Role of Ca^{2+} and protein kinase C in the receptor-mediated activation of Na^+/H^+ exchange in isolated liver cells

Angeles MARTÍN-REQUERO*, Francisco J. DAZA, Ofelia G. HERMIDA, Nora BUTTA and Roberto PARRILLA

Department of Pathophysiology and Human Molecular Genetics, Centro de Investigaciones Biológicas (CSIC), Velázquez 144, 28006-Madrid, Spain

This work aimed to study the relationship between agonist-induced changes in cytosolic free calcium levels, protein kinase C (PKC) activity and intracellular pH in isolated liver cells. We observed that, like α_1 -adrenergic agonists, the Ca^{2+} -mobilizing vasoactive peptides vasopressin and angiotensin II produced an extracellular- Na^+ -dependent, 5-(*N*-ethyl-*N*-isopropyl)amiloride-sensitive, intracellular alkalinization, indicative of Na^+/H^+ antiporter activation. Blocking the agonist-induced increase in the intracellular Ca^{2+} concentration using the calcium chelator bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) prevented all types of receptor-mediated intracellular alkalinization. Thus activation of the Na^+/H^+ exchanger by either α_1 -adrenergic agonists or vasoactive peptides relies on the mobilization of intracellular Ca^{2+} . In contrast, only the α_1 -

adrenergic-agonist-induced alkalinization was dependent on extracellular Ca^{2+} . Even though α_1 -adrenergic as well as vasoactive peptide agonists stimulated protein kinase C (PKC) activity in isolated liver cells, only the α_1 -adrenoreceptor-mediated intracellular alkalinization was dependent on PKC. According to these observations, Ca^{2+} -mobilizing agonists appear to activate the Na^+/H^+ exchanger by at least two different mechanisms: (1) the α_1 -adrenoreceptor-mediated activation that is dependent on extracellular Ca^{2+} and PKC; and (2) vasoactive-peptide-induced alkalinization that is independent of extracellular Ca^{2+} and PKC. The α_1 -adrenoreceptor-mediated, PKC-sensitive, activation of the Na^+/H^+ exchanger seems to be responsible for the distinct ability of these receptors to elicit the sustained stimulation of hepatic functions.

INTRODUCTION

There are numerous H^+ - and/or Ca^{2+} -sensitive cell functions, and thus the operation of effective mechanisms of control to maintain the concentrations of these ions within the appropriate limits is essential. The Na^+/H^+ exchanger plays a major role in controlling the intracellular pH (pH_i). Its activation increases the affinity of the allosteric modifier site for intracellular H^+ [1]. Receptor-mediated activation of cellular functions, induced by agents such as hormones, neurotransmitters and growth factors, is accompanied by changes in the intracellular concentrations of Ca^{2+} and/or H^+ , suggesting they might play a role in the mechanism(s) of response.

A distinct feature of the α_1 -adrenoreceptor-mediated activation of hepatic functions is activation of the Na^+/H^+ exchanger, resulting in H^+ release and intracellular alkalinization [2,3]. Moreover, the Na^+/H^+ and $\text{Na}^+/\text{Ca}^{2+}$ exchangers seem to be functionally coupled during α_1 -adrenergic agonist stimulation [2]. These α_1 -adrenergic effects were also observed in isolated liver cells, ruling out the possibility that changes in vascular resistance might contribute significantly to the cellular responses. We have previously reported that, in agreement with observations made in various types of cells [4,5], protein kinase C (PKC) may be the mediator of the hepatic α_1 -adrenoreceptor-induced activation of the Na^+/H^+ exchanger [6]. However, PKC-independent mechanisms of activation of the Na^+/H^+ antiporter [7,8], e.g. Ca^{2+} /calmodulin-dependent processes [9,10], have also been reported. The relative contributions of these two mechanisms of activation of the Na^+/H^+ exchanger seem to be cell type- and/or agonist-specific. Our present knowledge of the influence of receptor-mediated increases in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) on the activation of the hepatic

Na^+/H^+ exchanger is very limited. This is a controversial subject, since the reported data differ according to the experimental conditions. The actions of Ca^{2+} -mobilizing vasoactive peptides such as vasopressin and angiotensin II in perturbing pH_i are poorly defined. According to Anwer and Aktinson [11], the vasoactive peptides do not increase the basal pH_i , but they enhance the recovery of pH_i in acid-loaded hepatocytes by activating Na^+/H^+ exchange. This effect appears to be mediated in part by intracellular Ca^{2+} .

The present study aimed at elucidating the role played by Ca^{2+} and/or PKC activation in receptor-mediated changes in pH_i . Our data allow us to conclude that all Ca^{2+} -mobilizing agents induce intracellular alkalinization in an intracellular- Ca^{2+} -dependent manner. However, only α_1 -adrenergic agonists stimulate Na^+/H^+ exchange through the activation of a PKC- and extracellular Ca^{2+} -dependent signalling pathway.

EXPERIMENTAL

Hepatocyte preparation

Hepatocytes were isolated from the livers of fed male Wistar rats (body weight 180–220 g) by perfusion of the hepatic vascular system with collagenase, using previously described protocols [12].

Measurement of $[\text{Ca}^{2+}]_i$ and pH_i

$[\text{Ca}^{2+}]_i$ was determined using the fluorescent probe fura-2, as described previously [13]. It has been suggested that fura-2 may

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; PKC, protein kinase C; BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrapotassium salt; pH_i , intracellular pH; BCECF-AM, 2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein tetra-acetoxymethyl ester; H-7, 1-(5-isquinolinesulphonyl)-2-methylpiperazine; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.

* To whom correspondence should be addressed.

find its way into compartments other than the cytoplasm when the ester form of this indicator is used for loading [14]. However, for most studies, some degree of compartmentalization of the Ca^{2+} indicator does not necessarily obviate its use in experiments, because the fluorescence from the sequestered dye does not generally change during experimental manipulations. Cells were loaded with the intracellular indicator by a 15 min incubation with fura-2-AM (5 μM) in the presence of 0.0012% Pluronic F-127, followed by a 30 min incubation in standard incubation buffer (modified Krebs–Ringer buffer containing 20 mM Hepes, pH 7.4, 15 mM glucose, 1.3 mM CaCl_2 and 0.2% defatted BSA). After three washes with fresh buffer, portions of the cell suspension (~ 20 mg wet weight) were transferred to 2 ml of the incubation buffer in a cuvette prewarmed at 37 °C.

Fluorescence measurements were carried out using a Perkin–Elmer LS-50 B spectrofluorimeter equipped with a fast filter accessory that minimizes the interval delay between changing wavelengths. The emission wavelength was set at 510 nm and readings were integrated at 1 s intervals. Ca^{2+} concentration was estimated from the ratio of fluorescence excited at 340 nm and 380 nm (R_{340}/R_{380}) as described by Grynkiewicz et al. [15], using a K_d of 224 nM.

In some experiments, the intracellular calcium chelator bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrapotassium salt (BAPTA-AM; 40 μM) was added to the loading medium 5 min after the addition of fura-2-AM or 2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein tetra-acetoxymethyl ester (BCECF-AM); the cells were then washed as described above.

pH_i was determined using the intracellular trappable fluorescent pH indicator BCECF [16]. Liver cells were resuspended in buffer A (10 mM Hepes, pH 7.4, 140 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 and 1 mM KH_2PO_4) supplemented with 2% (w/v) Ficoll-70. Portions of approx. 40 mg wet weight of cells/ml were incubated at 37 °C with BCECF-AM (5 μM final concentration) followed by three washes with buffer A. The BCECF-loaded hepatocytes were then incubated at 25 °C for 15 min to allow for complete de-esterification of the indicator. Portions of the cell suspension (20 mg wet weight) were transferred to 2.3 ml of buffer A containing 0.2% Ficoll-70 in a cuvette prewarmed at 37 °C. The excitation wavelengths were 495 and 440 nm and the emission wavelength was 530 nm, using 5 and 10 nm slits respectively. Calibration of the fluorescent signal was carried out using the nigericin/ K^+ method of Thomas et al. [17]. The 495/440 nm fluorescence ratio was a linear function of pH over the pH range 6.8–7.6.

Acid-loading experiments were performed using the NH_4^+ prepulse technique [18], which decreased the basal pH_i from 7.05 ± 0.02 to 6.5. The buffering capacity of the cells was calculated using the formula: $\beta t = \Delta[\text{NH}_4]/\Delta\text{pH}_i$, where $\Delta[\text{NH}_4]$ represents the change in NH_4 concentration after abrupt removal of extracellular NH_3 and ΔpH_i represents the corresponding change in pH_i . The mean buffering capacity ($\pm$ S.E.M.) was $32 \pm 0.7 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{pH}^{-1}$ ($n = 12$). Hydrogen ion efflux rates were then determined by multiplying the rate of change in pH_i by the cellular buffering capacity.

PKC activity

PKC was assayed by measuring the incorporation of ^{32}P from [γ - ^{32}P]ATP into histone type III (Sigma), essentially as described [19]. The hepatocytes (100 mg wet wt./ml) were incubated in Krebs–Ringer bicarbonate buffer, pH 7.4 [20], containing 2% (w/v) Ficoll-70 for 15 min in the absence or in the presence of 10 μM phenylephrine, 10 nM vasopressin, 10 nM angiotensin II or 100 nM PMA. The cells were collected by centrifugation,

resuspended and homogenized in ice-cold buffer B (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 2 mM PMSF, 0.25 M sucrose, 20 $\mu\text{g}/\text{ml}$ trypsin inhibitor and 0.04 mM leupeptin). Homogenates were centrifuged at 12000 *g* for 20 min. The supernatants were used as the cytosol fraction, and the pellets were homogenized in buffer B containing 1% Triton X-100, incubated at 4 °C for 30 min and centrifuged at 12000 *g* for 30 min. The resulting supernatant was used as the solubilized membrane fraction. The cytosol and solubilized plasma membrane fractions were each applied to DEAE-cellulose columns (1 ml) equilibrated with buffer B without sucrose. Elution was performed with 0.1 M NaCl in buffer B.

The assay mixture (200 μl) contained 1 mM Tris, pH 7.5, 7.5 mM MgCl_2 , 1.5 mM CaCl_2 , 50 μg of histone, 25 μg of phosphatidylserine, 1 μg of 1,2-dioctanoylglycerol, 0.05 mM ATP, 0.25 μCi of [γ - ^{32}P]ATP and 50 μl of the enzyme preparation. Basal activity was obtained by replacing CaCl_2 and phospholipids with 5 mM EGTA. The mixture was incubated at room temperature for 10 min. The reaction was stopped by the addition of 2 ml of 5% (w/v) trichloroacetic acid containing 10 mM H_3PO_4 and 200 μg of BSA. The precipitates were collected and washed on Whatman GF/A glass-fibre filters, and radioactivity was counted using a liquid scintillation counter.

Materials

All reagents were of the highest purity available. Fura-2-AM and BAPTA-AM were obtained from Sigma (St. Louis, MO, U.S.A.). The enzymes and BCECF-AM were obtained from Boehringer. [γ - ^{32}P]ATP (5000 Ci/mmol) was obtained from Amersham. 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA) and calmidazolium chloride {1-[bis-(*p*-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]-ethyl]imidazolium chloride} were obtained from Research Biochemical Inc. (Natick, MA, U.S.A.).

Statistics

Comparisons between group means were carried out by two-tailed unpaired Student's *t* tests.

RESULTS

Effects of α_1 -adrenergic agonists, vasopressin and angiotensin II on $[\text{Ca}^{2+}]_i$ and pH_i in isolated hepatocytes

Isolated hepatocytes incubated in the presence of 1.3 mM CaCl_2 had a basal $[\text{Ca}^{2+}]_i$ of 213 ± 20 nM ($n = 58$). Addition of the α_1 -adrenergic agonist phenylephrine, or of the vasoactive peptides vasopressin or angiotensin II, elicited acute increments in $[\text{Ca}^{2+}]_i$ of 120 ± 21 , 180 ± 23 and 181 ± 17 nM respectively (Figure 1, left panels). $[\text{Ca}^{2+}]_i$ then fell rapidly, reaching within 2–3 min a plateau level slightly above the starting resting level. In agreement with previous reports [21,22], the slope of the decay of $[\text{Ca}^{2+}]_i$ was markedly increased by preincubating the cells in nominally Ca^{2+} -free medium (Figure 1, middle panels). The agonist-induced rise in $[\text{Ca}^{2+}]_i$ was virtually abolished in hepatocytes preloaded with the intracellular Ca^{2+} chelator BAPTA-AM [23] (Figure 1, right panels).

The rate of recovery of pH_i from an acid load was enhanced by phenylephrine, vasopressin and angiotensin II (Table 1). These agonists also induced intracellular alkalinization, although the patterns of response were different. The basal pH_i was 7.04 ± 0.01 ($n = 12$). The increases in pH_i , measured 15 min after the addition of phenylephrine, vasopressin or angiotensin II, were respectively 0.057 ± 0.001 , 0.060 ± 0.002 and 0.051 ± 0.002 pH unit (means $\pm$ S.E.M.; Figure 2, left panels). Unlike

Figure 1 Effects of phenylephrine, vasopressin and angiotensin II on $[Ca^{2+}]_i$

Liver cells were loaded with fura-2-AM in the absence or in the presence of the intracellular Ca^{2+} chelator BAPTA-AM, as described in the Experimental section, and then incubated in either buffer containing 1.3 mM $CaCl_2$ or nominally Ca^{2+} -free medium. Phenylephrine (PE; 10 μ M), vasopressin (VP; 10 nM) or angiotensin II (AII; 10 nM) was added as indicated (arrows). The calcium signal evoked by these agonists in hepatocytes incubated in the presence of 1.3 mM $CaCl_2$ (left panels) became more transient when hepatocytes were incubated in nominally Ca^{2+} -free medium (middle panels), and was blunted in BAPTA-AM-preloaded cells (right panels). Traces shown are representative of at least five different cell preparations.

Table 1 Effects of Ca^{2+} -mobilizing agonists on pH_i recovery in acid-loaded hepatocytes

Hepatocytes were acid-loaded by incubation in 40 mM NH_4Cl for 5 min. The recovery in pH_i was initiated by removing NH_4Cl in the absence or in the presence of the indicated concentrations of the agonists. Values shown represent means $\pm$ S.E.M. for 3–10 different cell preparations. Significant differences compared with controls: * $P < 0.05$, ** $P < 0.001$.

	ΔpH_i (unit/min)	H ⁺ efflux (mmol of H ⁺ /min per litre)
Control	0.083 ± 0.002	2.43 ± 0.06
Phenylephrine (10 μ M)	$0.110 \pm 0.010^*$	$3.48 \pm 0.03^*$
Vasopressin (10 nM)	$0.126 \pm 0.006^{**}$	$4.03 \pm 0.19^{**}$
Angiotensin II (10 nM)	$0.106 \pm 0.003^*$	$3.40 \pm 0.10^*$

that due to the vasoactive peptides, the α_1 -adrenoreceptor-induced alkalinization did not reach a plateau, at least during the time studied (Figure 2). In agreement with previous observations obtained with perfused liver [2], either removal of extracellular Na^+ or addition of $\leq 1 \mu$ M EIPA (a Na^+ /H⁺ exchange blocker) abolished the receptor-mediated changes pH_i (results not shown), indicating that the Na^+ /H⁺ exchanger had to be activated to elicit this response.

To investigate the influence of intracellular Ca^{2+} on the agonist-induced changes in pH_i , we studied the effects of incubating the cells in nominally Ca^{2+} -free medium or with BAPTA-AM. Like other cell-permeable indicators, this compound penetrates the cell membrane and becomes trapped in the cytosol upon de-esterification, acting as an intracellular sink for Ca^{2+} when used at high concentrations. In the absence of extracellular Ca^{2+} (Figure 2, middle panels), the responses to phenylephrine, vasopressin and angiotensin II were respectively 0.010 ± 0.004 , 0.053 ± 0.003 and 0.046 ± 0.001 pH unit/15 min. In contrast with the α_1 -adrenergic agonist, which produced only a mild increase

Figure 2 Effects of Ca^{2+} -mobilizing agonists on pH_i

Loading of hepatocytes with the pH indicator BCECF, in the absence or in the presence of the intracellular Ca^{2+} chelator BAPTA-AM, was performed as described in the Experimental section. The experimental conditions were similar to those described in the legend to Figure 1. Traces shown are representative of at least five different cell preparations. The basal pH_i was 7.04 ± 0.01 units ($n = 12$). The increases in pH_i (means $\pm$ S.E.M.) measured 15 min after the addition of phenylephrine (PE), vasopressin (VP) and angiotensin II (AII) were respectively 0.057 ± 0.001 , 0.06 ± 0.002 and 0.051 ± 0.002 unit in the presence of 1.3 mM Ca^{2+} , and 0.010 ± 0.004 , 0.053 ± 0.003 and 0.046 ± 0.001 unit in the absence of external Ca^{2+} . No increase in pH_i was observed in BAPTA-preloaded cells after hormone challenge.

in pH_i , the responses to the vasoactive peptides were not altered by the absence of Ca^{2+} , although the pattern of response was changed, with a delay in the initial rate of intracellular alkalinization from 0.0059 to 0.0036 pH unit/min and from 0.0050 to 0.0039 pH unit/min for the vasopressin- and angiotensin II-mediated effects respectively (Figure 2, middle and bottom panels). These data suggest that only intracellular alkalinization induced by α_1 -adrenergic agonists relies on the influx of extracellular Ca^{2+} .

When BAPTA-AM was used to blunt the increase in $[Ca^{2+}]_i$, the basal pH_i was reduced by 0.14 ± 0.03 unit ($n = 7$), suggesting that $[Ca^{2+}]_i$ might play a role in maintenance of the basal pH_i . Stimulation of cells preloaded with BAPTA-AM with either α_1 -adrenergic agonists or vasoactive peptides did not alter pH_i (Figure 2, right panels), indicating that the two processes, i.e. Ca^{2+} mobilization and pH_i response, are related.

Effects of non-receptor-mediated increases in $[Ca^{2+}]_i$ on pH_i

To study the influence of non-receptor-mediated increases in $[Ca^{2+}]_i$ on the activity of the Na^+ /H⁺ exchanger, cells were treated with the Ca^{2+} ionophore ionomycin. Previous reports have indicated that ionomycin induces intracellular alkalinization in a variety of cells [9,24]. In liver, it was suggested that the effect of ionomycin in increasing pH_i may be independent of the activation of Na^+ /H⁺ exchange [25]. Under our experimental conditions, the ionomycin-induced increase in pH_i was secondary to the activation of Na^+ /H⁺ exchange, since it was dependent on extracellular Na^+ and was inhibited by EIPA (Figure 3). The fact that ionomycin did not increase, but rather decreased, pH_i when the Na^+ /H⁺ exchanger was blocked either with EIPA or by lowering the extracellular Na^+ concentration (Figure 3) is consistent with the idea that an elevation of $[Ca^{2+}]_i$ might produce intracellular acidification due to competition between Ca^{2+} and H⁺ for calmodulin binding sites. The increased cytosolic concentration of H⁺ activates Na^+ /H⁺ exchange. Thus whether the elevation in $[Ca^{2+}]_i$ leads to intracellular acidification or alkalinization depends on the state of activation of the Na^+ /H⁺ antiporter.

Figure 3 Effect of ionomycin on pH_i in liver cells

Liver cells were loaded with BCECF as indicated in the Experimental section. The top trace depicts the alkalization that occurs upon exposing the cells to $1 \mu\text{M}$ ionomycin. The middle and bottom traces demonstrate the complete attenuation of alkalization observed on removal of extracellular Na^+ or on addition of the Na^+/H^+ antiport inhibitor EIPA ($5 \mu\text{M}$). Results shown are representative of three similar experiments.

Figure 4 Effects of ionomycin on $[\text{Ca}^{2+}]_i$ and pH_i profiles

Loading of liver cells with either fura-2-AM or BCECF-AM, in the absence or in the presence of the intracellular calcium chelator BAPTA-AM, was carried out as described in the Experimental section. The increases in both $[\text{Ca}^{2+}]_i$ and pH_i observed upon exposing the cells to $1 \mu\text{M}$ ionomycin (left panels) were markedly attenuated in the presence of BAPTA (right panels). Results shown are typical of four different experiments.

Figure 4 shows that ionomycin induced a rise in $[\text{Ca}^{2+}]_i$ of $\sim 200 \text{ nM}$ and a simultaneous increase in pH_i of ~ 0.05 unit. Preloading the cells with the Ca^{2+} chelator BAPTA-AM decreased by $\sim 50\%$ the ionomycin-induced elevations of $[\text{Ca}^{2+}]_i$ and pH_i (Figure 4). At the concentrations of BAPTA-AM and ionomycin used, we observed only a partial blockade of the ionomycin-induced elevation of $[\text{Ca}^{2+}]_i$. However, since the intracellular alkalization was inhibited proportionally, it seems plausible to conclude that a close relationship exists between Ca^{2+} and H^+ homeostasis.

Role of calmodulin-dependent processes in the stimulation of Na^+/H^+ exchange mediated by α_1 -adrenergic or vasoactive peptides

One mechanism by which Ca^{2+} can regulate the activity of Na^+/H^+ antiport is through the Ca^{2+} /calmodulin system. This possibility was investigated by assessing the effect of the calmodulin antagonist W-7 [*N*-(6-aminoethyl)-5-chloro-1-

Figure 5 Effects of the calmodulin antagonist W-7 on the intracellular alkalization induced by phenylephrine, vasopressin and angiotensin II

BCECF-loaded cells were incubated as described in the Experimental section for 10 min in the absence or in the presence of $25 \mu\text{M}$ W-7 before the addition of $10 \mu\text{M}$ phenylephrine (PE), 10 nM vasopressin (VP) or 10 nM angiotensin II (AII). Traces are representative of three different experiments.

naphthalenesulphonamide] [26] on intracellular alkalization induced by Ca^{2+} -mobilizing agents. Figure 5 shows that treatment of cells with W-7 for 10 min was sufficient to prevent the effects of Ca^{2+} -mobilizing agonists in increasing pH_i . To rule out a possible artifact of W-7, we tested the effect of another potent calmodulin antagonist, calmidazolium [27]. This antagonist exerted a similar effect to that of W-7 in preventing the agonist-mediated activation of the Na^+/H^+ exchanger (results not shown). This observation agrees with a previous report showing that calmidazolium inhibited the vasopressin-induced pH_i recovery in liver cells challenged with an acid load [28].

Role of PKC in stimulation of the Na^+/H^+ exchanger

In agreement with previous observations [3,6,29], treatment of cells with phenylephrine, vasopressin, angiotensin II or the phorbol ester PMA increased the membrane-associated PKC activity by 207 ± 20 , 146 ± 15 , 200 ± 40 and $184 \pm 17\%$ respectively over control activity, and decreased the cytosolic PKC activity proportionally.

The possibility that PKC-dependent processes are involved in the activation of Na^+/H^+ antiport was investigated by using H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine], an inhibitor of PKC [30]. Preincubation of liver cells with $10 \mu\text{M}$ H-7 for 5 min impaired PMA-induced intracellular alkalization (Figure 6a), but not the ionomycin-evoked rise in pH_i that occurs in the absence of PKC activation [31] (Figure 6b). This concentration of H-7 produced a marked attenuation of α_1 -adrenergic-agonist-induced intracellular alkalization (Figure 6c). In contrast, H-7 did not alter the responses to vasoactive peptides, although the early rate of increase of pH_i was decreased (Figures 6c and 6d). The increases in pH_i at 15 min after the addition of phenylephrine, vasopressin and angiotensin II in the presence of H-7 were respectively 0.019 ± 0.001 , 0.046 ± 0.003 and $0.042 \pm 0.004 \text{ pH unit/15 min}$ (means $\pm$ S.E.M.).

DISCUSSION

Activation of hepatocytes with α_1 -adrenergic agonists or the vasoactive peptides vasopressin and angiotensin II leads to a transient elevation in $[\text{Ca}^{2+}]_i$ as a result of increases in both Ca^{2+}

Figure 6 Effects of the PKC inhibitor H-7 on the intracellular alkalinization induced by PMA, ionomycin and Ca^{2+} -mobilizing agonists

BCECF-loaded hepatocytes were incubated for 5 min in the absence or in the presence of $10 \mu\text{M}$ H-7 before the addition of 100 nM PMA, 1 mM ionomycin (IONO), $10 \mu\text{M}$ phenylephrine (PE), 10 nM vasopressin (VP) or 10 nM angiotensin II (AII). Traces are representative of at least four different cell preparations. pH_i changes induced by the Ca^{2+} -mobilizing agonists in the absence of H-7 are given in the legend to Figure 2. The increases in pH_i (means $\pm$ S.E.M.) at 15 min after the addition of phenylephrine, vasopressin and angiotensin II in the presence of $10 \mu\text{M}$ H-7 were respectively 0.019 ± 0.001 , 0.046 ± 0.003 and 0.042 ± 0.004 unit.

influx and Ca^{2+} release from intracellular stores [21,26]. We have demonstrated previously that an important feature of hepatic α_1 -adrenoreceptor activation is activation of the Na^+/H^+ exchanger [2,3]. The present work confirms and extends these observations to show that increases in $[\text{Ca}^{2+}]_i$ mediated by vasopressin and angiotensin II are also accompanied by parallel increases in pH_i . This effect is mediated by activation of the Na^+/H^+ antiporter, since it is dependent on extracellular Na^+ and sensitive to EIPA (results not shown).

Relationship between $[\text{Ca}^{2+}]_i$ and changes in pH_i induced by Ca^{2+} -mobilizing agonists in isolated liver cells

The mechanism whereby the activation of Ca^{2+} -mobilizing receptors stimulates the Na^+/H^+ antiporter has not been yet elucidated. Several studies have suggested a temporal relationship between pH_i and $[\text{Ca}^{2+}]_i$ [5,9], but the role played by $[\text{Ca}^{2+}]_i$ in the regulation of pH_i is a matter of debate [32–34]. On the other hand, the activation of PKC has been shown to play an important role in controlling the state of activation of the Na^+/H^+ exchanger in different types of cells [4,5]. As far as we know, the precise roles played by Ca^{2+} and/or PKC in the receptor-mediated activation of the hepatic Na^+/H^+ antiporter have not been investigated previously. The following observations seem to support the conclusion that a Ca^{2+} -dependent process is involved in the intracellular alkalinization induced by Ca^{2+} -mobilizing agonists: (1) no changes in pH_i were detected when the receptor-mediated rise in $[\text{Ca}^{2+}]_i$ was inhibited by BAPTA; (2) the Na^+/H^+

exchanger was activated by a non-receptor-mediated increase in $[\text{Ca}^{2+}]_i$ using the ionophore ionomycin; and (3) the calmodulin antagonists W-7 and calmidazolium prevented the receptor-mediated intracellular alkalinization. $[\text{Ca}^{2+}]_i$ might also play a role in the modulation of pH_i under basal, non-activated, conditions. This assertion is based on our observation that the pH_i was lower in Ca^{2+} -depleted cells or in cells preloaded with BAPTA than in control cells maintained in 1.3 mM CaCl_2 . A role for Ca^{2+} in controlling pH_i has also been postulated in human platelets [24]. This possibility is consistent with the recent findings that NHE1 (Na^+/H^+ exchanger isoform 1) is a member of the family of calmodulin-binding proteins and that binding of Ca^{2+} /calmodulin to the high-affinity binding region of the antiporter may be a key event for its Ca^{2+} -induced activation [35,36].

The Na^+/H^+ exchanger is a phosphoprotein [37], and therefore calmodulin and PKC could act co-ordinately in controlling its activity. The C-terminus of NHE1 contains multiple putative protein kinase consensus sequences. Whether or not calmodulin-dependent kinases and PKC could affect the state of phosphorylation of the same or different sets of specific phosphopeptides has not been investigated. PKC might also control the activity of the Na^+/H^+ exchanger indirectly by modifying the calmodulin levels through its ability to phosphorylate and inactivate calmodulin-dependent kinase II as well as other calmodulin binding proteins [38].

Role of PKC in the modulation of pH_i by Ca^{2+} -mobilizing agonists

The non-receptor-mediated activation of PKC leads to intracellular alkalinization in isolated liver cells, and this effect is prevented by PKC inhibitors such as H-7 (Figure 6). This finding agrees with previous observations made in perfused liver, in which PMA was shown to induce H^+ release, although this effect was less pronounced than that mediated by receptor activation [6].

The activation of α_1 -adrenoreceptors as well as vasoactive peptide receptors led to the activation of PKC in liver cells. However, inhibition of PKC prevented α_1 -adrenergic-induced intracellular alkalinization, but not that induced by vasoactive peptides (Figure 6). This finding was not totally unexpected, since we have reported previously that inhibition of PKC activity [6] prevented α_1 -adrenergic-mediated, but not vasopressin-induced, actions in perfused liver. Based on these observations, the Ca^{2+} -mobilizing receptors can be classified into two distinct categories according to the mechanism(s) by which they activate Na^+/H^+ exchange: (1) the α_1 -adrenoreceptors show calcium sensitivity and PKC-dependence; and (2) vasoactive peptide receptors are calcium-sensitive and PKC-independent.

The ability of vasoactive peptides to induce intracellular alkalinization in the absence of PKC activation indicates that $[\text{Ca}^{2+}]_i$ elevation may be a sufficient signal to stimulate the hepatic Na^+/H^+ exchanger. It could also indicate that Ca^{2+} -mobilizing receptors activate specific PKC isoforms. This observation poses the question: what is the role of PKC activation in the vasoactive-peptide-mediated stimulation of hepatic function? A possibility to be considered is that PKC might control the sodium pump, since it has been shown that the catalytic α subunit of purified Na^+/K^+ -ATPase can be phosphorylated *in vitro* by PKC [39]. Thus the increase in Na^+/K^+ -ATPase activity that is observed in cells exposed to Ca^{2+} -mobilizing hormones or tumour-promoting phorbol esters [40–42] may, at least in part, be a consequence of the direct phosphorylation of Na^+/K^+ -ATPase by PKC.

Functional significance of the activation of hepatic Na^+/H^+ exchange by Ca^{2+} -mobilizing agonists

α_1 -Adrenergic agonists and vasoactive peptides induce similar acute and transient changes in $[\text{Ca}^{2+}]_i$ (Figure 1). Nevertheless, the hepatic actions of the α_1 -adrenergic agonists differ considerably from those induced by peptide hormones. The activation of hepatic α_1 -adrenoreceptors produces acute and sustained respiratory, ionotropic and vasopressive effects [2–4]. The biphasic nature of the hepatic α_1 -adrenoreceptor-mediated effects and their requirement for extracellular Ca^{2+} are distinct features of this Ca^{2+} -mobilizing receptor [43]. Unlike the α_1 -adrenergic agonists, the vasoactive peptides elicit only transient changes in respiration and extracellular acidification [2,6]. The sustained phase of the α_1 -adrenoreceptor-mediated stimulation of hepatic functions relies on the functional coupling between Na^+/H^+ and $\text{Na}^+/\text{Ca}^{2+}$ exchangers, and is PKC-sensitive [2,6]. These observations support the conclusion that the sustained extracellular acidification elicited by hepatic α_1 -adrenoreceptor activation is the result of a sustained activation of the Na^+/H^+ antiporter. Our data suggest the existence of at least two means of Na^+/H^+ exchanger activation induced by Ca^{2+} -mobilizing agonists: (1) a Ca^{2+} -dependent form that is responsible for the acute and transient stimulation of the exchanger, shared by all the agonists; (2) a PKC-sensitive form, induced by α_1 -adrenergic agonists, which is associated with the sustained activation of the Na^+/H^+ antiporter. This conclusion is based on the characteristic hepatic responses elicited by each type of Ca^{2+} -mobilizing agonist, as discussed above, as well as the distinct kinetic patterns of intracellular alkalinization in isolated liver cells (Figure 2). The intracellular alkalinization induced by vasoactive peptides and the non-receptor-mediated activation of the Na^+/H^+ antiporter approach a plateau in about 5 min, indicating that the activation of the antiporter has ceased. In contrast, the α_1 -adrenergic-induced alkalinization shows an acute rise followed by a sustained increase throughout the incubation period. Despite the reservations imposed by the different experimental models, particularly those derived from the markedly different volumes that the cells had to be equilibrated with, it seems plausible to conclude that these kinetic differences reflect the ability of each type of Ca^{2+} -mobilizing receptor to induce either transient and/or sustained stimulation of the exchanger.

In conclusion, the non-receptor-mediated elevation of $[\text{Ca}^{2+}]_i$ or PKC stimulation may activate the Na^+/H^+ antiporter in isolated liver cells. Moreover, a close relationship appears to exist between the basal $[\text{Ca}^{2+}]_i$ and pH_i . The Ca^{2+} -mobilizing receptors activate the Na^+/H^+ antiporter in a Ca^{2+} -dependent manner. However, the activation of the Na^+/H^+ antiporter induced by α_1 -adrenergic agonists, but not that due to vasoactive peptides, is prevented by PKC inhibitors. It is postulated that the receptor-mediated, PKC-sensitive, activation of the Na^+/H^+ antiporter is responsible for the distinct ability of α_1 -adrenergic agents to induce sustained H^+ efflux and intracellular alkalinization.

The skilful technical assistance of M. J. Arias-Salgado is greatly appreciated. This work was supported in part by grants from Fondo de Investigaciones Sanitarias (F.I.S.) of the Spanish Ministry of Public Health (94/0224), and Dirección General de Investigación Científica y Técnica (93/163).

REFERENCES

- Grinstein, S. and Rothstein, A. (1986) *J. Membr. Biol.* **90**, 1–12
- Urcelay, E., Butta, N., Ciprés, G., Martín-Requero, A., Ayuso, M. S. and Parrilla, R. (1994) *J. Biol. Chem.* **269**, 860–867
- Ciprés, G., Butta, N., Urcelay, E., Parrilla, R. and Martín-Requero, A. (1995) *Endocrinology* **136**, 468–475
- Moolenaar, W. H. (1986) *Annu. Rev. Physiol.* **48**, 363–373
- Ives, H. E. and Daniel, T. O. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1950–1954
- Urcelay, E., Butta, N., González-Manchón, C., Ciprés, G., Martín-Requero, A., Ayuso, M. S. and Parrilla, R. (1993) *Endocrinology* **133**, 2105–2115
- Berk, B. C., Brock, T. A., Gimbrone, Jr., M. A. and Alexander, R. W. (1987) *J. Biol. Chem.* **262**, 5065–5072
- Mitsuhashi, T. and Ives, H. E. (1988) *J. Biol. Chem.* **263**, 8790–8795
- Little, P. J., Weissberg, P. L., Cragoe, Jr., E. J. and Bobik, A. (1988) *J. Biol. Chem.* **263**, 16780–16785
- Wakabayashi, S., Bertrand, B., Ikeda, T., Pouyssegur, J. and Shigekawa, M. (1994) *J. Biol. Chem.* **269**, 13170–13175
- Anwer, M. S. and Atkinson, J. M. (1992) *Hepatology* **15**, 134–143
- Martín-Requero, A., Ayuso, M. S. and Parrilla, R. (1986) *Arch. Biochem. Biophys.* **246**, 114–127
- Kass, G. E. N., Duddy, S. E., Moore, G. A. and Orrenius, S. (1989) *J. Biol. Chem.* **262**, 15192–15198
- Glennon, M. C., Bird, G. St. J., Kwan, C.-Y. and Putney, Jr., J. W. (1992) *J. Biol. Chem.* **267**, 8230–8233
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Rink, T. J., Tsien, R. Y. and Pozzan, T. (1982) *J. Cell Biol.* **95**, 189–196
- Thomas, J. A., Buschbaum, R. N., Zimniak, A. and Racker, E. (1979) *Biochemistry* **18**, 2210–2218
- Ross, A. and Boron, W. E. (1981) *Physiol. Rev.* **61**, 296–434
- Kikkawa, U., Tokai, Y., Minakuchi, R., Inchara, S. and Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 13341–13348
- Krebs, H. A. (1943) *Adv. Enzymol.* **3**, 191–248
- Williamson, J. R. and Monck, J. R. (1989) *Annu. Rev. Physiol.* **51**, 107–124
- Berven, L. A., Hughes, B. P. and Barritt, G. J. (1994) *Biochem. J.* **299**, 399–407
- Tsien, R. Y. (1980) *Biochemistry* **19**, 2396–2404
- Poch, E., Botey, A., Gaya, J., Cases, A., Rivera, F. and Revert, L. (1993) *Biochem. J.* **290**, 617–622
- Anwer, M. S. (1993) *Hepatology* **18**, 423–439
- Tanara, T., Ohmura, T., Yamarado, T. and Hidaka, H. (1982) *Mol. Pharmacol.* **22**, 408–412
- Veigl, M. L., Kelevit, R. E. and Sedwick, W. D. (1994) *Pharmacol. Ther.* **44**, 181–230
- Anwer, M. S. (1994) *Hepatology* **20**, 1309–1317
- Hernández-Sotomayor, S. M. T. and García-Saiz, J. A. (1988) *Biochim. Biophys. Acta* **968**, 138–141
- Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) *Biochemistry* **23**, 5036–5041
- Fearon, C. W. and Tashjian, Jr., A. H. (1985) *J. Biol. Chem.* **260**, 8366–8371
- Frelin, C., Vigne, P., Ladoux, A. and Lazdunski, M. (1988) *Eur. J. Biochem.* **174**, 3–14
- Siffert, W. and Akkerman, J. W. N. (1987) *Nature (London)* **325**, 456–458
- Sage, S. O., Jobson, T. M. and Rink, T. J. (1990) *J. Physiol. (London)* **420**, 31–45
- Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J. and Shigekawa, M. (1994) *J. Biol. Chem.* **269**, 13703–13709
- Wakabayashi, S., Bertrand, B., Ikeda, T., Pouyssegur, J. and Shigekawa, M. (1994) *J. Biol. Chem.* **269**, 13710–13715
- Sardet, C., Counillon, L., Franchi, A. and Pouyssegur, J. (1990) *Science* **247**, 723–726
- Waxham, M. N. and Aronowsky, J. (1993) *Biochemistry* **32**, 2923–2930
- Lowndes, J. M., Hokin-Neaverson, M. and Bertics, P. J. (1990) *Biochim. Biophys. Acta* **1052**, 143–151
- Lynch, C. J., Bocchino, S. B., Blackmore, P. F. and Exton, J. H. (1987) *Biochem. J.* **248**, 807–813
- Capiod, T., Berthon, B., Poggioli, J., Burgess, G. M. and Claret, M. (1982) *FEBS Lett.* **141**, 49–52
- Smart, J. L. and Deth, R. C. (1988) *Pharmacology* **37**, 94–104
- Urcelay, E., Butta, N., Arias-Salgado, M. J., Ayuso, M. S. and Parrilla, R. (1993) *Biochim. Biophys. Acta* **1220**, 49–56