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The Relationships between Receptor Binding Capacity for Norepinephrine, Angiotensin II, and Vasopressin and Release of Inositol Trisphosphate, Ca^{2+} Mobilization, and Phosphorylase Activation in Rat Liver

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SUMMARY

Concentration-response relationships for norepinephrine-, angiotensin II-, and vasopressin-stimulated changes in cell Ca^{2+} content, phosphorylase activation, and cytosolic free Ca^{2+} and *myo*-inositol-P_3 levels were examined in isolated hepatocytes. The specific binding of radioligands to α_1-adrenergic, vasopressin, and angiotensin II receptors was also examined in rat liver plasma membranes.

Disparities occurred between the concentration-response curves for *myo*-inositol-P_3 formation and the Ca^{2+} and phosphorylase responses, with the greatest difference being observed with vasopressin and the smallest with norepinephrine. It was also observed that all three agonists produced the same maximum changes in phosphorylase, cell Ca^{2+}, and cytosolic Ca^{2+}, but the maximum capacity of each agonist to generate *myo*-inositol-P_3 varied greatly and was correlated with the maximum receptor binding capacity. The data indicated that a very small and submaximal elevation of *myo*-inositol-P_3 was sufficient to maximally elevate cytosolic Ca^{2+} and activate phosphorylase. In addition, the relationship between the accumulation of *myo*-inositol-P_3 and the elevation of cytosolic Ca^{2+} was similar, irrespective of whether the agonist was norepinephrine, angiotensin II, or vasopressin.

It is proposed that the large differences between the concentration-response curves for *myo*-inositol-P_3 formation and Ca^{2+} and phosphorylase changes observed with vasopressin and angiotensin II are due to the higher density of their receptors on liver cell plasma membranes compared with α_1-adrenergic receptors.

INTRODUCTION

Recently, a number of studies have provided experimental evidence indicating a role for IP_3[4] as a second messenger for receptor-mediated, intracellular Ca^{2+} mobilization in liver and other tissues (Refs. 1–9; see Ref. 10 for review). While most investigators now favor such a role for PIP_2 breakdown and IP_3 formation in the action of those agonists that act by raising cytosolic Ca^{2+}, there have been several problems with the theory. One problem relates to the Ca^{2+} dependence of the IP_3 response. In our earlier studies (11), we observed that vasopressin-induced PIP_2 breakdown in hepatocytes was abolished by EGTA treatment and restored by Ca^{2+} readdition. More recently, the ionophore A23187 has been found to stimulate IP_3 formation in a Ca^{2+}-dependent manner (8). These observations give rise to the possibility that PIP_2 breakdown is the result rather than the cause of Ca^{2+} mobilization (11). However, another explanation is that PIP_2 breakdown requires Ca^{2+}, but is not controlled by physiological changes in Ca^{2+}.

Another problem is that the agonist concentrations required to stimulate PIP_2 hydrolysis and subsequent IP_3 release are higher than those for Ca^{2+} mobilization and Ca^{2+}-dependent physiological events (3, 8, 11–13). This could be interpreted as indicating that PIP_2 hydrolysis and Ca^{2+} mobilization are not causally related. On the other hand, Michell and co-workers (12, 13) have suggested that the presence of spare receptors for agonists that mobilize Ca^{2+} could explain the disparity.

In this study, the maximal binding capacities of α_1-adrenergic, ANG, and AVP receptors on liver plasma

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[4] The abbreviations used are: IP_3, *myo*-inositol trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; NE, (−)-norepinephrine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AVP, [8-arginine]vasopressin; ANG, angiotensin II (human sequence).

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membranes have been measured. NE, ANG, and AVP stimulation of Ca^{2+} mobilization and phosphorylase activation, which are presumably IP_3-dependent events, have also been examined in hepatocytes incubated with supra- and submaximal concentrations of hormone.

The concentration-response studies on IP_3 release and other events confirmed the reported disparities between the concentration-response curves for hormone-stimulated IP_3 release and Ca^{2+} mobilization or phosphorylase *a* activation, which were greatest for AVP, intermediate for ANG, and smallest for NE. Since those discrepancies were in direct proportion to the relative receptor binding capacities for these agonists as determined from radioligand binding studies, it was concluded that the data are compatible with the concept of spare receptors for Ca^{2+}-dependent events in liver cells. In addition, we showed that the relationship between receptor-mediated changes in IP_3 release and the subsequent mobilization of calcium is similar for each of the agonists studied.

MATERIALS AND METHODS

Materials. Radiolabeled materials were purchased from New England Nuclear. (−)-Norepinephrine bitartrate, Trizma, (±)-propranolol, [8-arginine]vasopressin, angiotensin II, bacitracin, bovine serum albumin, penicillin K, and streptomyin sulfate were purchased from Sigma. Quin-2/AM was obtained from Calbiochem. Phentolamine was from CIBA-GEIGY Corp. Sprague Dawley rats were obtained from Harlan Industries. Percoll was purchased from Pharmacia. Sources of other materials have been described previously (8, 14, 15).

Methods for hepatocyte and liver plasma membrane isolation. Hepatocytes (30–50 mg/ml) were isolated from the livers of male rats (180–220 g) maintained on standard laboratory chow and water *ad libitum* and incubated as described (14). Liver plasma membrane vesicles were isolated using the rapid Percoll procedure of Prpic *et al.* (15). Isolated membrane vesicles were resuspended in the hypotonic binding buffer (see below), lysed by rapid freezing in liquid N_2, and used immediately. Protein content was determined by the method of Lowry *et al.* (16).

Dose-response curves on liver cells. Methods for the measurement of phosphorylase *a*, [^{3}H]IP_3 formation (in the presence or absence of 10 mM LiCl), total cell Ca^{2+}, and cytosolic free Ca^{2+} have been described previously (8, 14, 17–19). All cell incubations were performed in triplicate, and duplicate samples were assayed. Experiments representative of at least three are shown. All hormone solutions were prepared fresh daily and diluted with 0.1% bovine serum albumin in 0.9% NaCl and kept on ice until use. Propranolol (1 μM) was added in all the experiments with NE to eliminate possible effects mediated by β-adrenergic receptors.

Radioligand binding studies. Measurements of the specific binding of [^{3}H]prazosin (80.9 Ci/mmol) to liver plasma membranes were performed as described in detail elsewhere (20). The specific binding of [^{3}H]AVP (40–50 Ci/mmol) and [^{3}H]ANG (45.9 Ci/mmol) to hepatic plasma membranes (12–20 μg of protein/tube) was estimated as previously described (24–26). Briefly, the incubation mixture contained 50 mM Tris/HCl, (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml bacitracin, 1 mg/ml bovine serum albumin, and 0.05–29 nM [^{3}H]AVP or [^{3}H]ANG in a final volume of 200 μl. Following a 20-min incubation at 30° (21–23), samples were diluted to 4.2 ml with ice-cold solution containing 50 mM Tris/HCl (pH 7.4 at 2°), 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.1 mg/ml bacitracin, and 1 mM EGTA and rapidly filtered through 24-mm Whatman GF/C glass fiber filters under vacuum. The filters were washed with an additional 8 ml of dilution buffer within 10 sec of the initial dilution and transferred to vials containing 10 ml of Beckman Ready-solv EP, and the radioactive content was measured in a Beckman LS-1800 liquid scintillation counter. Nonspecific binding (typically 5–15% of total) was determined in the presence of 10 μM AVP for [^{3}H]AVP binding or 10 μM ANG for [^{3}H]ANG binding (21–23). Nonspecific [^{3}H]prazosin binding was determined using 10 μM phentolamine and was 7–30% of total binding. Scatchard (24) plots of all concentration-dependent radioligand binding measurements were linear (i.e., $r = 0.96$–0.99).

Analysis of concentration-response and binding data. In order to avoid the theoretical problems inherent in the use of linearized binding and concentration-response data (see Ref. 25 for review), all data from concentration-response and binding experiments were calculated, curve-fitted, and finally plotted as previously described in detail (20) using the mathematical modeling laboratory computer program, MLAB (26). The convergence factor for the sum of the squares (26) was less than 0.001 for all of the curve fits shown.

RESULTS

Concentration-response relationships. Fig. 1 shows the concentration dependencies for phosphorylase activation, cytosolic Ca^{2+} elevation, Ca^{2+} efflux, and IP_3 release induced in hepatocytes by NE, ANG, and AVP. Peak cytosolic Ca^{2+} values were measured 0.1–2 min after hormone addition. The other parameters were measured at 5 min for convenience, although the values were very similar to the peak values, as observed in other studies (3, 8, 27). Ca^{2+} efflux was measured by the decrease in

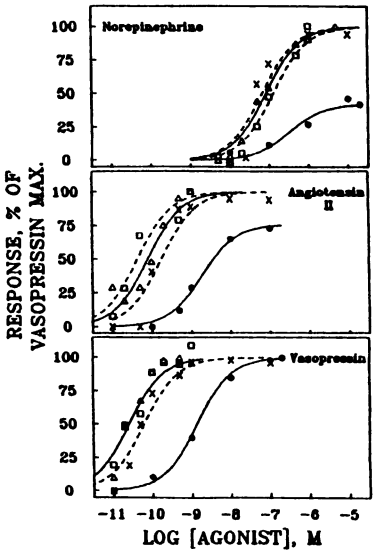

FIG. 1. *Dose responses of norepinephrine, angiotensin II, and vasopressin on hepatocyte phosphorylase activation, Ca^{2+} content, and myo-inositol-P_3 formation*

Hepatocyte suspensions (2 ml) were incubated in 25-ml Erlenmeyer flasks and continuously gassed with O_2/CO_2 (95:5). At zero time and after 5 min of incubation with hormones, samples were removed for the measurement of total cell Ca^{2+} (□) and phosphorylase *a* activity (△) as described in "Materials and Methods." The amount of [^{3}H]IP_3 in (●) cells previously incubated with [^{3}H]*myo*-inositol was measured at 0 and 5 min in the presence of 10 mM LiCl as described in "Materials and Methods." Each value is the mean from triplicate incubations and is representative of at least four separate experiments. For the measurement of changes in cytosolic Ca^{2+} (×), control and Quin-2-loaded hepatocytes were prepared as described previously (19). The maximum change in fluorescence (ΔmV·sec) induced by a given concentration of agonist was measured, with correction for the small changes (due to pyridine nucleotide fluorescence) occurring in control, i.e., not Quin-2-loaded, cells. The concentration of free cytosolic calcium was calculated as previously described (36). Each value is the mean from three to four different cell preparations assayed in duplicate.

total cell Ca^{2+}: this decrease comes about because Ca^{2+} mobilized from internal stores by agonists enters the cytosol and is then extruded from the cell due to the action of the plasma membrane Ca^{2+}-ATPase pump (8, 18, 19, 28). Propranolol (1 μM) was included in the experiments with NE to eliminate a possible contribution to phosphorylase activation mediated by β-adrenergic receptors. In other experiments (not shown), the addition of 1 μM prazosin completely abolished the effects of 10 nM to 1 μM NE on IP_3, Ca^{2+} efflux, and cytosolic Ca^{2+} (see below), indicating that these were mediated by α_1-adrenergic receptors. In any event, norepinephrine was chosen as the α_1-adrenergic agonist in these studies to minimize interaction with the hepatic β-adrenergic receptor, which is of the β_2 subtype and is much less responsive to norepinephrine than epinephrine (29). Measurements of cAMP (not shown) revealed no increases with ANG or AVP.

As shown in Fig. 1, each hormone exhibited similar EC_{50} values for stimulation of Ca^{2+} efflux, elevation of cytosolic Ca^{2+}, and phosphorylase activation. In contrast, as observed in previous studies (3, 8),[5] the concentration-dependence curves for hormone-stimulated release of IP_3 consistently lay to the right. For example, the ratio of the EC_{50} for hormone-stimulated IP_3 formation to that for the other responses studied simultaneously on the same batch of cells was 2.5–3.9 for NE, 17–30 for ANG, and 52–53 for AVP. Another feature observed was that, while each of the agonists studied produced the same maximal increase in cytosolic Ca^{2+} (Fig. 2), cellular Ca^{2+} efflux (Table 1), and phosphorylase *a* (Table 1), the maximal IP_3 release was different depending on the agonist studied (Table 1). For instance, supramaximal concentrations of NE consistently elicited the smallest release of IP_3, whereas ANG released an intermediate amount, and the largest release was observed with vasopressin.

In the preceding studies, Li^+ was included to increase the accumulation of IP_3 induced by the agonists (3, 8) and thus improve the measurements at low agonist concentrations. However, the inclusion of Li^+ did not significantly alter the EC_{50} values for each agonist on IP_3 formation (Table 2). Li^+ also does not alter the EC_{50} values for these agonists on cytosolic Ca^{2+} or phosphorylase *a* (8). As expected, the IP_3 levels achieved with maximally effective concentrations of agonists were less in the absence of Li^+ (Table 1). However, the order of efficacy of the agonists on IP_3 remained the same as in the presence of Li^+, i.e., AVP still produced the largest increase and NE the smallest increase.

[5] In the study of Thomas *et al.* (3), it is shown that when the *rates* of phosphorylase activation, cytosolic Ca^{2+} elevation, and IP_3 formation are plotted as a function of vasopressin concentration, the Ca^{2+} and IP_3 curves are superimposable, whereas the phosphorylase curve lies to the left. However, when their data are plotted in terms of absolute changes the pattern shown in Fig. 1 emerges. Since substantial time lags (up to 44 sec) are observed before low concentrations of agonists begin to elevate cytosolic Ca^{2+} or phosphorylase *a* (R. Charest, P. F. Blackmore, and J. H. Exton, unpublished observations), the basis upon which the rates reported by Thomas *et al.* (3) were calculated and hence the resulting conclusions are unclear. In the present study, measurements were made at times when maximum values were obtained in order to eliminate this problem.

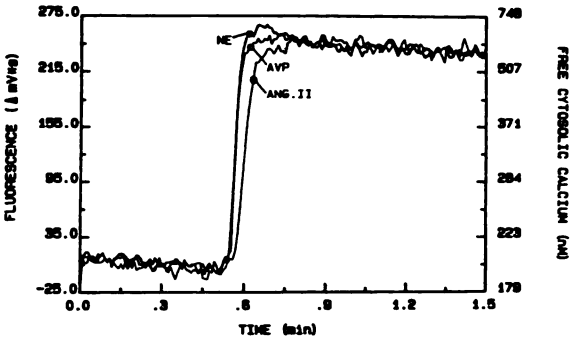

FIG. 2. *Changes in cytosolic free calcium following stimulation of isolated hepatocytes with norepinephrine, vasopressin, or angiotensin II*

Control and Quin-2-loaded hepatocytes were prepared as described previously (2). NE (1 μM), AVP (10 nM), or ANG II (10 nM) was added at 0.5 min. The results are presented as the change in fluorescence (ΔmV·sec) from the time of hormone addition. The concentration of free cytosolic calcium was calculated as described in detail elsewhere (19). Each tracing is the mean from two different cell preparations assayed in duplicate.

TABLE 1

Hormone-stimulated phosphorylase a, Ca^{2+} efflux, and [^{3}H]myo-inositol-P_3 in isolated hepatocytes

All parameters were measured during the 5-min period following the addition of saline control or supramaximal concentrations of agonist as described in "Materials and Methods." Basal amounts are *not* subtracted from the values shown in this table.

	NE (10 μM)	ANG (0.1 μM)	AVP (0.1 μM)
Phosphorylase *a* (units/g wet wt)[a]	23.7 ± 0.6	19.9 ± 0.2	21.5 ± 0.7
Total cell Ca^{2+} (nmol/mg wet wt)[b]	0.24 ± 0.01	0.24 ± 0.06	0.25 ± 0.05
Radioactivity in IP_3, 10 mM LiCl present (cpm/ml cells)[c]	480 ± 9	653 ± 16[d]	838 ± 78[d]
Radioactivity in IP_3, no LiCl present (cpm/ml cells)[e]	170 ± 10	273 ± 23[d]	430 ± 23[d]

[a] Basal phosphorylase *a* was 11.2 ± 0.3 units/g wet weight.
[b] Basal cell Ca^{2+} content was 0.35 ± 0.02 nmol/mg wet weight.
[c] Basal IP_3 release was 216 ± 4 cpm/ml of cells. Cell density was 45 mg wet weight/ml.
[d] Indicates a value significantly higher than NE value as determined by Student's *t*-test, $p < 0.05$.
[e] Basal IP_3 release was 120 ± 11 cpm/ml of cells.

Radioligand binding studies. Michell and co-workers (12, 13) have suggested that the differences in concentration dependence between AVP- and ANG-stimulated PIP_2 hydrolysis and IP_3-dependent events may be the consequence of a receptor reserve. Since in our experiments the ratio between the EC_{50} values for IP_3 release and the other hormone events varied with the hormone used, the spare-receptor hypothesis would predict that the binding capacities for NE, ANG, and AVP would be

TABLE 2

Lack of effect of 10 mM LiCl on concentrations of hormones required for half-maximal stimulation of IP_3 formation in hepatocytes

IP_3 formation was measured as described in "Materials and Methods" in hepatocytes incubated for 5 min with hormones at the concentrations shown in Fig. 1.

IP_3 formation	NE	EC_{50}	
		ANG II	AVP
		nM	
−LiCl	160 ± 40	1.9 ± 0.4	1.3 ± 0.1
+LiCl	320 ± 140	1.3 ± 0.5	1.3 ± 0.2

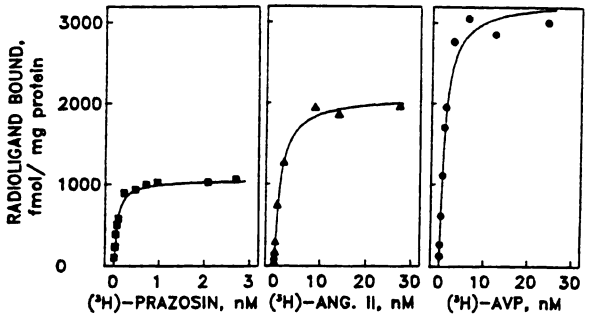

FIG. 3. *Concentration-dependent specific binding of [³H]prazosin, [³H]angiotensin II, and [³H]vasopressin*

Binding studies were performed and analyzed as described in "Materials and Methods" using 12–20 μg of membrane protein/tube. In order to achieve bound/free radioligand ratios less than 5%, [³H] prazosin binding was assayed in a 1250-μl rather than 200-μl total volume. The specific binding parameters determined and drawn by the MLAB program (26) were: for [³H]prazosin, B_{max} = 1060 ± 18 fmol/mg, K_d = 0.068 ± 0.0058 nM; for [³H]angiotensin II, B_{max} = 2088 ± 45 fmol/mg, K_d = 1.22 ± 0.12 nM; and for [³H]vasopressin, B_{max} = 3121 ± 170 fmol/mg, K_d = 0.98 ± 0.15 nM. Each panel shows an experiment which is representative of four or more such studies, and each point is the average of triplicate determinations.

different. In order to test this, concentration-dependent specific binding to α_1-adrenergic, ANG, and AVP receptors was measured using liver plasma membranes. As can be seen in Fig. 3, binding reached different maximal levels for each of the agents tested. This pattern was consistently observed using different batches of plasma membranes and radioligands. Thus, α_1-adrenergic receptors, characterized using [³H]prazosin, exhibited the lowest binding capacity; ANG receptors, studied with [³H] ANG, showed an intermediate B_{max}; while [³H]AVP binding exhibited the highest capacity. NE (100 μM) completely inhibited the α_1-adrenergic specific binding of 1 nM [³H]prazosin (data not shown). As presented in detail elsewhere (20), the NE concentration-dependence inhibition curves exhibit high and low affinity components in the absence of guanine nucleotides, and a single low affinity component in the presence of 0.2 mM guanyl-5′-yl imidodiphosphate. Guanine nucleotides have similar effects on AVP and ANG binding.[6]

Relationship between B_{max} for binding and concentration-dependent hormone responses. Fig. 4 indicates that there was a very close correlation (r = 0.993) between maximal agonist binding to membrane receptors and the

[6] C. J. Lynch and J. H. Exton, unpublished observations.

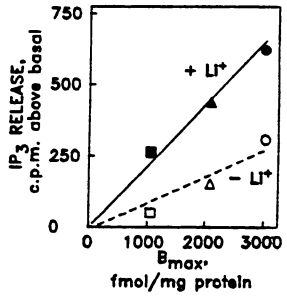

FIG. 4. *Relationship between receptor binding capacities and the release of IP_3 stimulated by supramaximal concentrations of agonists in the presence and absence of Li^+*

B_{max} values are in fmol/mg of liver membrane protein, and IP_3 release is expressed as maximally stimulated release-basal release in cpm/ml of cells during 5 min in the presence (——) or absence (– – –) of 10 mM LiCl (data from Table 1). The agonist concentrations for maximal stimulation of IP_3 release were as follows: □ and ■, α_1-adrenergic receptor (10 μM NE); △ and ▲, angiotensin II receptor (0.1 μM ANG); ○ and ●, vasopressin receptor (0.1 μM AVP).

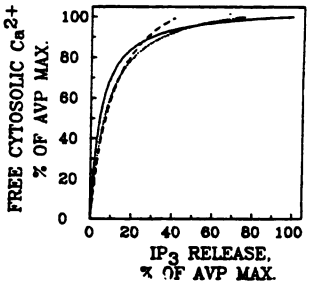

FIG. 5. *Relationship between IP_3 concentrations and free cytosolic Ca^{2+} levels*

The ordinates from the curve-fitted line for free cytosolic Ca^{2+} increases (monitored with the fluorescent indicator Quin-2) from Fig. 1 are plotted against the ordinate from the curve-fitted line for [³H]IP_3 release for each of the agonists studied. The *broken line* (– – –) represents NE, the very *short dashed line* (-----) represents ANG, and the *solid line* (——) represents AVP. The curves for NE and ANG are not complete since maximum IP_3 release was less than that with AVP (Fig. 1).

maximal capacity of the receptors to generate IP_3 in hepatocytes in the presence of Li^+. There was also a high correlation coefficient (0.992) between these parameters in the absence of Li^+. Furthermore, half-maximal binding was achieved with 1 nM ANG or AVP (Fig. 3) which was very close to the EC_{50} for IP_3 generation (Fig. 1 and Table 2). Likewise, the K_d for NE at the high affinity α_1-adrenergic binding site was 30–60 nM (20), which was close to the EC_{50} for NE on IP_3 formation (Fig. 1).

On the other hand, the relationships between IP_3 concentration and free cytosolic Ca^{2+} with different agonists (Fig. 5) indicated that maximum activation of the enzyme could be achieved with submaximal concentrations of IP_3 and that the relationship was not influenced by the agonist employed. These facts suggest that the IP_3 generated in response to different agonists is equally effective in raising cytosolic Ca^{2+} and that a small increase in IP_3 (3–6% of AVP maximum) is sufficient to half-maximally increase cytosolic Ca^{2+}.

DISCUSSION

Several investigators have recently presented data supporting the concept that IP_3 is a second messenger for intracellular Ca^{2+} mobilization in liver (for review see Ref. 10). The evidence includes the finding that the increase in IP_3 induced by maximally effective concentrations of AVP or epinephrine occurs within 3-sec, which is coincident with the increase in cytosolic Ca^{2+} (8). IP_3 also releases Ca^{2+} from saponin- and digitonin-permeabilized cells from several tissues including liver (1, 2, 4–7, 9) and from subcellular fractions enriched in mitochondria or endoplasmic reticulum (2, 7, 9). These fractions also show the largest loss of Ca^{2+} in perfused livers exposed to α_1-adrenergic agonists, AVP, and ANG (28). The half-maximally effective concentration of IP_3 to release Ca^{2+} from permeabilized cells or isolated organelles is approximately 0.1 μM (2, 9). This is within the range estimated for cytosolic IP_3 in liver cells (8). The Ca^{2+} released by IP_3 is rapidly taken back up into the organelles, due in part to the rapid dephosphorylation of IP_3 to inactive *myo*-inositol bis- and monophosphate (7).

In our experiments, stimulation of Ca^{2+} efflux, cytosolic Ca^{2+} elevation, and phosphorylase activation by each hormone typically displayed similar concentration dependencies. In contrast, stimulation of IP_3 formation required higher hormone concentrations (Fig. 1). This is in agreement with previous studies on the effects of AVP and ANG on IP_3 release and PIP_2 hydrolysis (8, 11–13). On the other hand, Thomas *et al.* (3) have claimed that the initial *rates* of stimulated IP_3 release, PIP_2 hydrolysis, and cytosolic Ca^{2+} elevation induced by vasopressin show similar concentration dependencies, while the *rate* of phosphorylase activation is at least an order of magnitude more sensitive. However, as pointed out in Footnote 5, it is unclear how Thomas *et al.* (3) calculated some of these rates since very large time lags are observed before low concentrations of vasopressin initiate increases in cytosolic Ca^{2+} and phosphorylase *a*. Furthermore, the accurate measurement of IP_3 changes at very low agonist concentrations is exceedingly difficult.

In addition to the concentration-response differences shown in Fig. 1, the maximal amounts of IP_3 released with the hormones were very different (Table 1), in agreement with previous studies on phosphatidylinositol and PIP_2 hydrolysis (12, 13, 30). On the other hand, the changes in cell Ca^{2+} content, cytosolic Ca^{2+}, and phosphorylase *a* displayed similar E_{max} values.

The preceding findings might be interpreted as indicating that IP_3 generation is secondary to or not involved in Ca^{2+} mobilization and subsequent events. Instead, Michell and co-workers (12, 13) have suggested that they are due to the presence of spare receptors for each hormone. While this explanation is plausible, the binding data presented by Michell's group (12, 13) were taken from cell binding studies performed by others (31), and only data for [^{3}H]AVP were presented. Additionally, the explanation was based on hormone binding affinities rather than maximal binding capacities measured at equilibrium, and the hormone responses were measured at considerably shorter times.

While comparisons between the EC_{50} values for agonist binding to isolated membranes and those for responses in intact cells are often attempted (see for instance Ref. 32), they may not be valid because AVP as well as ANG and α_1-adrenergic receptors may couple to transducing (guanine nucleotide-binding) proteins which alter receptor affinity for agonists, but do not affect B_{max} (20, 21, 23, 31, 33, 34). As noted above, GTP and its analogues convert the high affinity α_1-adrenergic specific binding of NE to liver membranes (K_d = 30–60 nM) to low affinity binding (K_d = 1–3 μM) (20). They also increase the K_d values for ANG and AVP from 0.5–4 to 4–10 nM and from 0.5–2 to 1–11 nM, respectively (23, 31, 34). The K_d values for ANG and AVP binding to liver membranes in the presence of the guanine nucleotides are comparable to those for binding to intact hepatocytes (23, 31) which are 30 and 15 nM, respectively.[7] These data suggest that the affinity of binding of ANG and AVP to intact hepatocytes is influenced by endogenous guanine nucleotides. To re-emphasize a point made above, the effects of these nucleotides would influence correlations based on the affinity of plasma membrane receptors for agonists, but not those based on the maximum number of binding sites.

Another approach to the evaluation of spare receptors involves the use of agents which irreversibly inactivate the receptors such as phenoxybenzamine, which covalently binds to α_1-adrenergic receptors (see Refs. 35–37). In this way, it is possible to lower and measure receptor availability by radioligand binding while simultaneously evaluating receptor-mediated responses. Previous studies of this nature on liver (36) indicate a close relationship between α_1-adrenergic receptor density and responses to α_1-adrenergic agonists and suggest that the hepatic α_1-adrenergic receptor reserve is small. Similar studies have not been done for ANG and AVP.

Another consideration in the present study is the chemical nature of the IP_3 generated by agonists in the absence or presence of Li^+. Essentially all reported studies in liver (1, 2, 7, 8) and other tissues have assumed this to be *myo*-inositol-1,4,5-P_3 since this is the active isomer for Ca^{2+} mobilization (38), and the only phosphatidylinositol bisphosphate reported in mammalian tissues so far is phosphatidylinositol-4,5-P_2. Furthermore, the separation and quantitation of the different IP_3 isomers are extremely difficult and time-consuming, and only one reported study has attempted to characterize the IP_3 accumulated in response to agonists (39). This study of carbachol-stimulated IP_3 formation in parotid glands indicated that *myo*-inositol-1,3,4-P_3 accumulated along with *myo*-inositol-1,4,5-P_3. However, the source of the *myo*-inositol-1,3,4-P_3 was unclear, and it could have been derived from the *myo*-inositol-1,4,5-P_3 by isomerization during the 15-min incubation. If other forms of IP_3 besides *myo*-inositol-1,4,5-P_3 also accumulate in liver because of isomerization, this would not alter the major conclusions of this study unless the agonists differentially affected the isomerization, i.e., altered the proportion of *myo*-inositol-1,4,5-P_3 in the total IP_3. As shown

[7] Corresponding whole cell binding studies have not been reported for NE.

in fig. 5, the effectiveness of IP_3 as a stimulator of cytosolic Ca^{2+} elevation appeared to be the same regardless of the agonist employed to generate it.[8]

As noted in "Results," Li^+ increased the accumulation of IP_3 in response to the three agonists used, but did not alter the concentration-response curves for the agonists on cytosolic Ca^{2+} elevation and phosphorylase activation. At first sight, these facts would appear to be at odds with the conclusion that the IP_3 concentration controls the cytosolic Ca^{2+} level and consequently phosphorylase activation. A possible explanation could be that the elevation in IP_3 attributable to Li^+ is due to an increase in an isomer(s) other than *myo*-inositol-1,4,5-P_3. In support of this explanation is the observation that Li^+ does not inhibit the rat liver phosphatase which degrades *myo*-inositol-1,4,5-P_3 (40). If, in fact, the increase in IP_3 induced by Li^+ were due to the accumulation of an isomer(s) which does not mobilize intracellular Ca^{2+}, this would account for the failure of Li^+ to alter the concentration dependence for agonists on cytosolic Ca^{2+} and phosphorylase. Such an effect of Li^+ would also explain why the concentration dependence for agonists on IP_3 is not altered by Li^+ (Table 2), if it is again assumed that the other isomer(s) is derived from *myo*-inositol-1,4,5-P_3. As illustrated in Fig. 4, the inclusion of Li^+ in some of the experiments of the present study does not alter the conclusion that maximum receptor occupancy and maximum IP_3 generation are correlated, but it obviously alters the quantitative relationship between these two parameters.

The major conclusions from our study are that, for NE, ANG, and AVP in liver, there is a close correlation (r = 0.993, Fig. 4) between receptor number and the capacity for generation of IP_3, which is the putative second messenger for intracellular Ca^{2+} mobilization, and that maximal Ca^{2+} mobilization and the resultant activation of phosphorylase can be elicited by submaximal concentrations of IP_3. These conclusions suggest that the disparity between the concentration-response curves for hormone-stimulated phosphorylase *a* or Ca^{2+} efflux and IP_3 release may be due at least in part to receptor reserve. However, further experiments, e.g., with compounds which irreversibly inactivate AVP and ANG receptors, will be required in order to prove this hypothesis. The data of Fig. 5 indicate that a very small increase of IP_3 (approximately 20%) in liver cells is sufficient to fully mobilize intracellular Ca^{2+} and fully activate phosphorylase. They also indicate that the relationship between IP_3 release and Ca^{2+} mobilization is independent of the type of receptor which mediates these responses. The data of Fig. 4 suggest that this elevation of IP_3 can be achieved with occupancy of a small fraction of the total number of receptors for any of the agonists tested.

REFERENCES

1. Streb, H., R. F. Irvine, M. J. Berridge, and I. Schulz. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* **306:** 67–69, 1983.
2. Joseph, S. K., A. P. Thomas, R. J. Williams, R. F. Irvine, and J. R. Williamson. *myo*-Inositol 1,4,5-trisphosphate. A second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver. *J. Biol. Chem.* **259:** 3077–3081, 1984.
3. Thomas, A. P., J. Alexander, and J. R. Williamson. Relationship between inositol polyphosphate production and the increase of cytosolic free Ca^{2+} induced by vasopressin in isolated hepatocytes. *J. Biol. Chem.* **259:** 5574–5584, 1984.
4. Burgess, G. M., P. P. Godfrey, J. S. McKinney, M. J. Berridge, R. F. Irvine, and J. W. Putney, Jr. The second messenger linking receptor activation to internal Ca release in liver. *Nature* **309:** 63–66, 1984.
5. Prentki, M., T. J. Biden, D. Janjic, R. F. Irvine, M. J. Berridge, and C. B. Wollheim. Rapid mobilization of Ca^{2+} from rat insulinoma microsomes by inositol-1,4,5-trisphosphate. *Nature* **309:** 562–564, 1984.
6. Suematsu, E., M. Hirata, T. Hashimoto, and H. Kuriyama. Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. *Biochem. Biophys. Res. Commun.* **120:** 481–485, 1984.
7. Dawson, A. P., and R. F. Irvine. Inositol (1,4,5)trisphosphate-promoted Ca^{2+} release from microsomal fractions of rat liver. *Biochem. Biophys. Res. Commun.* **120:** 858–864, 1984.
8. Charest, R., V. Prpic, J. H. Exton, and P. F. Blackmore. Stimulation of inositol triphosphate formation in hepatocytes by vasopressin, adrenaline, and angiotensin II and its relationship to changes in cytosolic free Ca^{2+}. *Biochem. J.* **227:** 79–90, 1985.
9. Exton, J. H. Mechanisms involved in α-adrenergic phenomena. *Am. J. Physiol.* **248** (*Endocrinol. Metab. II*): E633–E647, 1985.
10. Berridge, M. J. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220:** 345–360, 1984.
11. Rhodes, D., V. Prpic, J. H. Exton, and P. F. Blackmore. Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in hepatocyes by vasopressin. *J. Biol. Chem.* **258:** 2770–2773, 1983.
12. Kirk, C. J., J. A. Creba, C. P. Downes, and R. H. Michell. Hormone-stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. *Biochem. Soc. Trans.* **9:** 377–379, 1981.
13. Creba, J. A., C. P. Downes, P. T. Hawkins, G. Brewster, R. H. Michell, and C. J. Kirk. Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca^{2+}-mobilizing hormones. *Biochem. J.* **212:** 733–747, 1983.
14. Hutson, N. J., F. T. Brumley, F. D. Assimacopoulos, S. C. Harper, and J. H. Exton. Studies on the α-adrenergic activation of hepatic glucose output. I. Studies on the α-adrenergic activation of phosphorylase and gluconeogenesis and inactivation of glycogen synthase in isolated rat liver parenchymal cells. *J. Biol. Chem.* **251:** 5200–5208, 1976.
15. Prpic, V., K. C. Green, P. F. Blackmore, and J. H. Exton. Vasopressin-, angiotensin II-, and α_1-adrenergic-induced inhibition of Ca^{2+} transport by rat liver plasma membrane vesicles. *J. Biol. Chem.* **259:** 1382–1385, 1984.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275, 1951.
17. Blackmore, P. F., and J. H. Exton. Assessment of effects of vasopressin, angiotensin II and glucagon on Ca^{2+} fluxes and phosphorylase activity in liver. *Methods Enzymol.* **109:** 550–558, 1985.
18. Blackmore, P. F., T. F. Brumley, J. L. Marks, and J. H. Exton. Studies of hepatic glucose output. Relationship between α-adrenergic stimulation of calcium efflux and activation of phosphorylase in isolated rat liver parenchymal cells. *J. Biol. Chem.* **253:** 4851–4858, 1978.
19. Charest, R., P. F. Blackmore, B. Berthon, and J. H. Exton. Changes in free cytosolic Ca^{2+} in hepatocytes following α_1-adrenergic stimulation. Studies on Quin-2-loaded hepatocytes. *J. Biol. Chem.* **258:** 8769–8773, 1983.
20. Lynch, C. J., R. Charest, P. F. Blackmore, and J. H. Exton. Studies on the hepatic α_1-adrenergic receptor. Modulation of guanine nucleotide effects by calcium, temperature, and age. *J. Biol. Chem.* **260:** 1593–1600, 1985.
21. Fahrenholz, F., R. Boer, P. Crause, G. Fritzsch, and Z. Grzonka. Interactions of vasopressin agonists and antagonists with membrane receptors. *Eur. J. Pharmacol.* **100:** 47–58, 1984.
22. Campanile, C. P., J. K. Crane, M. J. Peach, and J. C. Garrison. The hepatic angiotensin II receptor. I. Characterization of the membrane-binding site and correlation with physiological response in hepatocytes. *J. Biol. Chem.* **257:** 4951–4958, 1982.
23. Keppens, S., H. DeWulf, P. Clauser, S. Jard, and J. L. Morgat. The liver angiotensin receptor involved in the activation of glycogen phosphorylase. *Biochem. J.* **208:** 809–817, 1982.
24. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51:** 660–672, 1949.
25. Burgisser, E. Radioligand-receptor binding studies. What's wrong with the Scatchard analysis. *Trends Pharmacol. Sci.* **5:** 142–144, 1984.
26. Knott, G. MLAB—a mathematical modeling tool. *Comp. Prog. Biomed.* **10:** 271–280, 1969.
27. Blackmore, P. F., L. E. Waynick, G. E. Blackman, C. W. Graham, and R. S. Sherry. α- and β-adrenergic stimulation of parenchymal cell Ca^{2+} influx. Influence of extracellular pH. *J. Biol. Chem.* **259:** 12322–12325, 1984.
28. Reinhart, P. H., W. M. Taylor, and F. L. Bygrave. The role of calcium ions in the mechanism of action of α-adrenergic agonists in rat liver. *Biochem. J.* **223:** 1–13, 1984.

[8] This finding also argues against the possibility that the agonists differentially stimulate the breakdown of other PIP_2 isomers besides phosphatidylinositol-4,5-P_2.

29. Morgan, N. G., P. F. Blackmore, and J. H. Exton. Age-related changes in the control of hepatic cAMP levels by α_1- and β_2-adrenergic receptors in male rats. *J. Biol. Chem.* **258:** 5103–5109, 1983.
30. Prpic, V., P. F. Blackmore, and J. H. Exton. Phosphatidylinositol breakdown induced by vasopressin and epinephrine in hepatocytes is calcium-dependent. *J. Biol. Chem.* **257:** 11323–11331, 1982.
31. Cantau, B., S. Keppens, H. DeWulf, and S. Jard. [^{3}H]Vasopressin binding to isolated rat hepatocytes and liver membranes. Regulation by GTP and relation to glycogen phosphorylase activation. *J. Receptor Res.* **1:** 137–168, 1980.
32. Sastre, A., K. K. Griendling, M. M. Rusher, and W. R. Milnor. Relation between α-adrenergic receptor occupation and contractile response. Radioligand and physiologic studies in canine aorta. *J. Pharmacol. Exp. Ther.* **229:** 887–896, 1984.
33. Goodhardt, M., N. Ferry, P. Geynet, and J. Hanoune. Hepatic α_1-adrenergic receptors show agonist-specific regulation by guanine nucleotides. Loss of nucleotide effect after adrenalectomy. *J. Biol. Chem.* **257:** 11577–11583, 1982.
34. Crane, J. K., C. P. Campanile, and J. C. Garrison. The hepatic angiotensin II receptor. II. Effect of guanine nucleotides and interaction with cyclic AMP production. *J. Biol. Chem.* **257:** 4959–4965, 1982.
35. Minneman, K. P., and P. W. Abel. "Spare" α_1-adrenergic receptors and the potency of agonists in rat vas deferens. *Mol. Pharmacol.* **25:** 56–63, 1983.
36. Lynch, C. J., R. C. Deth, and M. L. Steer. Simultaneous loss and reappearance of [^{3}H]prazosin binding sites and α_1-adrenergic events in rat liver after phenoxybenzamine administration. *Biochim. Biophys. Acta* **757:** 156–163, 1983.
37. Guellaen, G., and J. Hanoune. Hepatic α-adrenoceptor specific and irreversible labeling by [^{3}H]phenoxybenzamine. *Biochem. Biophys. Res. Commun.* **89:** 1178–1185, 1979.
38. Burgess, G. M., R. F. Irvine, M. J. Berridge, J. S. McKinney, and J. W. Putney, Jr. Actions of inositol phosphates on Ca^{2+} pools in guinea-pig hepatocytes. *Biochem. J.* **224:** 741–746, 1984.
39. Irvine, R. F., A. J. Letcher, D. J. Lander, and C. P. Downes. Inositol trisphosphates in carbachol-stimulated rat parotid glands. *Biochem. J.* **223:** 237–243, 1984.
40. Seyfred, M. A., L. E. Farrell, and W. W. Wells. Characterization of D-*myo*-inositol 1,4,5-trisphosphate phosphatase in rat liver plasma membranes. *J. Biol. Chem.* **259:** 13204–13208, 1984.

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