Time Course of \( \alpha_1 \)-Adrenergic and Vasopressin Actions on Phosphorylase Activation, Calcium Efflux, Pyridine Nucleotide Reduction, and Respiration in Hepatocytes*

Peter F. Blackmore‡, Bernard P. Hughes, Robert Charest, Edwin A. Shuman, IV, and John H. Exton§

From the Laboratories for the Studies of Metabolic Disorders, Howard Hughes Medical Institute and Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

(Received for publication, August 6, 1982)

Calcium efflux from rat liver perfused with nonrecirculating medium was observed at 1.4 s following \( 10^{-5} \) M (-)-epinephrine infusion, when the perfusate Ca\(^{2+} \) was 60 \( \mu \)M. Net calcium efflux was also seen in livers perfused with 1.3 mM Ca\(^{2+} \) at approximately 8 s. In isolated rat hepatocytes, phosphorylation, a cytosolic enzyme, was activated significantly at 3 s and maximally at approximately 15 s by phenylephrine \( (10^{-6} \) M), epinephrine \( (10^{-6} \) M), and vasopressin \( (10^{-8} \) M). Hexose phosphates were elevated at between 3 and 6 s with vasopressin.

Phenylephrine and vasopressin stimulated hepatocyte respiration relatively slowly. The effects took 10 s to become evident, were dependent on the presence of Ca\(^{2+} \), and were probably the result of increased total cellular reduced pyridine nucleotide observed at 5 s. The slowness of the increase in respiration indicates that it cannot be the cause of the Ca\(^{2+} \) mobilization, but is more likely to be a consequence of it.

From these studies, it is proposed that, following binding of catecholamines to \( \alpha_1 \)-adrenergic receptors, Ca\(^{2+} \) is first mobilized from the plasma membrane resulting in an elevation of the free Ca\(^{2+} \) ion concentration in the cytosol \( (\text{Charest, R., Blackmore, P. F., Berthon, B., and Exton, J. H. (1983) J. Biol. Chem. 258, 8769-8773}) \) which stimulates phosphorylase kinase and, hence, phosphorylase. These events begin to occur within the first 2 to 3 s. Following this, the concentration of reduced pyridine nucleotide(s) increases at 5 s resulting in the stimulation of respiration seen at 10 s. These events occur more slowly than the mobilization of cell Ca\(^{2+} \) and activation of phosphorylase, and may be secondary to the rise in cytosolic Ca\(^{2+} \). The time at which mitochondrial Ca\(^{2+} \) decreases is not known, but it accounts for most of the Ca\(^{2+} \) mobilized. Evidence that net cellular Ca\(^{2+} \) influx is not a primary event and that internal stores of Ca\(^{2+} \) are mobilized \( (1, 2) \). Many studies have shown that mitochondrial Ca\(^{2+} \) content is decreased following \( \alpha_1 \)-adrenergic stimulation \( (3-11) \), while some studies show the opposite \( (12-14) \). The studies of Berthon et al. \( (10) \) using female rats have indicated that perfusion of livers with 5 \( \mu \)M (-)-norepinephrine in the presence of 5 \( \mu \)M (2)-propranolol causes a rapid \( (30 \) s), small \( (21 \pm 10\%) \), but transient increase in mitochondrial Ca\(^{2+} \) followed by a large decrease. These results imply that the first pool of Ca\(^{2+} \) to be mobilized is not localized to mitochondria but to either the plasma membrane and/or endoplasmic reticulum.

Our earlier studies on the effects of \( \alpha \)-adrenergic agonists, vasopressin, and angiotensin II on mitochondrial Ca\(^{2+} \) content in male rats were performed at times ranging from 1 to 6 min of stimulation \( (3-6) \). Any early transient mitochondrial Ca\(^{2+} \) influx \( (10) \) would not have been seen in these studies, especially since perfusate Ca\(^{2+} \) was lowered to approximately 50 \( \mu \)M (total) to facilitate the measurement of Ca\(^{2+} \) efflux. We showed that the effects of \( \alpha \)-agonists, vasopressin, and CCCP\(^{1} \) on phosphorylase activation could be seen at 2 s or less, while near maximal effects were seen at 15 s \( (1) \). Also, Ca\(^{2+} \) mobilization in perfused liver and isolated hepatocytes could be seen at less than 10 s \( (1) \).

It was the purpose of the present study to examine the time course of activation of phosphorylase, an enzyme in the cytosolic compartment, and of changes in Ca\(^{2+} \) fluxes, pyridine nucleotides, and respiration. The effects of various mitochondrial inhibitors on glycogenolysis and glycolysis were also examined.

The findings dispute several conclusions of a recent publication \( (15) \) in which it is claimed that there is a "tight association" between agonist-induced glucose output and oxygen uptake in the perfused rat liver.

**EXPERIMENTAL PROCEDURES**

*Rat Liver Perfusion and Isolation of Hepatocytes—Livers from 200-220-g fed male rats were perfused as previously described \( (4) \). Isolated liver parenchymal cells were prepared and incubated as described elsewhere \( (16, 17) \). In the perfusion time course experiments, (-)-[\(^{3} \)H]epinephrine was included with unlabeled (-)-epinephrine as previously described \( (1) \). Samples of perfusate \( (4 \) drops of which were equivalent to 1.4 s) were collected in polypropylene test tubes \( (12 \times 75 \) mm) in an LKB Redirac fraction collector (model 2112). Samples of perfusate were monitored for radioactivity by counting 0.05 ml in 10 ml of ACS scintillation fluid (Amersham). The perfusate sample preceding the appearance of radioactivity was set as time 0 (see Fig. 1). Total perfusate Ca\(^{2+} \) was

---

*The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DNP, 2,4-dinitrophenol.

---

A preliminary report of this study was presented at the International Symposium on Isolation, Characterization, and Use of Hepatocytes held October 22-24, 1982 in Indianapolis, IN. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Recipient of National Institutes of Health Grant AM 20693.

§Recipient of National Institutes of Health Grant AM 18660.

Associate Investigator, Howard Hughes Medical Institute.

Investigator, Howard Hughes Medical Institute.
determined by atomic absorption spectroscopy as previously described (4).

Analytical Methods—Samples for phosphorylase (0.5 ml) were pipetted directly into polypropylene tubes (17 × 100 mm) immersed in liquid N2 and assayed as described before (16, 17). The tubes also contained approximately 3 ml of liquid nitrogen; within 1 s, samples were frozen solid. The times quoted for phosphorylase are the times at which the sample was added to the tubes.

Hepatocyte glycogen was measured by the method of Chan and Epton (18). Lactate was measured by the method of Hohorst (19) and glucose was determined using a Beckman Glucose Analyzer 2. The concentrations of glucose 6-P and fructose 6-P were determined using the bacterial luciferase method (20) on neutralized perchlorate extracts of hepatocyte suspensions.

Hepatocyte Respiration—The conditions employed for the measurement of hepatocyte respiration were those previously described (5). Measurements of O2 partial pressure were made with a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH). The output from this device was channeled into a Hewlett-Packard analog/digital converter to give a digitalized signal (i.e. in millivolt·seconds, sampled twice each second). The data were stored for 2 min before the addition of hormone and for 5 min after. The results are expressed as change in millivolt·seconds from control, i.e. no addition, with 1 mV·s being equivalent to 0.626 µl of O2. As seen in Fig. 3, the addition of 0.9% NaCl (control) resulted in a decrease in signal which became steady again after approximately 20 s.

Pyridine Nucleotide Fluorescence—Pyridine nucleotide fluorescence of hepatocyte suspensions (3 ml) was measured in a Varian spectrofluorometer model SF-330 fitted with a magnetically stirred cuvette. Excitation wavelength was 340 nm and emission wavelength was 470 nm. The fluorescence signal (in millivolts) was channeled into a Hewlett-Packard analog/digital converter and the data, sampled twice each second, were stored in a Hewlett-Packard 3356 computer (Laboratory Automation System). The data are represented as millivolt·seconds and the results are expressed as change from 0, i.e. immediately before hormone addition. Additions of agents to the cuvette were made by injection through a short length of polyethylene tubing without opening the door to the cell housing. The injection and mixing time was 1.5 s, as determined by addition of a solution of NADH to hepatocyte suspensions.

Sources of Materials—Antimycin A, and atractyloside were from Sigma, St. Louis, MO. Sources of other materials have been described previously (1, 4, 16, 17).

RESULTS

Time Course of α-Adrenergic-mediated Mobilization of Hepatocyte Ca2+—In previous studies, we showed that activation of phosphorylase in isolated hepatocytes by epinephrine, vasopressin, CCCP, DNP, and oligomycin could be observed as early as 2 s, with maximal effects being observed between 15 and 20 s (1). If it is postulated that mobilization of intracellular Ca2+ is responsible for this activation (e.g. Ref. 21), then it must be shown that this occurs before or at the same time as phosphorylase activation. The data in Fig. 1 demonstrate that epinephrine-induced Ca2+ mobilization (as indicated by Ca2+ efflux) occurred as early as 1.4 s in livers perfused with medium containing 60 µM Ca2+. When livers were perfused with a medium containing a more physiological concentration (1.3 mM) of Ca2+, efflux was seen again (Fig. 2). These findings confirm our previous conclusion that net Ca2+ influx is not involved in the initial activation of phosphorylase (1, 22).

Epinephrine perfusions always increased Ca2+ above the control, even as early as 1.4 s. However, the increase did not become statistically significant (p < 0.05) until 8.4 s. The rapid nature of the Ca2+ change observed in these perfusions (Figs. 1 and 2) suggests to us the possibility that Ca2+ was released first from the plasma membrane, before being mobilized from other intracellular stores such as endoplasmic reticulum and mitochondria (e.g. Refs. 1, 4, and 10).

![Fig. 1. Time course of the effect of 10−8 M (−)epinephrine on perfusate Ca2+ and 3H. Rat livers were perfused with nonrecir-culating low Ca2+ medium as previously described (4) and samples of perfusate were collected at 1.4-s intervals for the measurement of Ca2+ and 3H. Results show as the mean ± S.E. from four separate perfusions. p values were determined using a 1-tailed paired t test. The fraction preceding the first one to have significant 3H in it (p < 0.025 compared with background) was set as zero time. ns, not significant.](https://i.imgur.com/3H.png)

![Fig. 2. Time course of the effect of 10−8 M (−)epinephrine (EPI) on perfusate Ca2+. Rat livers were perfused with nonrecir-culating medium containing 1.27 ± 0.11 mM Ca2+, and samples of perfusate were collected at 1.4-s intervals for the measurement of Ca2+ and 3H. The number of perfusions for each group is shown in parentheses. For the control (CONT) perfusions, carrier-free (−)−(3)Hepinephrine was infused with 10−5 M phenolamine and 10−4 M (2)propranolol. Other details are in Fig. 1.](https://i.imgur.com/2H.png)
Time Course and Ca\(^{2+}\) Dependency of Changes in Respiration Induced by Vasopressin and Epinephrine—Increased hepatocyte respiration induced by phenylephrine, vasopressin, and angiotensin II can be observed after approximately 15 s of exposure (5). This was also shown by Scholz and Schwabe (23) using perfused liver and recently was reconfirmed by others (15) using female rat livers and a nonrecirculating perfusion system with no erythrocytes or albumin present in the perfusate (15). These latter workers reported that it took 9.9 ± 0.4 s following phenylephrine treatment before respiration was stimulated. Since phosphorylase activation was seen at 2 s with a maximal effect seen at 10–20 s (1), one can speculate that the increased respiration may be secondary to a rise in cytosolic Ca\(^{2+}\). To show more precisely the relationship between the time course of phosphorylase activation and the onset of respiration, we utilized an analog/digital converter to transform the O\(_2\) electrode signal into a digital form (see “Experimental Procedures”). The data in Fig. 3 show that vasopressin began to stimulate respiration at approximately 10 s and that the effect became statistically significant (p < 0.05) at 17 s. The decrease in signal seen with the saline control was an “addition” artifact. When phosphorylase activity was measured under the same conditions of incubation and that vasopressin began to stimulate respiration at approximately 10 s and that the effect became statistically significant (p < 0.05) at 17 s. The decrease in signal seen with the saline control was an “addition” artifact. When phosphorylase activity was measured under the same conditions of incubation as the O\(_2\) measurements, significant activation occurred at 3 s (p < 0.05), which is clearly much earlier than the respiration change. Similar results were obtained with phenylephrine (10\(^{-5}\) M) and epinephrine (10\(^{-6}\) M) as the stimuli (data not shown). Phosphorylase activation by vasopressin or phenylephrine could not be observed at 1 s, but significant activation could be seen above control at 2 s (p < 0.05) (data not shown).

The contention that the stimulation of respiration was secondary to and/or dependent on calcium was supported by the finding that the increase in respiration seen with vasopressin was completely blocked in Ca\(^{2+}\)-depleted hepatocytes (21) and was restored upon Ca\(^{2+}\) readition (data not shown). Hormonally induced changes in hepatic pyridine nucleotides have been described (24–26). However, the precise time of onset of these changes has not been determined, although they have been shown to be relatively rapid (25). The data in Fig. 4 show that vasopressin, phenylephrine, and epinephrine promoted an increase in the level of cellular pyridine nucleotide which began at 5–7 s. The effect was maximal by 15–20 s and was not observed in Ca\(^{2+}\)-depleted hepatocytes (21) (data not shown).

Effect of Respiratory Chain Inhibitors, Uncouplers, Oligomycin, and Atractyloside on Epinephrine Actions—The data in Fig. 5 show that glucose release is a poor index of hepatic glycogenolysis when agents like rotenone, oligomycin, antimycin A, CCCP, DNP, and atractyloside are added to isolated hepatocytes. When added alone to hepatocytes, these agents stimulated glycogenolysis almost equally as judged by glycolysis (Fig. 5A) and their effects were slightly greater than that observed with epinephrine alone. Glucose release due to these agents was somewhat variable, but always less than that observed with epinephrine alone (Fig. 5D). Phosphorylase \(a\) activity was also variably increased, with the uncouplers DNP and CCCP producing the largest increase (Fig. 5C). When lactate output was measured (Fig. 5B), it was seen that each agent stimulated this very markedly, e.g. between 12- and 19-fold. Thus, the somewhat variable and low glucose release observed with these agents (Fig. 5D) could be partly accounted for by the greatly increased rate of glycolysis as indicated by the increased lactate levels. When epinephrine was combined with each of these agents, the changes in glycogen depletion and phosphorylase \(a\) were less

**Fig. 4. Effect of vasopressin (Vaso, 10\(^{-8}\) M), epinephrine (Epi, 10\(^{-8}\) M), and phenylephrine (Phenyl, 10\(^{-8}\) M), on hepatocyte pyridine nucleotide reduction.** Total cellular pyridine nucleotide fluorescence was measured as described under “Experimental Procedures.” Values shown are means ± S.E. from six separate incubations. Sal, saline.

**Fig. 3. Time course of vasopressin (10\(^{-8}\) M)-induced increase in the rate of hepatocyte oxygen uptake and phosphorylase activity.** A dilute suspension of hepatocytes (approximately 5 mg wet wt/ml) was used to measure both oxygen utilization and phosphorylase activity. The increase in respiration stimulated by vasopressin was evident at 12 s and was statistically significant (p < 0.05) by 17 s. The statistical significance of the increase in phosphorylase activity above control is shown at each time point. The electrode response and mixing time were very rapid as indicated by the fact that when 50 µl of a saturated dithionite solution was added to the 5-ml mixing chamber an increase in electrode response was observed at 0.5 s.

---

\(^2\) The mitochondrial inhibitors could also have increased the AMP level. This would have stimulated phosphorylase \(b\) allosterically in the intact cells (27), but would not have resulted in an increase in phosphorylase activity detectable in our assay system since caffeine was included.
Time Course of α1-Adrenergic Actions

Fig. 5. Effect of (-)epinephrine (10^{-5} M), rotenone (10^{-5} M), CCCP (10^{-5} M), antimycin A (2 \times 10^{-5} M), oligomycin (10^{-5} M), atracyloside (10^{-4} M), and DNP (10^{-5} M) on hepatocyte glycogen content, lactate production, phosphorylase activity, and glucose output. Hepatocytes from fed animals were incubated in normal (2.5 mM) Ca^{2+}-containing medium for 30 min with the agents shown. The experiment shown is a representative of three and the values shown are from duplicate incubations.  

(1) Propranolol (2 \times 10^{-5} M) was present in all incubations to inhibit the small β-adrenergic component of epinephrine action. Rotenone, CCCP, antimycin A, oligomycin, and DNP were dissolved in dimethyl sulfoxide.

Fig. 6. Time course of vasopressin (10^{-8} M)-induced increases in the levels of hepatocyte, glucose 6-P, and fructose 6-P. Hepatocyte suspensions (5 ml) from fed rats were pre-incubated for 15 min, after which time either vasopressin (VASO) (10^{-8} M final concentration) or saline (control) (CONT) was added and samples (0.5 ml) were removed at the times shown and added to 0.5 ml of 0.6 M HClO_{4}. Results are means of triplicate incubations, each one being assayed in duplicate. Internal standards were included to correct for quenching.

than additive. However, this was due to the fact that the effects produced by the agents alone were substantial or close to maximal, and not because the agents inhibited phosphorylase activation. These results also illustrate the danger of using glucose release as a measure of glycogenolysis in the presence of agents which stimulate glycolysis (15).

Time Course of Vasopressin-induced Increases in Glucose 1-P, Glucose 6-P, and Fructose 6-P—Further evidence to support the fact that glucose release is a poor index of glycogenolysis when studying early time courses is shown in Fig. 6. Since the activation of the rate-limiting step in glycogenolysis, namely phosphorylase, can be seen at 3 s (Fig. 3) with phenylephrine and vasopressin as stimuli, one would predict that the first intermediate to be increased would be glucose 1-P followed by glucose 6-P and fructose 6-P. Fig. 6 shows that glucose 6-P and fructose 6-P increased at between 3 and 6 s; similar results were obtained with phenylephrine and epi-

nephrine as agonists (data not shown). These changes are clearly more rapid than those seen on glucose release (1, 4, 15) and respiration (Fig. 3). Glucose 1-P increased slightly (data not shown), but did not accumulate to the same extent as glucose 6-P and fructors 6-P, presumably because the equilibrium of phosphoglucomutase favors glucose 6-P.
DISCUSSION

The data presented here show that Ca\(^{2+}\) mobilization elicited by (10\(^{-6}\) M) epinephrine can be detected at 1.4 s at 37 °C in rat livers perfused with nonrecirculating medium. This is sufficiently rapid to account for the activation of phosphorylase which can be seen as early as 2 s (Fig. 3 and Ref. 1) and the increase in the hexose phosphate pool seen at 3–6 s (Fig. 6). Other agents which activate phosphorylase within 2 s are FCCP, oligomycin (1), antimycin A, and rotenone (data not shown). Previous evidence supporting a role for Ca\(^{2+}\) in the activation of phosphorylase by a stimulation of phosphorylase kinase is the very close correlation between Ca\(^{2+}\) fluxes and phosphorylase activation induced by a variety of hormones (e.g. Refs. 17 and 28) and the finding that, in phosphorylase kinase-deficient hepatocytes, no activation of phosphorylase can be observed, despite the mobilization of intracellular Ca\(^{2+}\) by vasopressin, epinephrine, and A23187 (29). Recently, Chrisman et al. (30) have purified rat liver phosphorylase kinase 30,000-fold and have shown that it has a similar subunit composition to skeletal muscle phosphorylase kinase, including the δ subunit (calmodulin), and that its activity can be modulated by 10\(^{-6}\) to 10\(^{-6}\) M Ca\(^{2+}\).

The changes in respiration which are seen approximately 10 s after addition of phenylephrine or vasopressin are possibly secondary to the Ca\(^{2+}\) fluxes and the increase in reduced pyridine nucleotide (Fig. 4) since they are both dependent on the presence of Ca\(^{2+}\) (data not shown). They are clearly too slow to be the cause of phosphorylase activation (Fig. 3). This conclusion is contrary to that reached in a recent study (15). These workers postulate a "tight association between this oxygen uptake response and agonist-induced glucose output," since the onset of glucose release followed the stimulation of respiration by approximately 2 s. Other evidence presented to support their claim was the observation that inhibitors of the mitochondrial respiratory chain inhibited the action of phenylephrine, vasopressin, and angiotensin II on glycogenolysis. However, glucose output was used as a temporal and quantitative index of glycogenolysis and, as discussed below, this is erroneous.

First, the increase in glucose release from the perfused rat liver in response to Ca\(^{2+}\)-dependent hormones lags about 30 s after the efflux of Ca\(^{2+}\) begins (1, 4). Since Ca\(^{2+}\) efflux is coincident with phosphorylase activation (Figs. 1 and 3), this indicates that it takes about 30 s for the glucosyl residues of glycogen to be converted by phosphorylase, phosphoglucone isomerase (Fig. 6), and glucose 6-phosphatase to glucose and transferred into the bloodstream. Thus, glucose release is a relatively late temporal index of glycogenolysis. Second, glucose release may not be a valid quantitative index of glycogenolysis in the presence of agents which stimulate glycolysis. As shown in Fig. 5B, rotenone, FCCP, antimycin A, oligomycin, strychnisine, and DNP markedly stimulate hepatic glycogenolysis, as indicated by lactate output (31). Thus, any inhibition of glucose output caused by these agents may be attributable to the increased conversion of glucose-6-P to lactate. As illustrated in Fig. 5A, the most valid way to measure glycogenolysis is to measure changes in glycogen content. Third, as shown in Fig. 5C, when added alone, the inhibitors produce a near-maximal stimulation of phosphorylase which is the rate-limiting enzyme in glycogenolysis. Thus, the increase in phosphorylase and glucose release seen when phenylephrine, vasopressin, and epinephrine are added together with the inhibitors has to be less additive to that observed with the hormones and agents alone. A likely reason why Reinhart et al. (15) observed that the inhibitors did not affect the stimulation of glucose output by glucagon is that this hormone markedly stimulates gluconeogenesis, whereas agents such as phenylephrine, vasopressin, and angiotensin II only have very weak effects on gluconeogenesis (e.g. Ref. 32).

Concerning the source of Ca\(^{2+}\) mobilized in liver cells by α-agonists, we and others originally proposed that the major source was mitochondria (17, 21, 33) and this was confirmed by several studies (3–11). We also suggested "the involvement of another pool" (3) since we calculated that, in the nonrecirculating perfused liver with low (50 μM) Ca\(^{2+}\) in the perfusate, mitochondria release only 60% of the cellular Ca\(^{2+}\) mobilized by hormones. Subsequently, it was demonstrated that subcellular fractions enriched in 5'-nucleotidase and glucose 6-phosphatase also had lower Ca\(^{2+}\) content, showing the involvement of endoplasmic reticulum and/or plasma membranes, although the temporal sequence was not elucidated (4). It is now clear that there is a very rapid mobilization of intracellular Ca\(^{2+}\) which can be seen as early as 1.4 s (Fig. 1). This suggests an initial release of Ca\(^{2+}\) from a site close to the α-adrenergic receptor, which is localized in the plasma membrane (6, 28, 34–36). Some of this Ca\(^{2+}\) is extruded from the cell and released into the extracellular space while some enters the cytoplasm to elevate cytosolic Ca\(^{2+}\). The result of this is that phosphorylase kinase is stimulated and this in turn activates phosphorylase within 3 s or less (Fig. 3) and raises the concentration of hexose phosphates at 3 to 6 s (Fig. 6).

Respiration increases in a Ca\(^{2+}\)-dependent fashion and is also relatively slow, taking approximately 10 s to become evident and 17 s to become statistically significant. These data suggest that this occurs later than the increase in cytoplasmic free Ca\(^{2+}\) and is secondary to the increase in cellular reduced pyridine nucleotide (Fig. 4). It has been proposed that the increase in hepatic pyridine nucleotide fluorescence in response to hormones indicates an increase in the influx of reducing equivalents into the mitochondrial respiratory chain resulting in a higher level of mitochondrial NADH (24–26). The data in Figs. 3 and 4 are consistent with this proposal since the increase in reduced pyridine nucleotide fluorescence induced by vasopressin and α1-adrenergic stimulation precedes the onset of respiration by approximately 6 s. The reaction(s) producing the reducing equivalents at these times is not known, but is presently under investigation in this laboratory. The increased transfer of reducing equivalents to the mitochondria is also consistent with a stimulation of the an-glycero-3-phosphate dehydrogenase shuttle by an increase in cytosolic Ca\(^{2+}\) ions (37, 38). The Ca\(^{2+}\) dependency of the pyridine nucleotide and respiration changes induced by α1-agonists and vasopressin are consistent with this proposal.

The nature of the plasma membrane Ca\(^{2+}\)-pool which is postulated above to be modified by α1-agonists and vasopressin may be speculated upon. Two possible candidates are Ca\(^{2+}\)-bound to calmodulin and to phosphatidylinositol-4,5-P\(_2\). Regarding phosphatidylinositol-4,5-P\(_2\), this binds Ca\(^{2+}\), but for it to release Ca\(^{2+}\) it must first be hydrolyzed by a specific phospholipase C, then dephosphorylated to release the Ca\(^{2+}\) which is chelated between the phosphates on the 4 and 5 carbons of the inositol ring (41). Phosphatidylinositol-4,5-P\(_2\) degradation can be stimulated by pharmacological doses of vasopressin at 15 s (approximately 15% decrease) (39, 40). This breakdown is certainly faster than that of phosphatidylinositol, which is seen at 2 min (42), but is much

---

2 B. P. Hughes and P. F. Blackmore, unpublished observations.
slower than the effects seen on Ca\(^{2+}\) mobilization and phosphorylase activation (Figs. 1 and 3). The release of Ca\(^{2+}\) from phosphatidylinositol-4,5-P\(_2\), during its breakdown may contribute to the mobilization of Ca\(^{2+}\), but it appears unlikely that it can account for all the Ca\(^{2+}\) (42). Alternatively, phosphatidylinositol-4,5-P\(_2\) may control the release of a “plasma membrane-bound Ca\(^{2+}\) pool,” or that products of phosphatidylinositol-4,5-P\(_2\) breakdown, namely diacylglycerol and myo-inositol-1,4,5-P\(_3\), may alter the Ca\(^{2+}\)-transporting systems located in the mitochondria, endoplasmic reticulum, and plasma membrane (43, 44). These possibilities are presently being explored by us.

A role for calmodulin in the initial \(\alpha\)-adrenergic response in liver has been proposed (45) but substantial evidence against this has been presented (46-48) since it was shown that in liver phenothiazines act as \(\alpha\)-adrenergic antagonists (46). The \(\alpha\)-adrenergic antagonists (phentolamine, dibenamine, and phenoxybenzamine) like the phenothiazines can inhibit calmodulin-stimulated cAMP phosphodiesterase, and it has been proposed “that calmodulin inhibition may be responsible for the \(\alpha\)-adrenergic blocking activity of both classes of compounds” (49). Since the \(\alpha\)-adrenergic antagonists phenotolamine, dibenamine, and phenoxybenzamine are structurally related to the phenothiazines and Ca\(^{2+}\) channel blockers (50) it is not unreasonable to expect these drugs to have the same activity as \(\alpha\)-adrenergic antagonists. Prazosin, a highly potent and specific \(\alpha\)-adrenergic antagonist, which is not structurally related to the other \(\alpha\)-adrenergic antagonists, phenothiazines, and Ca\(^{2+}\) channel blockers, is not able to block calmodulin-stimulated cAMP phosphodiesterase up to a concentration of 10\(^{-4}\) M. Thus, it is unlikely that \(\alpha\)-adrenergic antagonists act by inhibiting calmodulin and there is presently no valid evidence that calmodulin plays any role in the initial events in the \(\alpha\)-adrenergic or vasopressin responses (46).

Most studies of \(\alpha\)-adrenergic stimulation of hepatic Ca\(^{2+}\) fluxes have shown a decrease in total mitochondrial Ca\(^{2+}\) (3-11) while one study (10) showed an initial small increase followed by a slower decrease. These latter workers used perfused livers or hepatocytes from mature female rats in which catecholamines mainly act by activating \(\beta\)-adrenergic receptors with a resultant large increase in cAMP (51). We have recently shown (52) that, when both \(\alpha\)- and \(\beta\)-adrenergic receptors are stimulated in juvenile rats, mitochondrial Ca\(^{2+}\) content increases up to 2-fold at 5 min and 6-fold at 15 min. This is very different from the situation in mature male rats (200 g or larger) in which \(\beta\)-receptor-mediated cAMP accumulation is minimal or absent (53) and activation of \(\alpha\)-receptors leads to mitochondrial Ca\(^{2+}\) release (3-11). These considerations illustrate that one has to be extremely careful when comparing data obtained in different experimental situations. The system we have used in almost all our studies is perfused liver or hepatocytes from fed male rats with body weights in the range 200-250 g in normal (2.4 mM) Ca\(^{2+}\)-containing media. Under these conditions, there is virtually no \(\alpha\)-receptor-mediated increase in cAMP and only a very small \(\beta\)-adrenergic component (16, 53).

Our data are consistent with the following hypothesis regarding the mechanism of action of \(\alpha\)-adrenergic agonists and vasopressin in hepatocytes from male rats. Following binding of catecholamines to \(\alpha\)-adrenergic receptors (28), Ca\(^{2+}\) ions are released from plasma membrane or endoplasmic reticulum (10, 12, 14) thereby elevating Ca\(^{2+}\) in the cytoplasm at 1 s (8, 54). This effect may be mediated by hydrolysis of phosphatidylinositol-4,5-P\(_2\) (39, 40, 43, 44). Some of this Ca\(^{2+}\) is excluded from the cell by the plasma membrane Mg-Ca-ATPase (55) while the remainder elevates the free cytoplasmic pool. This elevation causes the activation of phosphorylase kinase and, hence, activation of phosphorylase (29, 50). In a “second cascade” (9), total mitochondrial Ca\(^{2+}\) falls to reach a minimum by approximately 4 to 5 min (3-11) by an as yet unknown mechanism. The precise time at which mitochondrial Ca\(^{2+}\) begins to fall has not been determined since current methods used to isolate mitochondria do not permit the isolation of these organelles within 2-3 s after hormone exposure, the time at which Ca\(^{2+}\) and phosphorylase changes occur. It is conceivable that mitochondrial Ca\(^{2+}\) does change at 1-2 s and that products of phosphatidylinositol-4,5-P\(_3\) hydrolysis, namely myo-inositol-1,4,5-P\(_3\), act on mitochondria to release Ca\(^{2+}\) (7).

Acknowledgments—We would like to thank Dr. A. Borle for allowing us see Ref. 51 before its publication. We would also like to thank Bill Billings and Thomas Lukas for assistance with the Hewlett-Packard computer.

REFERENCES

1. Blackmore, P. F., Hughes, B. P., Shuman, E. A., and Exton, J. H. (1982) J. Biol. Chem. 257, 190-197

2. Williamson, J. R., Cooper, R. H., and Hoek, J. B. (1981) Biochim. Biophys. Acta 661, 245-295.

3. Blackmore, P. F., Dehaye, J.-P., Strickland, W. G., and Exton, J. H. (1979) FEBS Lett. 100, 117-120

4. Blackmore, P. F., Dehaye, J.-P., and Exton, J. H. (1979) J. Biol. Chem. 254, 6945-6950.

5. Dehaye, J.-P., Hughes, B. P., Blackmore, P. F., and Exton, J. H. (1981) Biochem. J. 194, 949-956.

6. Dehaye, J.-P., Blackmore, P. F., Venter, J. C., and Exton, J. H. (1980) J. Biol. Chem. 255, 3905-3910.

7. Babcock, D. F., Chen, J.-L. Yip, B. P., and Lardy, H. A. (1979) J. Biol. Chem. 254, 8117-8120.

8. Murphy, E., Coll, K., Rich, T. L., and Williamson, J. R. (1980) J. Biol. Chem. 255, 6600-6608.

9. Kimura, S., Kugai, M., Tada, R., Kojima, L., Abe, K., and Ogata, E. (1982) Hormone Metab. Res. 14, 133-138.

10. Berthon, B., Poggioli, J., Capiod, T., and Claret, M. (1981) Biochem. J. 199, 177-180.

11. Barratt, G. J., Parker, J. C., and Wadsworth, J. C. (1981) J. Physiol. (Lond.) 312, 29-55.

12. Poggioli, J., Berthon, B., and Claret, M. (1980) FEBS Lett. 115, 243-246.

13. Foden, S., and Randle, P. J. (1978) Biochem. J. 170, 615-625.

14. Altman-Salzman, M., Carafoli, E., and Jakob, A. (1980) Eur. J. Biochem. 106, 241-248.

15. Reinhart, P. H., Taylor, W. M., and Bygrave, F. L. (1982) J. Biol. Chem. 257, 1906-1912.

16. Hutson, N. J., Brumley, F. T., Assimacopoulos, J. D., Harper, S. C., and Exton, J. H. (1978) J. Biol. Chem. 253, 5200-5208.

17. Blackmore, P. F., Brumley, F. T., Marks, J. L., and Exton, J. H. (1978) J. Biol. Chem. 253, 4851-4858.

18. Chan, T. M., and Exton, J. H. (1975) Anal. Biochem. 71, 96-105.

19. Hohorst, H. J. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 266-270, Academic Press, New York.

20. Golden, S., and Katz, J. (1980) Biochem. J. 188, 799-805.

21. Assimacopoulos-Jeannet, F. D., Blackmore, P. F., and Exton, J. H. (1977) J. Biol. Chem. 252, 2662-2669.

6 Following submission of this study a paper by Reinhart et al. (57) appeared in which they showed that Ca\(^{2+}\) efflux from the perfused liver was observed at 7.1 s following \(\alpha\)-adrenergic stimulation. Our study clearly shows that \(\alpha\)-adrenergic stimulation can be seen at 1.4 s (Fig. 1); thus, the metabolic integrity of their preparation is in question. These workers performed an experiment with very low (10 mM) Ca\(^{2+}\) and showed that \(\alpha\)-agonists mobilize Ca\(^{2+}\) from mitochondria and endoplasmic reticulum at 2 s, thus confirming our perfused liver studies (3-6).

7 Myo-inositol-1,4,5-P\(_3\) increases by 10% in isolated hepatocytes at 5 s after stimulation with 10\(^{-7}\) M vasopressin (unpublished observations).
Time Course of $\alpha_1$-Adrenergic Actions
Time course of alpha1-adrenergic and vasopressin actions on phosphorylase activation, calcium efflux, pyridine nucleotide reduction, and respiration in hepatocytes.

P F Blackmore, B P Hughes, R Charest, E A Shuman, 4th and J H Exton

J. Biol. Chem. 1983, 258:10488-10494.

Access the most updated version of this article at http://www.jbc.org/content/258/17/10488

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/17/10488.full.html#ref-list-1