Noradrenaline, vasopressin and angiotensin increase Ca^{2+} influx by opening a common pool of Ca^{2+} channels in isolated rat liver cells

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The effects of the Ca^{2+} -mobilizing hormones noradrenaline, vasopressin and angiotensin on the unidirectional influx of Ca^{2+} were investigated in isolated rat liver cells by measuring the initial rate of $^{45}\text{Ca}^{2+}$ uptake. The three hormones increased Ca^{2+} influx, with EC_{50} values (concentrations giving half-maximal effect) of $0.15\ \mu\text{M}$, $0.44\ \text{nM}$ and $0.8\ \text{nM}$ for noradrenaline, vasopressin and angiotensin respectively. The actions of noradrenaline and angiotensin were evident within seconds after their addition to the cells, whereas the increase in Ca^{2+} influx initiated by vasopressin was slightly delayed (by 5–15 s). The activation of Ca^{2+} influx was maintained as long as the receptor was occupied by the hormone. The measurement of the resting and hormone-stimulated Ca^{2+} influxes at different external Ca^{2+} concentrations revealed Michaelis–Menten-type kinetics compatible with a saturable channel model. Noradrenaline, vasopressin and angiotensin increased both K_m and $V_{\max}$ of Ca^{2+} influx. It is proposed that the hormones increase the rate of translocation of Ca^{2+} through a common pool of Ca^{2+} channels without changing the number of available channels or their affinity for Ca^{2+} .

The transmembrane electrochemical gradient for Ca^{2+} is very large in rat liver cells, as for other tissues (Thomas, 1982). Recent detection of cytoplasmic Ca^{2+} by the null-point method (Murphy *et al.*, 1980) or by the fluorescent Ca^{2+} indicator quin 2 (Charest *et al.*, 1983; Berthon *et al.*, 1984) indicates that $[\text{Ca}^{2+}]_i$ (intracellular $[\text{Ca}^{2+}]$) is about $100\text{--}200\ \text{nM}$. For a negative membrane potential (Claret & Mazet, 1972; Haylett & Jenkinson, 1972) and at millimolar concentration of external Ca^{2+} , the passive Ca^{2+} influx is necessarily much larger than the passive Ca^{2+} efflux. In the liver, the resulting net influx is balanced by the ATP-dependent Ca^{2+} pump (Claret-Berthon *et al.*, 1977; Lotersztajn *et al.*, 1981; Iwasa *et al.*, 1982). Very few results have been reported on the properties of Ca^{2+} influx in the mammalian liver (Claret-Berthon *et al.*, 1977; Barritt *et al.*, 1981; Studer & Borle, 1983) although, given the transmembrane Ca^{2+} gradient, it should be involved in the control of $[\text{Ca}^{2+}]_i$. The Ca^{2+} -mobilizing hormones nor-

adrenaline, vasopressin and angiotensin cause a rapid and sustained increase in cytosolic Ca^{2+} (Murphy *et al.*, 1980; Charest *et al.*, 1983; Berthon *et al.*, 1984). We have observed (Berthon *et al.*, 1984) that the rise in $[\text{Ca}^{2+}]_i$ is affected by removing external Ca^{2+} from isolated rat hepatocytes, suggesting a contribution of Ca^{2+} influx either directly in increasing $[\text{Ca}^{2+}]_i$ or in reloading the internal Ca^{2+} stores mobilized by the hormones. In the present work we have analysed the unidirectional Ca^{2+} influx into isolated rat liver cells and examined the effects of the Ca^{2+} -mobilizing hormones. The results confirm that noradrenaline, vasopressin and angiotensin stimulate unidirectional Ca^{2+} influx in rat hepatocytes, as previously observed by others (Assimakopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977; Barritt *et al.*, 1981). The effect is evident within seconds after the addition of the agonists, is sustained as long as the agonist is bound to its receptor and is rapidly reversed by the antagonists. The measurement of the influx as a function of $[\text{Ca}^{2+}]_o$ (extracellular $[\text{Ca}^{2+}]$) indicates that Ca^{2+} enters the cell via a saturable process and that the three hormones increase both K_m and $V_{\max}$ of Ca^{2+} influx through a common pool of Ca^{2+} channels (or carriers).

Abbreviation used: d(CH₂)₅tyr(et)VAVP, [1-(β -mercapto- $\beta\beta$ -cyclopentamethylenepropionic acid), 2-(α -ethyltyrosine), 4-valine, 8-arginine]vasopressin.

Experimental

Preparation of dispersed rat liver cells

Parenchymal cells were isolated from the liver of fed female Wistar rats (200–220 g) by a collagenase method as previously described (Burgess *et al.*, 1981). Unless otherwise indicated, the cells were incubated at 37°C in Eagle's medium containing (mM): NaCl, 116; KCl, 5.4; CaCl₂, 1.8; MgSO₄, 0.81; NaH₂PO₄, 0.96; NaHCO₃, 25; and (mg/l): glutamine, 292; amino acids, 805; vitamins, 8.1; glucose, 1000; supplemented with 2% bovine serum albumin and gassed with O₂/CO₂ (19:1).

Measurement of ⁴⁵Ca²⁺ influx

The influx measurement was started by adding 500 µl of the cell suspension (2 × 10⁶–3 × 10⁶ cells/ml, i.e. about 4.4–6.6 mg dry wt./ml) to trace amounts of ⁴⁵CaCl₂ (2–3 µCi/ml) and to the hormone. In some experiments (see the Results section), the cells were preincubated for 50–60 min in Eagle's medium containing 150 µM-Ca²⁺ and the influx was assessed by adding the desired Ca²⁺ concentration, ⁴⁵Ca²⁺ and the hormone to the cell suspension. At Ca²⁺ concentrations lower than 150 µM, EGTA (75 µM) was present. The concentration of free Ca²⁺ was calculated by using the dissociation constant of the EGTA–Ca²⁺ complex (227 nM) proposed by Bartfai (1979). The ⁴⁵Ca²⁺ uptake was determined by taking 100 µl samples at 15, 45, 75 and 105 s. Each was immediately diluted in 4 ml of an ice-cold 'washing solution' containing (mM): NaCl, 144; CaCl₂, 5; Tris/HCl (pH 7.4), 5. The mixture was then filtered through a Whatman GF/C glass filter and washed with 3 × 4 ml of the ice-cold 'washing solution'. Care was taken that the filter did not get into contact with air before the termination of the washing procedure. In these conditions, the cellular K⁺ content determined by flame spectrophotometry was 250–260 nmol/mg dry wt., which is 90% of the K⁺ content determined in cells from the same suspension washed by centrifugation at 12000 g for 30 s. This indicates that the filtration procedure did not alter the ionic permeabilities of rat hepatocytes. The radioactivity associated to the filter was then counted in a scintillation spectrometer after addition of the appropriate scintillation liquid.

Analysis of the results

The unidirectional influx of Ca²⁺ was determined from the initial rate of ⁴⁵Ca²⁺ uptake by the cells. The sampling period (0–105 s) was short enough as compared with the time constant (10–20 min) of ⁴⁵Ca²⁺ exchange in rat liver (Claret-Berthon *et al.*, 1977; Studer & Borle, 1983) to minimize the underestimation of Ca²⁺ influx. It may be estimated from the correction factor

$(t/\tau)/[1 - \exp(-t/\tau)]$, where τ is the time constant of Ca²⁺ exchange and t the loading time (Keynes, 1954), that after 105 s the measured influx was underestimated by 6–8%. This is reasonably in the range of the experimental error associated with flux measurements. This was experimentally confirmed by the observation of linear ⁴⁵Ca²⁺ uptakes over 105 s periods as estimated by linear-regression analysis (see Fig. 1).

Extrapolation of Ca²⁺ uptake to the ordinate gave values different from zero, indicating that a small amount of ⁴⁵Ca²⁺ was not displaced by the 'washing solution' containing 5 mM non-radioactive Ca²⁺. Similar results were found when the solution contained 0.1 mM-La³⁺ or 2 mM-EGTA instead of Ca²⁺. Of this fraction of ⁴⁵Ca²⁺ resistant to repeated washings, 60% resulted from ⁴⁵Ca²⁺ bound to the glass-fibre filter, and the remaining 40% presumably from binding to permeabilized cells. In agreement with this conclusion, if the cells were incubated at 0°C to block ⁴⁵Ca²⁺ exchange through plasma membranes, the radioactivity remaining on the filter was not different from the values obtained by extrapolation to zero time in experiments at 37°C.

Materials

⁴⁵Ca²⁺ was supplied by I.R.E., Fleurus, Belgium; amino acids and vitamins were from Gibco Europe; collagenase was from Boehringer; (–)-noradrenaline, [arginine]vasopressin, angiotensin, (±)-propranolol, carbonyl cyanide *m*-chlorophenylhydrazone and albumin (fraction V) were from Sigma; phenoxybenzamine was given by Smith, Kline and French; phentolamine was given by Ciba-Geigy; d(CH₂)₅tyr(et)VAVP and [1-sarcosine, 8-isoleucine]angiotensin were generously given by Dr. S. Jard and Dr. G. Guillon (Centre CNRS-INSERM Pharmacologie-Endocrinologie, Montpellier, France). All other materials were of analytical grade.

Results

Effects of noradrenaline, vasopressin and angiotensin on the unidirectional influx of Ca²⁺

The uptake of ⁴⁵Ca²⁺ was measured 15, 45, 75 and 105 s after the addition of trace amounts of ⁴⁵Ca²⁺ to the cells incubated in the presence of 1.8 mM-CaCl₂ (Fig. 1). The basal Ca²⁺ influx as calculated from the slope of the regression lines was 339 ± 15 pmol/min per mg cell dry wt. ($n = 18$). Maximal concentrations of noradrenaline (1 µM), vasopressin (10 nM) and angiotensin (10 nM) added at the same time as ⁴⁵Ca²⁺ increased the influx by 35, 160 and 150% respectively (see Fig. 1 and Table 1).

Table 1 shows the effect of the antagonists on the

Table 1. Effect of noradrenaline, vasopressin and angiotensin on Ca²⁺ influx in isolated rat hepatocytes

The cells were incubated and Ca²⁺ influx was measured as indicated in the legend to Fig. 1. When used, the antagonists were added 6 min before ⁴⁵Ca²⁺. Results are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses.

Addition	Ca ²⁺ influx (pmol/min per mg dry wt.)
0.9% NaCl	339 $\pm$ 15 (18)
Noradrenaline (1 μ M)	458 $\pm$ 55 (8)
Noradrenaline (1 μ M) + phenoxybenzamine (50 μ M)	349 $\pm$ 59 (10)
Vasopressin (10 nM)	881 $\pm$ 85 (9)
Vasopressin (10 nM) + d(CH ₂) ₅ tyr(et)VAVP (50 nM)	329 $\pm$ 68 (3)
Angiotensin (10 nM)	848 $\pm$ 51 (6)
Angiotensin (10 nM) + [1-sarcosine, 8-isoleucine]angiotensin (10 nM)	339 $\pm$ 17 (6)

Fig. 1. Uptake of ⁴⁵Ca²⁺ into rat hepatocytes as a function of time

Rat hepatocytes (4.3 mg dry wt./ml) were incubated in Eagle's medium containing 2% albumin and 5 μ M-propranolol under O₂/CO₂ (19:1) at 37°C. Trace amounts of ⁴⁵Ca²⁺ were added at zero time, and samples were taken at the indicated time, washed and counted for radioactivity as indicated in the Experimental section. When tested, the hormones were added at the same time as ⁴⁵Ca²⁺. The lines drawn were determined by linear-regression analysis. The correlation coefficient were: 0.991 for control uptake (●), and 0.991, 0.997 and 0.998 in the presence of, respectively, 1 μ M-noradrenaline (○), 10 nM-vasopressin (□) and 10 nM-angiotensin (■). Each point is the mean of triplicate determinations in one typical experiment.

α -adrenergic activity of noradrenaline in isolated rat liver cells. Similarly, the antagonists d(CH₂)₅tyr(et)VAVP (50 nM) and [1-sarcosine, 8-isoleucine]angiotensin (10 nM) totally abolished the stimulatory effects of vasopressin and angiotensin.

Ca²⁺ influx is not mediated by Ca²⁺/Ca²⁺ exchange

The following experiments were done to exclude the possibility that the increase in Ca²⁺ influx was secondary to an increase in [Ca²⁺]_i. This could have occurred if the rapid rise in cytosolic Ca²⁺ concentration (resulting from the release of Ca²⁺ from internal stores initiated by the hormones) had promoted an influx of Ca²⁺ mediated by a Ca²⁺/Ca²⁺ exchange mechanism. This was tested by examining the influence of different agents which are known to mimic the hormone action by increasing [Ca²⁺]_i (Chen *et al.*, 1978; Murphy *et al.*, 1980; Blackmore *et al.*, 1982) on Ca²⁺ influx. The mitochondrial inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (10 μ M) or the uncoupler 2,4-dinitrophenol (100 μ M) applied at zero time altered neither the basal nor the hormone-stimulated Ca²⁺ influx (results not shown). These results clearly indicate that the Ca²⁺ influx initiated by noradrenaline, vasopressin and angiotensin was not a secondary consequence of changes in [Ca²⁺]_i.

Time courses of the action of hormones

It is noteworthy that the effects of noradrenaline and angiotensin on Ca²⁺ influx were evident as early as 15 s. In contrast, Fig. 1 shows that the action of vasopressin cannot be detected at this time. If vasopressin was added 30 s before ⁴⁵Ca²⁺, the delay in the hormone action was no longer apparent. However, in any case the duration of the lag phase cannot be precisely determined, since the Ca²⁺ influx was not measured between 0 and 15 s.

Fig. 2 shows the time course of the effects of maximal concentrations of noradrenaline and vasopressin added at the same time as, or 2, 4 and 6 min before, ⁴⁵Ca²⁺. The stimulated influx was maintained as long as the cells were in the presence

hormone-stimulated Ca²⁺ influx in isolated rat hepatocytes. The antagonists were added 6 min before the agonist to ensure maximal binding to membrane receptors. The activation of Ca²⁺ influx by noradrenaline (measured in the presence of 5 μ M-propranolol to eliminate any β -adrenergic effect) was inhibited by the α -adrenergic antagonist phenoxybenzamine (50 μ M). This confirms the

of each hormone. Fig. 2 also shows that the α -adrenergic antagonist phentolamine used at a concentration ($10\ \mu\text{M}$) sufficient to abolish the reassociation of noradrenaline to its receptor rapidly reversed the stimulation. This effect was evident as early as 15s and was complete within 2min. The Ca^{2+} influx measured in control cells or in the presence of vasopressin was not significantly altered by $10\ \mu\text{M}$ -phentolamine. These results indicate that stimulated Ca^{2+} influx is directly dependent on the activation of membrane receptors by the hormones.

Dose-response curves for the hormone-stimulated Ca^{2+} influx

Fig. 3 shows dose-responses curves for noradrenaline (in the presence of $5\ \mu\text{M}$ -propranolol), vasopressin and angiotensin. The respective concentrations giving half-maximal responses were $0.15\ \mu\text{M}$, $0.44\ \text{nM}$ and $0.80\ \text{nM}$, which are in close agreement with those obtained for Ca^{2+} -dependent phosphorylase activation (El-Refai *et al.*, 1979; Cantau *et al.*, 1980; Keppens *et al.*, 1982).

Fig. 2. Time course of the effect of $1\ \mu\text{M}$ -noradrenaline and $10\ \text{nM}$ -vasopressin

Rat hepatocytes ($3.6\ \text{mg dry wt./ml}$) were incubated as indicated in the legend of Fig. 1. Noradrenaline ($\circ$) or vasopressin ($\square$) was added at zero time, and the influx was measured for 105s from the indicated time. When tested, $10\ \mu\text{M}$ -phentolamine was added at the time indicated by the arrow; the influx was then measured in the presence of noradrenaline and phentolamine ($\blacktriangledown$). The stimulated Ca^{2+} influx is expressed as a percentage of control Ca^{2+} influx ($\bullet$) ($294 \pm 28\ \text{pmol/min per mg dry wt.}$). Each point is the mean $\pm$ S.E.M. for three determinations.

Ca^{2+} -dependency of the Ca^{2+} influx

The dependency of Ca^{2+} influx on external Ca^{2+} concentration was investigated in the range $75\ \mu\text{M}$ – $4.8\ \text{mM}$. The cells were first preincubated in Eagle's medium containing $150\ \mu\text{M}$ - Ca^{2+} for 30–60 min. In preliminary experiments it was checked that the preincubation period did not alter Ca^{2+} influx subsequently determined at another Ca^{2+} concentration. Table 2 shows that identical resting and vasopressin-stimulated Ca^{2+} influxes were measured at $1.8\ \text{mM}$ - Ca^{2+} for a 12-fold change in the Ca^{2+} concentration of the preincubation medium.

Fig. 4(a) shows that Ca^{2+} influxes determined in both control cells and hormone-treated cells were non-linearly related to external Ca^{2+} concentration. The hormones noradrenaline ($1\ \mu\text{M}$), vasopressin ($10\ \text{nM}$) and angiotensin ($10\ \text{nM}$) stimulated Ca^{2+} influx at external Ca^{2+} concentrations higher than $150\ \mu\text{M}$. The activated Ca^{2+} influx increased up to about $2\ \text{mM}$ - Ca^{2+} with noradrenaline and was not saturated at $4.8\ \text{mM}$ - Ca^{2+} with the peptide hor-

Fig. 3. Dose-response curves for noradrenaline, vasopressin and angiotensin on Ca^{2+} influx

Ca^{2+} influxes were determined at the indicated concentrations of noradrenaline ($\circ$), vasopressin ($\square$) or angiotensin ($\blacksquare$) as described in the legend to Fig. 1. The stimulated Ca^{2+} influx is expressed as a percentage of control influx ($202 \pm 17\ \text{pmol/min per mg dry wt.}$). Each point is the mean $\pm$ S.E.M. for six determinations.

Table 2. *Effect of preincubation in low-Ca²⁺ medium on Ca²⁺ influx*

Rat hepatocytes (6 mg dry wt./ml) were preincubated for 60 min in Eagle's medium containing the indicated Ca²⁺ concentration. The influx was measured by adding ⁴⁵Ca²⁺ and CaCl₂ to give 1.8 mM-Ca²⁺ in the incubation medium. When tested, vasopressin was added at the same time as ⁴⁵Ca²⁺. Results are means ± S.E.M. for three determinations.

Addition	[Ca ²⁺] ₀ (mM) during preincubation ...	Ca ²⁺ influx (pmol/min per mg dry wt.)	
		0.15	1.8
0.9% NaCl		191 ± 23	179 ± 9
Vasopressin (10nM)		448 ± 47	436 ± 30

Fig. 4. *Influence of external Ca²⁺ concentration on Ca²⁺ influx*

Rat hepatocytes were incubated in Eagle's medium containing 2% albumin and 150 μM-CaCl₂, under O₂/CO₂ (19:1). The influx was measured with or without the agonists after the addition of trace amounts of ⁴⁵Ca²⁺ and the concentration of CaCl₂ or EGTA required to give the indicated Ca²⁺ concentrations. (a) Influxes were measured at the indicated Ca²⁺ concentration in control cells (●) or in the presence of 1 μM-noradrenaline (○), 10 nM-vasopressin (□) or 10 nM-angiotensin (■). Each point is the mean of triplicate determinations in one typical experiment. (b) Hofstee plots of the data of Fig. 4(a). Linear-regression analysis yielded straight lines with correlation coefficients of 0.970, 0.960, 0.996 and 0.997 for control cells or cells incubated with noradrenaline, vasopressin and angiotensin respectively. In this typical experiment *K_m* and *V_{max}* for Ca²⁺ were respectively 0.114 mM and 315 pmol/min per mg dry wt. for control cells, 0.190 mM and 499 pmol/min per mg for noradrenaline-treated cells, 0.710 mM and 1217 pmol/min per mg for vasopressin-treated cells and 1.46 mM and 2177 pmol/min per mg for angiotensin-treated cells. Mean values are given in Table 3.

Table 3. *Effect of noradrenaline, angiotensin and vasopressin on *K_m* and *V_{max}* of Ca²⁺ influx in rat hepatocytes*
K_m and *V_{max}* were determined by Hofstee analysis as described in Fig. 4. Each value is the mean ± S.E.M. for *n* experiments.

Addition ...	0.9% NaCl	Noradrenaline (1 μM)	Vasopressin (10nM)	Angiotensin (10nM)
<i>K_m</i> (mM)	0.121 ± 0.012	0.167 ± 0.030	0.82 ± 0.15	1.48 ± 0.10
<i>V_{max}</i> (pmol/min per mg dry wt.)	320 ± 22	421 ± 27	1270 ± 170	2140 ± 250
<i>n</i>	11	4	4	3

mones. The data of Fig. 4(a) can be linearized by Hofstee plots (Fig. 4b). This indicates that Ca²⁺ influx in resting isolated liver cells or after stimulation by the hormones followed Michaelis-Menten kinetics. Table 3 gives the mean values of *K_m* and

V_{max} determined in control cells and hormone-treated cells. It shows that noradrenaline, angiotensin and vasopressin increased both kinetic parameters of Ca²⁺ influx, but in different ways for each hormone.

Additivity of cell responses

A possible explanation for these discrepancies between the hormone effects on $V_{\max}$ and K_m was that different kinds of Ca^{2+} channels or carriers underlie the Ca^{2+} influxes caused by the three hormones. This was examined by testing the additivity of the cell responses. In these experiments maximal doses of hormones were used. Addition of vasopressin (10 nM) plus angiotensin (10 nM) or of vasopressin (10 nM) plus noradrenaline (1 μM) resulted in Ca^{2+} influx which was equivalent to the response to the peptide hormones alone. It appears then that the three Ca^{2+} -mobilizing hormones seem to use a common pool of Ca^{2+} channels or carriers to increase Ca^{2+} influx in isolated rat hepatocytes.

Discussion

In the present work we have studied the kinetic properties of the unidirectional Ca^{2+} influx in isolated rat hepatocytes by determining the $^{45}\text{Ca}^{2+}$ uptake within 105 s after the addition of the tracer. This period is short enough as compared with the time constant of exchangeable cell Ca^{2+} (10–20 min) (Claret-Berthon *et al.*, 1977; Studer & Borle, 1983) to prevent significant labelling of intracellular Ca^{2+} . Under these conditions, the influx of Ca^{2+} is not altered by a possible redistribution of exchangeable cell Ca^{2+} promoted by the hormones (Chen *et al.*, 1978; Blackmore *et al.*, 1978; Burgess *et al.*, 1981). Ca^{2+} influx is then only dependent on extracellular Ca^{2+} and on the permeability of cell membrane (Claret-Berthon *et al.*, 1977; Borle, 1981). Since $[\text{Ca}^{2+}]_o$ was experimentally fixed, any change in the initial rate of $^{45}\text{Ca}^{2+}$ uptake initiated by the Ca^{2+} -mobilizing hormones directly reflected the modifications of the translocation rate of Ca^{2+} through the plasma membrane. In agreement, linear $^{45}\text{Ca}^{2+}$ uptake was observed over 15–105 s in the hormone-treated or in the control cells.

The hormones that increased cytosolic Ca^{2+} (Murphy *et al.*, 1980; Charest *et al.*, 1983; Berthon *et al.*, 1984) also stimulated Ca^{2+} influx, as previously reported by others (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Barritt *et al.*, 1981). At 1.8 mM external Ca^{2+} concentration, the effect of noradrenaline on Ca^{2+} influx (135% of control) was substantially lower than those initiated by the peptide hormones (200–300% of control). These actions were dose-dependent and blocked by the α -adrenergic antagonist phenoxybenzamine and by the respective antagonists of vasopressin and angiotensin, namely $\text{d}(\text{CH}_2)_5\text{tyr}(\text{et})\text{VAVP}$ and [1-sarcosine, 8-isoleucine] angiotensin. This confirms that they were mediated by activation of the membrane receptors

to hormones. It was found that noradrenaline and angiotensin stimulated Ca^{2+} influx with no apparent lag. This property was not shared by vasopressin, since its action was delayed by about 5–15 s. This is in keeping with the observation that the α -adrenergic-mediated increase in cytosolic Ca^{2+} as monitored by the fluorescent Ca^{2+} indicator quin 2 is complete within 5 s, whereas the action of vasopressin has not reached its peak value by this time (Charest *et al.*, 1983; Berthon *et al.*, 1984). The kinetics of association of [^3H]lysine]vasopressin and [^3H]angiotensin to rat hepatocytes do not differ significantly (Cantau *et al.*, 1980; Keppens *et al.*, 1982). The observation of a delay in the action of vasopressin thus raises the possibility that the coupling mechanism between the receptor and the Ca^{2+} channel or carrier may differ from one hormone to another in isolated rat liver cells. Once it has been stimulated by the α -adrenergic agonists or by vasopressin, Ca^{2+} influx is sustained as long as the agonist is bound to its receptor. The stimulating effect of noradrenaline was reversed within 2 min if phentolamine was added to prevent reassociation of the agonist to its receptor (Fig. 2). Thus it appears that the increase in Ca^{2+} influx is directly related to the activation of membrane receptors by the hormones.

In the present work, we have also studied the dependence of the Ca^{2+} influx on external $[\text{Ca}^{2+}]$ and determined the effects of the hormones on the parameters that define the Ca^{2+} influx. In resting cells, Ca^{2+} influx depends on external $[\text{Ca}^{2+}]$ in the range of 75 μM to about 1 mM and reaches saturation at higher concentrations. In the presence of the hormones, the increase in Ca^{2+} influx occurs between 0.15 and 1.5 mM- Ca^{2+} for noradrenaline and between 0.3 and above 5 mM for the peptide hormones. The finding that Ca^{2+} influx is very dependent on $[\text{Ca}^{2+}]_o$ in this range of concentration might account for the controversies about the dependency on external Ca^{2+} of the hormonal responses in the liver (see Williamson *et al.*, 1981; Exton, 1981).

Hofstee plots of the data of Fig. 4(a) fit straight lines (Fig. 4b). This indicates that the influx of Ca^{2+} follows Michaelis–Menten kinetics. We can thus rule out a process of simple passive diffusion of Ca^{2+} through the plasma membrane. Saturable processes have also been found for the Ca^{2+} entry in both excitable cells (Hagiwara & Byerly, 1981; Kostyuk, 1982) and non-excitable cells, e.g. kidney cells (Borle, 1970) or pancreatic cells (Kondo & Schulz, 1976). In contrast, non-saturable Ca^{2+} influx has been reported in isolated liver cells (Parker *et al.*, 1983). However, those authors analysed the Ca^{2+} influx by curve peeling of $^{45}\text{Ca}^{2+}$ uptake, whereas this method cannot be used at non-steady state (see Claret-Berthon *et al.*, 1977; Borle, 1981).

Noradrenaline, angiotensin and vasopressin increased both $V_{\max}$ and K_m of Ca²⁺ influx, as determined by Hofstee plots. It is noteworthy that the increases in K_m and $V_{\max}$ initiated by the hormones were directly correlated. In other words, the order of efficiency of the hormones in increasing K_m was the same as the order of efficiency in increasing $V_{\max}$, i.e. angiotensin > vasopressin > noradrenaline (Table 3). This observation, together with the suggestion that the hormone apparently used a common pool of Ca²⁺ channels or carriers, may be explained by the model previously proposed for the effects of parathyryl in kidney cells (Borle, 1970) or the effect of pancreozymin or carbamoylcholine in pancreatic cells (Kondo & Schulz, 1976). The Ca²⁺ transport through the Ca²⁺ channel or carrier may be described as follows:

where Ca_o^{2+} and Ca_i^{2+} are the extra- and intracellular Ca²⁺ and X is the Ca²⁺ channel or carrier located in the membrane. In this form

$$V_{\max} = k_2[\text{X}_t] \text{ and } K_m = \frac{k_{-1} + k_2}{k_{+1}}$$

One explanation involving the modification of a single factor is that the three hormones increase k_2 . This could account for the parallel increases in K_m and $V_{\max}$. According to this hypothesis, the role of noradrenaline, vasopressin and angiotensin could be to stimulate the Ca²⁺ influx in rat hepatocytes by increasing the transfer rate of Ca²⁺ through the channel or carrier after its binding to its recognition site and not by increasing the total number of channels nor their binding affinity for Ca²⁺.

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