiring protein kinases in the hepatic response to vasopressin, hepatocytes were stimulated with the Ca\(^{2+}\) ionophore A23187. Care was taken to keep the time of exposure short (90–180 s) to minimize toxic effects of this compound on mitochondrial function.

The lower right section of Fig. 2 shows that treatment of the cells with 30 \(\mu\)M A23187 for 3 min changes the phosphorylation of 7 cytoplasmic proteins. These proteins are identified by arrowheads. Note that these 7 proteins comprise the rest of the 10 proteins affected by treating cells with vaso-
proteins (spots 4, 35, and 36) was similar with spot 34 lagging esters and the autoradiographs were computer-analyzed. As shown in Table II below, the effect of A23187 on three phosphoproteins (spots 13, 17, and 23) is small. Therefore, all subsequent experiments were performed with a dose of 20-30 \( \mu M \) A23187 for 2-3 min. One \( \mu M \) ionophore caused about a 30% response and maximal responses were observed following doses of 10-30 \( \mu M \). The response of three proteins (spots 4, 35, and 36) was similar with spot 34 lagging somewhat behind the others (\( n = 3 \)). In light of these results, all subsequent experiments were performed with a dose of 20-30 \( \mu M \) A23187 for 2-3 min.

Quantitative Phosphorylation Changes Following Treatment with A23187 and PMA—The data of Fig. 2 suggest that stimulation of hepatocytes with the combination of A23187 and PMA should mimic the response of the cell to vasopressin. To test this hypothesis, \([^{32}P]PO_4\)-labeled cells were stimulated with maximal doses of vasopressin, A23187, PMA, or A23187 + PMA and the autoradiographs were subjected to computer analysis. Table II presents the quantitative effects of vasopressin on the phosphorylation of 10 proteins identified in the upper right quadrant of Fig. 2. As anticipated, PMA mimics the effects of vasopressin on only 3 proteins (spots a, b, and 29) while A23187 mimics the effects on the other 7 (spots 4, 13, 17, 23, 34, 35, and 36). While A23187 may have caused a small increase in the phosphorylation of spot 29, the effects were of borderline significance (\( p \approx 0.05 \)). The far right column shows that the combination of ionophore and phorbol ester is able to quantitatively reproduce the effects of vasopressin on protein phosphorylation. This result suggests that stimulation of hepatocytes with a hormone such as angiotensin II or vasopressin activates at least two distinct events within the cell. Apparently, phorbol esters and calcium ionophores can be used as probes to activate the separate segments of the hormone response.

**Effects of Other Phorbol Esters**—The effects of phorbol esters other than PMA have been examined in a wide variety of assay systems with very consistent results. PMA is usually the most potent compound with 4\( \beta \)-phorbol-didecanoate, 4\( \beta \)-phorbol-dibenzoate, and 4\( \beta \)-phorbol-dibutyrate being about 10-20-fold less potent. 4\( \alpha \)-Phorbol-didecanoate and 4\( \beta \)-phorbol-13-monoacetate are usually inactive (41). The expected structure activity pattern was observed when these compounds were tested for their ability to activate purified protein kinase C (28). In order to support the hypothesis that phorbol esters were activating protein kinase C in hepatocytes, the effects of the six analogues on the phosphorylation state of spots a, b, and 29 were examined. Table III demonstrates that

### Table II

Quantitative effects of vasopressin, PMA, and A23187 on the phosphorylation state of 10 cytoplasmic proteins

Hepatocytes were labeled with \([^{32}P]PO_4\), stimulated with 24 \( nM \) vasopressin for 3 min, 1 \( \mu g/ml \) of PMA for 2 min, 30 \( \mu M \) A23187 for 3 min, or both PMA and ionophore for 3 min and the cytoplasmic proteins were resolved on two-dimensional gels. Autoradiographs were prepared and computer-analyzed as described under "Materials and Methods." The density information is presented below as -fold over control. All other details are as described in the legend to Table I. The values reported in A are significantly different from the control (\( p < 0.025 \)).

| Spot No. | Changes in phosphorylation (-fold over control) | Vasoressin \( (n = 6) \) | PMA \( (n = 7) \) | A23187 \( (n = 6) \) | A23187 + PMA \( (n = 3) \) |
|----------|-----------------------------------------------|--------------------------|----------------|--------------------------|--------------------------|
| A. Proteins whose phosphorylation is changed | | | | | |
| 4 | 6.3 ± 1.1 | 1.7 ± 0.0 | 6.2 ± 0.4 | 6.0 ± 0.8 |
| a | 7.4 ± 1.0 | 11.7 ± 2.0* | —a* | 8.8 ± 0.1 |
| b | 9.9 ± 0.8 | 6.8 ± 0.6 | —a* | 7.6 ± 0.5 |
| 13 | 2.0 ± 0.2 | —a | 2.0 ± 0.1 | 2.0 ± 0.1 |
| 17 | 1.9 ± 0.1 | —a* | 1.7 ± 0.1 | 1.7 ± 0.1 |
| 23 | 1.8 ± 0.1 | —a* | 1.7 ± 0.1 | 1.8 ± 0.1 |
| 29 | 9.2 ± 2.9 | 11.7 ± 3.0 | 1.7 ± 0.2 | 7.8 ± 3.8 |
| 34 | 13.2 ± 0.6 | —a | 12.8 ± 0.6 | 13.1 ± 5.4 |
| 35 | 2.0 ± 0.2 | —a | 1.8 ± 0.1 | 1.9 ± 0.1 |
| 36 | 0.4 ± 0.07 | —a | 0.5 ± 0.05 | 0.5 ± 0.03 |
| B. Benchmark proteins* | | | | | |
| 6 | 0.98 ± 0.06* | 0.94 ± 0.04 | 0.98 ± 0.06 | 0.93 ± 0.03 |
| 10, 11, 12, 14, 15, 16, 17, 21, 22, 24 | 0.90 ± 0.05 | 1.02 ± 0.05 | 1.07 ± 0.06 | 1.05 ± 0.09 |
| 24 | 1.03 ± 0.05 | 1.05 ± 0.05 | 1.03 ± 0.05 | 1.11 ± 0.08 |
| 30 | 1.04 ± 0.05 | 1.02 ± 0.05 | 0.98 ± 0.03 | 1.01 ± 0.09 |
| 31 | 0.98 ± 0.04 | 0.99 ± 0.03 | 0.94 ± 0.04 | 0.96 ± 0.09 |
| 33 | 0.95 ± 0.04 | 1.00 ± 0.04 | 1.02 ± 0.05 | 1.11 ± 0.07 |

*Not different from control (\( p > 0.2 \)).

*While spots a, b, 29, and 34 are minor proteins in the pattern, the agonists tested cause large changes in their phosphorylation state. This occurs because the proteins contain an undetectable amount of phosphate in control cells. Therefore, when the change is expressed as -fold/control, the small integral for the blank film from the control is divided into the much larger integral from the hormone-treated cells yielding a large estimate change. See Ref. 32 for a full discussion.

* A23187 caused a consistent but small stimulation of spot 29 that was of borderline significance (\( p \approx 0.05 \)) in this series of experiments.
were inactive at doses up to 10 μg/ml. These results are very similar to the structure-activity relationships observed for the activation of purified protein kinase C and support the hypothesis that proteins a, b, and 29 are substrates for this enzyme in the intact cell.

**Effect of Phorbol Esters on Ca²⁺ Fluxes**

*Effect on Phosphorylase—* A number of studies have shown that the phosphorylation state and activity of phosphorylase in hepatocytes closely follows the level of free Ca²⁺ in the cytosol (46, 47). Table II demonstrates that phorbol esters do not increase the phosphorylation state of phosphorylase (spot 4) in hepatocytes, suggesting that these compounds do not raise Ca²⁺ levels in liver cells. Since there are inconclusive reports on the effect of phorbol esters on Ca²⁺ levels in other types of cells (41), the effects of phorbol esters on Ca²⁺ fluxes were tested in hepatocytes. These experiments were performed indirectly, by assaying their effects on phosphorylase, and directly, by monitoring Ca²⁺ fluxes in intact cells. Table IV presents the effects of a series of phorbol esters on the activity of phosphorylase in hepatocytes. The controls in the top portion of the table show that vasopressin and A23187 provide the expected stimulation of phosphorylase activity. However, none of the phorbol esters increased phosphorylase activity. These results confirm the prediction made from the data in Table II.

**Effect on Ca²⁺ Fluxes—** When hepatocytes are completely equilibrated with ⁴⁶Ca²⁺ and stimulated with hormones such as angiotensin II or vasopressin, a Ca²⁺ efflux is observed over the following 6-15 min (10, 11). Fig. 3 presents the effect of various concentrations of vasopressin and PMA on Ca²⁺ fluxes in cells equilibrated with ⁴⁶Ca²⁺. As expected, vasopressin increased Ca²⁺ fluxes about 30% over the dose range of 0.24-24 nM. Ten nm angiotensin II produced similar effects and 1-30 μM A23187 caused an even larger stimulation of Ca²⁺ efflux (50-60% greater than the control rate, data not shown). In contrast, PMA did not cause a statistically significant efflux of Ca²⁺ over the dose range that caused maximal

### Table III

A structure-activity study of the effects of various phorbol esters on protein phosphorylation.

| Compound                      | Concentration (μg/ml) | Change in phosphorylation (fold over control) | Change in phospho-activity (n) |
|-------------------------------|-----------------------|---------------------------------------------|-----------------------------|
| A. Active phorbol esters      |                       |                                             |                             |
| PMA                           | 1 (7)                 | 11.7±6.8                                    | 11.7±6.8                    |
| 4β-Phorbol-12,13-didecanoate  | 1 (2)                 | 3.1±2.6                                     | 2.3±0.8                     |
| 4α-Phorbol-12,13-dibenzoate   | 10 (2)                | 12.6±4.0                                    | 4.2±0.8                     |
| 4β-Phorbol-12,13-dibutyrate   | 10 (2)                | 7.7±2.1                                     | 3.7±0.8                     |
| 4α-Phorbol-12,13-dibutyrine   | 10 (1)                | 16.5±3.0                                    | 4.5±0.8                     |
| 4β-Phorbol-12,13-didecanoate  | 20 (2)                | 11.6±7.6                                    | 6.1±0.8                     |
| B. Inactive phorbol esters    |                       |                                             |                             |
| 4α-Phorbol-12,13-dicanoate    | 1 (3)                 | 1.10±1.20                                   | 1.02±0.8                    |
| 4β-Phorbol-13-monoaceate      | 10 (2)                | 0.96±0.86                                   | 0.90±0.8                    |
| 4α-Phorbol-12,13-dibutyrine   | 10 (2)                | 1.00±0.75                                   | 0.98±0.8                    |

* Data taken from Table II.

### Table IV

The effect of vasopressin, A23187, and various phorbol esters on the activity of phosphorylase in hepatocytes.

| Agonist added | Concentration (μM) | phosphorylase activity (n) |
|--------------|--------------------|-----------------------------|
|              |                    | μmol/mg protein/15 min      |
| A. Controls  |                     |                             |
| None         | 24 μM              | 0.30±0.03 (9)               |
| Vasopressin  | 10 μM              | 0.67±0.05 (9)               |
| B. Active phorbol esters | |                             |
| PMA          | 1 μg/ml            | 0.24±0.02 (6)               |
| 4α-Phorbol-12,13-didecanoate | 1 μg/ml | 0.23±0.04 (4)               |
| 4β-Phorbol-12,13-dibenzoate   | 1 μg/ml | 0.31±0.06 (5)               |
| 4β-Phorbol-12,13-dibutyrate   | 1 μg/ml | 0.33±0.03 (3)               |
| 4α-Phorbol-12,13-dicanoate    | 10 μg/ml | 0.28±0.02 (5)               |
| 4β-Phorbol-13-monoaceate      | 10 μg/ml | 0.29±0.03 (3)               |
| 4α-Phorbol-12,13-dibutyrine   | 20 μg/ml | 0.30±0.02 (3)               |

* Significantly different from glucose alone (p < 0.005).
* None of the phorbol esters tested increased phosphorylase activity (p > 0.1).

PMA, 4β-phorbol-dicanoate, 4β-phorbol-dibutyrate, and 4β-phorbol-dibenzoate were about equally effective in stimulating the phosphorylation of the three proteins. However, 10-20-fold more of the latter three compounds was required. 4α-Phorbol-dicanoate and 4β-Phorbol-13-monoaceate
Ca$^{2+}$-sensitive Protein Kinases in Hepatocytes

increases in protein phosphorylation. This result is in complete agreement with the data of Tables II and IV and, taken together, the data provide strong evidence that phorbol esters do not elicit Ca$^{2+}$ fluxes in hepatocytes.

**DISCUSSION**

The ability of angiotensin II, vasopressin, and α$_1$-adrenergic agonists to stimulate hepatic carbohydrate metabolism through a Ca$^{2+}$-requiring, cyclic AMP-independent mechanism is well recognized (1-3, 51). In addition, previous work has shown that important regulatory enzymes in the glycolytic and gluconeogenic pathways can be controlled by these hormones via Ca$^{2+}$-dependent protein phosphorylation reactions (15-17). The object of this study was to explore the role of various Ca$^{2+}$-sensitive protein kinases in the response of hepatocytes to hormones such as angiotensin II or vasopressin. The data support the conclusion that these hormones activate a minimum of three Ca$^{2+}$-sensitive protein kinases: phosphorylase kinase, protein kinase C and at least one other kinase in the intact cell. This information helps to define the role of the particular protein kinase in the overall metabolic response of the cell.

Phosphorylase kinase is functionally inactive in the liver of the gsd/gsd rat (27, 36). Therefore, hepatocytes prepared from these animals provide an excellent experimental system to examine the role of this kinase in the response of the cell to glucagon or the Ca$^{2+}$-linked hormones. The data presented in Fig. 1 and Table I clearly show that phosphorylase (spot 4) is not phosphorylated in response to hormones in hepatocytes from the gsd/gsd animal. However, the potentially more interesting result is that phosphorylase is the only substrate observed for phosphorylase kinase in these experiments. Quantitation of the effects of glucagon, angiotensin, or vasopressin on 15 other cytoplasmic phosphoproteins in hepatocytes from Wistar or gsd/gsd animals provided no measurable differences between the response of these two cell types to hormones (Table I). These results suggest that phosphorylase kinase has a very restricted role in the biochemical response of the hepatocyte to a hormone such as glucagon or vasopressin. The data support and extend the observations of Clark et al. (52) showing that catecholamines still inactivate pyruvate kinase in hepatocytes from gsd/gsd animals. Moreover, they provide confirmation, in the intact cell, of the data obtained by Chrisman et al. (24) with purified hepatic phosphorylase kinase showing that the enzyme has a very restricted set of substrates in vitro. On the other hand, it must be cautioned that the data in this report cannot be used to argue that phosphorylase is the only substrate in the cell for phosphorylase kinase. Only cytoplasmic proteins were examined in these experiments and even within this set of proteins, certain important enzymes such as glycogen synthase and acetyl-CoA carboxylase do not enter the focusing dimension of the gel system (17). How many other potential substrates exist in other fractions of the cell or outside the M, and pI limits used in this work is unknown. However, since in vitro work with purified skeletal muscle phosphorylase kinase has shown that it can phosphorylate a number of substrates including glycogen synthase (53-55) and troponin (56), studies are planned to answer these important questions.

Protein kinase C is a Ca$^{2+}$-requiring kinase that has a wide distribution in mammalian tissues (57, 58). While the complete role of this kinase in the hormonal response of cells is unknown, it is noteworthy that this protein kinase can bind to membranes (39, 42) and is markedly stimulated by the first product of phosphatidylinositol breakdown, diacylglycerol (23, 38). Therefore, this enzyme is thought to participate in signal transduction for hormones that elicit the phosphatidylinositol response (59, 60). Since phorbol esters have been demonstrated to activate this protein kinase in the intact cell (28, 29), these compounds were used to explore the role of the enzyme in the response of hepatocytes to hormones such as angiotensin II or vasopressin. The data obtained yield five important conclusions. First, stimulation of the cells with a phorbol ester elicits only a part of the vasopressin response (5 of 10 proteins, substrates a, b, and 29). Second, all of these protein substrates are unique for Ca$^{2+}$-linked hormones; none of them are phosphorylated in response to glucagon (Table I). Third, spots a, b, and 29 are relatively low in concentration in the cell and are not visible in gels stained with Coomassie blue (they can be seen if the gels are stained with silver, data not shown). Fourth, as is obvious from Table I, these proteins do not represent any of the regulatory enzymes so far identified in the gel pattern. Finally, stimulation of the cells with Ca$^{2+}$ ionophores does not cause an increase in the phosphorylation state of these proteins (Table II).

Taken together, these results suggest that protein kinase C is responsible for a portion of the phosphorylation response observed following stimulation of the cells with a Ca$^{2+}$-linked hormone. However, the present data do not identify any role for this enzyme in the regulation of glycogen metabolism. Indeed, no effects of phorbol esters were observed on phosphorylase activity or on Ca$^{2+}$ fluxes in the cell (Table IV and Fig. 3). Although a yet undiscovered role in gluconeogenesis cannot be eliminated, no effect of phorbol esters was observed on phosphorylases known to regulate the gluconeogenic pathway (Tables I and II). These observations suggest that protein kinase C does not participate directly in the regulation of carbohydrate metabolism and therefore must be involved in some other aspect of hepatic function.

Another intriguing result is that Ca$^{2+}$ influx does not increase the phosphorylation of the putative protein kinase C substrates, suggesting that some other messenger activates this kinase. An obvious candidate would be a diacylglycerol released during the breakdown of phosphatidylinositol induced by angiotensin II or vasopressin. In this regard, it is encouraging that synthetically prepared 1-oleoyl-2-acetyl-glycerol, a diacylglycerol analogue that can cross the cell membrane (61), exactly mimics the effects of phorbol esters on the phosphorylation of spots a, b, and 29 in hepatocytes.4 This observation clearly supports the idea that angiotensin and vasopressin can activate protein kinase C via changes in diacylglycerol levels. Finally, it must be realized that while the results suggesting that hormones or phorbol esters activate protein kinase C in the intact cell are convincing, it has not been proven that this is the only effect of phorbol esters in cells. Indeed, a host of effects of these compounds have been described (41). Thus, additional proof that protein kinase C is involved in the phosphorylation of substrates a, b, and 29 in liver will have to await in vitro studies with purified kinases and substrates.

The majority of the cytosolic proteins (spots 13, 17, 23, 34, 35, 36) phosphorylated in response to angiotensin II and vasopressin are substrates for protein kinases other than phosphorylase kinase or protein kinase C. An important discovery is that the quantitative effects of hormones on these substrates can be mimicked by addition of the Ca$^{2+}$ ionophore

4 J. C. Garrison and C. P. Campanile, manuscript in preparation.
that one of the enzymes activated by this event is phosphorylase kinase, since this enzyme is known to be stimulated by Ca++ and calmodulin (24). A similar situation exists in platelets (61, 62) and smooth muscle (48, 50, 63) where A23187 or Ca++-linked hormones increase the phosphorylation of myosin light chain via another enzyme known to require Ca++ and calmodulin for full activity, myosin light chain kinase (43–45). Based on the above and consideration of the literature (64, 65), it is tempting to argue that the hepatic proteins phosphorylated in response to A23187 are substrates for Ca++-calmodulin-regulated kinases or phosphatases. While there is no direct experimental proof of this hypothesis, a number of known calmodulin-requiring enzymes may explain the observed results.

One known Ca++-calmodulin-requiring protein kinase in liver is glycogen synthase kinase (25, 26). When studied in vitro with purified substrates, this enzyme can phosphorylate glycogen synthase, smooth muscle myosin light chain, casein, and phosvitin. Whether pyruvate kinase (spot 13), phenylalanine hydroxylase (spot 17), and other cytosolic proteins are substrates for this enzyme has not been reported. Another consideration is that, to date, this enzyme has only been studied in rabbit liver (25, 26) and information about the rat liver enzyme is lacking. Therefore, conclusions about the role of this protein kinase in the response of rat hepatocytes to angiotensin or vasopressin must await further studies. Another interesting discovery is that calciurein appears to be a Ca++-calmodulin-stimulated phosphatase (66). This finding is intriguing because hormones and A23187 decrease the phosphorylation of one protein, spot 36 (Tables I and II). If a similar protein phosphatase exists in liver, this enzyme may participate in the response to hormones along with the protein kinases discussed above. While the nature of all the enzymes mediating the response of the cell to a Ca++ influx is not known, it is clear at least that some of the substrates affected by these enzymes are involved in regulating carbohydrate metabolism. For example, phosphorylase (spot 4) and pyruvate kinase (spot 13), proteins important in controlling glycogenolysis and gluconeogenesis, are both substrates for the Ca++-calmodulin-requiring enzymes. This finding is consistent with the observation that the effects of angiotensin II or vasopressin on the activity of these enzymes require Ca++ ion in the bathing medium (1–3, 16). Further work with purified enzymes will be necessary to fully understand the nature of all the Ca++-sensitive enzymes involved in this segment of the hormone response.

Perhaps the most interesting implication of the data obtained in this study is that binding of a hormone such as vasopressin to a cell membrane appears to elicit responses within the cell via two different messengers, diacylglycerol and Ca++ ion. Each of these signals can activate distinct protein kinases which phosphorylate only a portion of the substrates affected by the hormone itself. Apparently, phorbol esters can activate one segment of this response by mimicking the effect of diacylglycerol on protein kinase C. One potentially important conclusion from this line of reasoning is that activation of protein kinase C does not appear to initiate Ca++ fluxes in the hepatocyte (Fig. 2, 3, and Table IV). This observation may help explain the apparent lack of correlation between phosphatidylinositol metabolism and phosphorylase activation observed in some studies (67–69). A23187 appears to activate the other portion of the hormone response by activating at least one Ca++-calmodulin-requiring enzyme (phosphorylase kinase) and perhaps other calmodulin-regulated enzymes acting on their respective substrates. Recent evidence suggests that these biochemical mechanisms may exist in a number of cell types and are capable of regulating a variety of intracellular events. Extensive data have been accumulated in platelets where thrombin appears to stimulate protein kinase C via diacylglycerol and myosin light chain kinase via Ca++ ion (61, 62). In addition, thyrotropin-releasing hormone appears to regulate protein phosphorylation and prolactin release in GH3 cells through very similar mechanisms (70, 71). Clearly, the cellular responses to hormones such as angiotensin II, thrombin, thyrotropin-releasing hormone, or vasopressin are complex and new insights into the role of protein phosphorylation in cell function will be gained by further study of these systems.

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