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Modulation of the hepatic α_1-adrenoceptor responsiveness by colchicine: dissociation of free cytosolic Ca^{2+}-dependent and independent responses

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1 The cytoskeletal depolymerizing agent, colchicine, prevents the hepatic α_1-adrenoceptor-mediated stimulation of respiration, H^+ and Ca^{2+} release to the effluent perfusate, intracellular alkalosis, and glycogenolysis. Unlike the other parameters, colchicine does not perturb the α_1-agonist-induced stimulation of gluconeogenesis or phosphorylase 'a' activation, and enhances the increase in portal pressure response. The lack of effect of colchicine on the hepatic α_2-adrenoceptor-mediated effects indicates that its actions are α_1-specific.

2 Colchicine enhances the acute α_1-adrenoceptor-mediated intracellular Ca^{2+} mobilization and prevents the activation of protein kinase C. This differential effect on the two branches of the α_1-adrenoceptor signalling pathway is a distinctive feature of the colchicine action.

3 The lack of effect of colchicine in altering the α_1-adrenoceptor ligand binding affinity suggests that it might interact with some receptor-coupled regulatory element(s).

4 The acuteness of the colchicine effect and the ability of its isomer β-lumicolchicine to prevent all the α_1-adrenoceptor-mediated responses but the increase in vascular resistance, indicate that its action cannot be merely ascribed to its effects in depolymerizing tubulin.

5 Colchicine perturbs the hepatic responses to vasoactive peptides. It enhances the vasopressin-induced rise of cytosolic free Ca^{2+} in isolated hepatocytes and prevents the sustained decrease of Ca^{2+} in the effluent perfusate. It also inhibits the stimulation of glycogenolysis, without altering the stimulation of gluconeogenesis.

6 It is concluded that there are at least two major α_1-adrenoceptor signalling pathways. One is colchicine-sensitive, independent of variations in free cytosolic Ca^{2+}, and protein kinase C-dependent; the other one is colchicine-insensitive, dependent on variations in free cytosolic Ca^{2+}, and protein kinase C-independent.

Keywords: Colchicine; phenylephrine; α_1-agonist; protein kinase C; free cytosolic Ca^{2+}; intracellular pH; glycogenolysis; gluconeogenesis; portal pressure; perfused liver

Introduction

The stimulation of hepatic α_1-adrenoceptors elicits guanine nucleotide-binding regulatory (G) protein-coupled hydrolysis of membrane phospholipids leading to cellular increases in *myo*inositol 1,4,5,-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). The rise in IP_3 appears to be responsible for the acute mobilization of Ca^{2+} from intracellular stores and the subsequent activation of Ca^{2+}-dependent processes (Berridge, 1993). An increase in DAG may also control numerous hepatic processes by activating protein kinase C (Kikkawa & Nishizuka, 1986).

In rat liver, the α_1-adrenoceptor ligand-binding affinity is modulated by a pertussis toxin-sensitive guanine nucleotide-binding regulatory protein(s) (G_i protein) (Butta *et al.*, 1993). ADP-ribosylation of hepatic G-proteins also prevents the receptor-induced inflow of Ca^{2+} (Butta *et al.*, 1993). Recent work implicates the cytoskeleton in the control of ion fluxes across the plasma membrane (Cantiello *et al.*, 1991). The molecular basis for the relationship between the cytoskeleton and the receptor-operated ion channels may lie in the analogies and functional similarities between the G-proteins and cytoskeletal proteins. Tubulin itself is a GTP-binding protein (Cantiello *et al.*, 1991) and forms macromolecular complexes with G-proteins (Higashi & Ishibashi, 1985; Sternlicht *et al.*, 1987). On the other hand tubulin is, like the G-proteins, a substrate for pertussis or cholera toxin catalysed ADP-ribosylation (Amir-Zaltsman *et al.*, 1982; Wang *et al.*, 1990) and seems to control cellular Ca^{2+} fluxes (Lim *et al.*, 1985). Based on these and other (Taylor *et al.*, 1973; Omann *et al.*, 1987) observations that suggest the implication of the cytoskeleton in the signal transduction mechanism of plasma membrane receptors, we found it interesting to determine the role of the cytoskeleton on the hepatic α_1-adrenoceptor signalling pathway. For this purpose, we used the microtubular depolymerizing agent colchicine (Wilson *et al.*, 1974) and determined its effect on the α_1-adrenoceptor-mediated responses of isolated perfused livers. In order to differentiate between cytoskeleton-dependent and independent actions of colchicine, we used the isomer β-lumicolchicine, which does not interfere with tubulin polymerization (Wilson & Friedkin, 1967).

Methods

Animals

Male rats of the Wistar strain, 180–220 g in body weight, were used and maintained under controlled conditions of light and temperature and had free access to food and water. When indicated, animals were starved 48 h before their experimental use. All the animals were treated in a manner that complied with the European guidelines for the care and use of laboratory animals.

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Liver perfusion and isolation of liver cells

Livers were perfused in a non-recirculating system with Krebs-Ringer bicarbonate buffer (KRB) of the following composition (mM): (NaCl 124.76, $NaHCO_3$ 19.04, KH_2PO_4 1.19, $MgSO_4$ 1.19, KCl 4.79 and $CaCl_2$ 1, at 36.5°C, equilibrated with a 95:5 O_2:CO_2 mixture and a flow rate of 30 ± 2 ml min^{-1}. The basal outflow perfusate Po_2 was about 350 mmHg. Details of surgical procedures and perfusion techniques were previously described (Martín-Requero *et al.*, 1992; Butta *et al.*, 1993). Gluconeogenic substrates and agonists were dissolved into the perfusion medium. Colchicine and β-lumicolchicine were dissolved in KRB and infused into the portal vein at a rate of 70 μl min^{-1}. Control experiments showed that infusion of KRB alone did not alter either basal or agonist-stimulated parameters.

Portal pressure, as well as Po_2, PCa^{2+}, and pH of the effluent perfusate were continuously monitored in most of the experiments. A Statham (Spectramed Inc., USA), model P23XL pressure transducer and an electromanometer Hugo Sach Elektronik (March-Hugstetten, Germany) were used to determine portal pressure. Ion activities were determined with commercially available ion specific electrodes. Po_2 was measured with a Clark-type platinum electrode. Electrodes were placed in specially designed flow-through cells mounted in series, located close to the hepatic outflow. The calibration of the sensors and their signal processing were carried out as previously described (Butta *et al.*, 1993).

Liver cells were isolated by perfusion of the vascular system with collagenase (Berry & Friend, 1969). The experimental details were as previously noted (Menaya *et al.*, 1988).

Determination of intracellular pH, free cytosolic Ca^{2+} concentration, protein kinase C activity and α_1-agonist binding to plasma membranes

Intracellular pH was determined with the fluorescent pH indicator 2'-7'-*bis* (carboxyethyl)-5,6 carboxyfluorescein (BCECF) (Rink *et al.*, 1982). The experimental details for loading hepatocytes with BCECF were as described by Urcelay *et al.* (1994). Fluorescence measurements were performed using a Perkin Elmer LS-50 B spectrofluorimeter equipped with a fast filter accessory that minimized the interval delay between changing wavelengths. For BCECF measurement, the excitation wavelengths were 495 and 440 nm and that of the emission 530 nm, using 5 and 10 nm slits, respectively. Calibration of the BCECF fluorescent signal was carried out by the nigericin/K^+ method of Thomas *et al.* (1979). The 495/440 ratio was a linear function of pH over the range of 6.8–7.6 pH units.

Cytosolic free Ca^{2+} was determined with the fluorescent probe Fura-2 as previously described (Grynkiewicz *et al.*, 1985). Fluorescence of Fura-2 was determined as described above using the excitation wavelengths 340 and 380 nm and that of the emission 510 nm.

Protein kinase C activity was determined in isolated hepatocytes or in perfused livers. In the first case, experiments were performed as followed: hepatocytes (100 mg wet wt. ml^{-1}) were incubated, at 36.5°C, in KRB-bicarbonate buffer pH 7.4 containing 2% Ficoll-70 for 20 min in the absence or in the presence of colchicine or β-lumicolchicine. Then, phenylephrine was added. After 10 min of additional incubation, 1 ml aliquots of the cell suspension were collected by centrifugation, resuspended and homogenized in ice-cold buffer A (composition, mM: Tris 20, pH 7.5, EDTA 2, EGTA 0.5, DTT 2, PMSF 2, sucrose 0.25 M, trypsin inhibitor 20 μg ml^{-1} and leupeptin 0.04). When the activity of PKC was determined in perfused livers, the procedure for the liver perfusion was as described above. Three liver biopsies (1 g each) were taken from each liver. One at zero time, a second just before the addition of phenylephrine and a third 10 min thereafter. The liver biopsies were immediately frozen using aluminium clamps cooled in liquid nitrogen and homogenated in buffer A. Homogenates prepared from liver biopsies or from hepatocytes were centrifuged at 40,000 r.p.m. for 1 h. The supernatants were used as the cytosol fraction and the pellets were homogenized in buffer A containing 1% Triton X-100, incubated at 4°C for 30 min and centrifuged at 40,000 r.p.m. for 1 h. The supernatant was used as solubilized membrane fraction. Cytosol and solubilized plasma membranes were applied to DEAE-cellulose columns (1 ml) equilibrated with buffer A without sucrose. Elution was performed with 0.1 M NaCl in buffer A without sucrose. Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}]-ATP into histone (type III, Sigma) essentially as described by Piccoletti *et al.* (1990). The results were normalized according to the protein concentration of the eluted fractions, and expressed as a percentage of the value obtained just before the addition of phenylephrine. The maximum effect of phenylephrine on PKC translocation to the plasma membrane was observed in perfused livers after 5 min of incubation, when this maximal value was maintained for at least 5 additional minutes.

The isolation of liver plasma membranes, the binding of [^{3}H]-prazosin to the hepatic α_1-receptors and its displacement by phenylephrine were performed as previously described (Tohkin *et al.*, 1990). Non-specific binding was determined in the presence of 1 mM prazosin. Values for maximal binding (B_{max}) and K_d were calculated from Scatchard plots.

Metabolite and enzymatic analysis

Samples of the outflow perfusate were collected at 2 or 5 min intervals and analysed immediately thereafter. Metabolites were determined spectrophotometrically by previously described enzymatic procedures (Bergmeyer, 1975). Phosphorylase activity was determined as previously described (Buxton *et al.*, 1982).

Statistical evaluation of the data

Values are expressed as mean ± s.e.mean of *n* number of experiments and the statistical significance of the differences was assessed by Student's unpaired, two tailed, *t* test.

Materials

Glucose-6-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, collagenase, phenylmethyl-sulphonyl fluoride (PMSF) and leupeptin were obtained from Boehringer Mannheim (Germany). Phenylephrine hydrochloride, colchicine, β-lumicolchicine, clonidine hydrochloride, [Arg^8]-vasopressin (acetate salt), sodium fluoride, Fura 2AM, 2'-7'-*bis* (carboxyethyl)-5,6 carboxyfluorescein, ascorbate, catechol, adenosine 5'-triphosphate (ATP), dithiothreitol, sucrose, glycogen, histone (type III), prazosin hydrochloride and 1,2 dioctanoyl-sn-glycerol (C:8) were purchased from Sigma (St Louis, MO, U.S.A.). Pluronic was obtained from Calbiochem (La Jolla, U.S.A.) and Bio Rad Protein Assay from Bio Rad (Munchen, Germany). [7-methoxy-^{3}H]-prazosin (S.A. 70 Ci $mmol^{-1}$) and [γ-^{32}P] ATP (S.A. 3,000 Ci $mmol^{-1}$) were obtained from Amersham International (Little Chalfont, England) and [^{14}C]-D-glucose 1-phosphate (S.A. 233 mCi $mmol^{-1}$) from New England Nuclear (Bad Homburg, Germany). All other reagents, of the highest purity grade available, were purchased from commercial sources.

Results

Effect of colchicine on the α_1-adrenoceptor-mediated stimulation of hepatic metabolism

Figure 1 (left panels) depicts the hepatic responses to the α_1-adrenoceptor agonist phenylephrine. As previously described (González-Manchón *et al.*, 1988; Butta *et al.*, 1993) the α_1-agonist elicited characteristic biphasic effects in stimulating hepatic respiration, glucose mobilization, H^+ and Ca^{2+} release

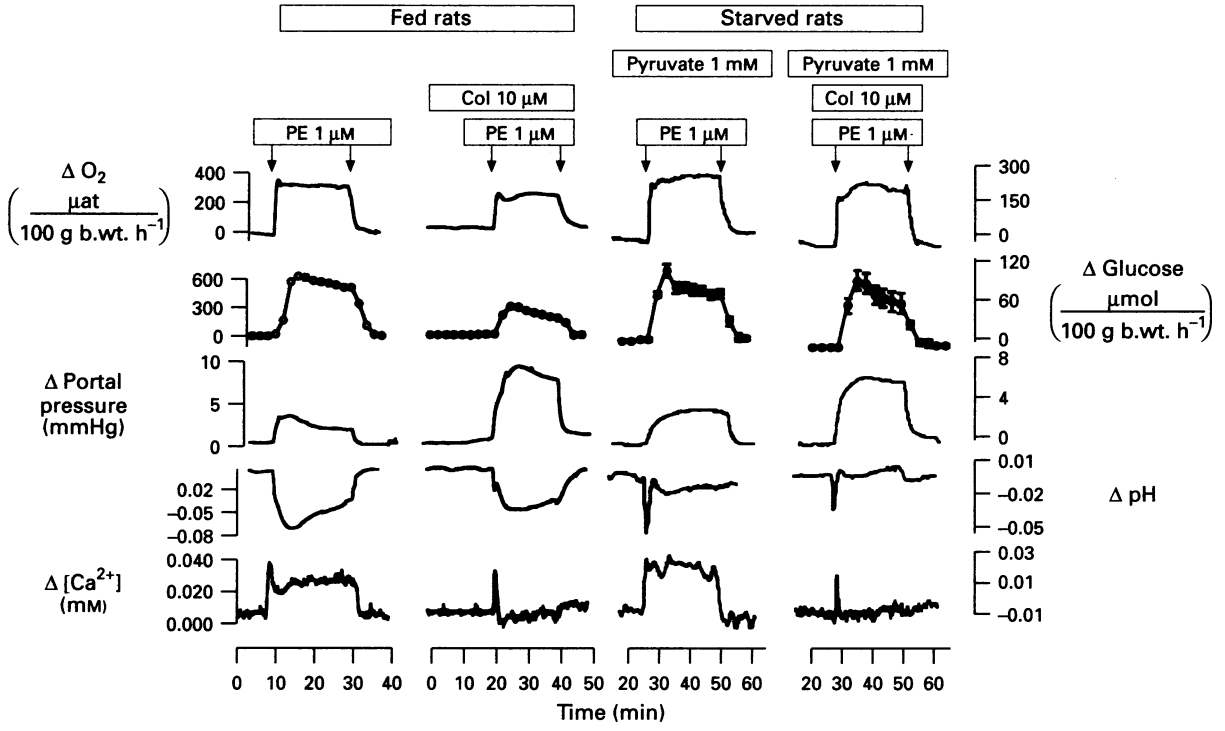

Figure 1 Effect of colchicine on basal and α_1-adrenoceptor-induced hepatic responses in livers from fed or starved rats. Livers isolated from fed rats or from 48 h starved rats were perfused as described in Methods. After an equilibration time of ≅30 min to attain steady rates of O_2 consumption, colchicine dissolved in KRB was infused into the portal vein; 20 min later phenylephrine (PE, 1 μM) was added to the perfusion medium for the periods indicated by the arrows. In starved rats, the livers were perfused with pyruvate, 1 mM. Details on data collection and processing are described in Methods. Each experiment was repeated four to six times obtaining reproducible results and data from representative experiments are shown. The basal average absolute values ± s.e. in the control group and in the presence of colchicine (Col, 10 μM) were, in livers from fed rats, respectively: oxygen uptake 954 ± 10 and 997 ± 55, 737 μatom 100 g^{-1} body wt. h^{-1}; pH: 7.444 ± 0.027 and 7.407 ± 0.074 units; [Ca^{2+}]: 1.000 ± 0.001 and 1.000 ± 0.001 mM; glucose production: 350 ± 29 and 343 ± 29 μmol 100 g^{-1} body wt. h^{-1}. In livers from starved rats, the basal average absolute values ± s.e. obtained after 20 min of pyruvate infusion in the absence and in the presence of 10 μM colchicine were, respectively: oxygen uptake: 855 ± 21 and 787 ± 12 μatom 100 g^{-1} body wt. h^{-1}; pH: 7.434 ± 0.031 and 7.391 ± 0.110 units; [Ca^{2+}]: 1.002 ± 0.002 and 1.000 ± 0.001 mM; glucose production: 89 ± 7 and 65 ± 18 μmol 100 g^{-1} body wt. h^{-1}.

reflected by the increases in $[H^+]$ and $[Ca^{2+}]$ in the effluent perfusate, and increasing vascular resistance. The sustained phase of stimulation lasted as long as the agonist was present. The addition of 10 μM colchicine to perfused livers did not alter any of the functional parameters studied (Figure 1). However, colchicine decreased the α_1-adrenoceptor-induced stimulation of respiration, glucose release and acidification of the extracellular medium (Figure 1). Colchicine did also abolish the α_1-agonist-induced sustained increase of $[Ca^{2+}]$ in the effluent perfusate, although the average intensity and duration of the acute and transient phase was similar to the control. Despite its inhibitory action on the other parameters, colchicine enhanced the vasopressive action of phenylephrine. The maximum stimulation of the portal pressure was increased by more than 100% (from 4 ± 0.75 to 7.2 ± 0.5 mmHg). These effects of colchicine are specific for the α_1-adrenoceptor-mediated responses. The hepatic stimulation of respiration, glycogenolysis and increase in portal pressure induced by the α_2-agonist clonidine, in the presence of the α_1-blocker, prazosin, were not prevented by colchicine (Figure 2). This finding indicates that colchicine acts primarily on the α_1-adrenoceptor signalling pathway.

Colchicine did not inhibit the α_1-agonist-induced stimulation of gluconeogenesis in livers from starved animals perfused with pyruvate as the glucose precursor (Figure 1, right panels). The inhibition of respiration and extracellular acidification were less than in livers from fed rats, presumably due to the overlapping effects of substrate and metabolites transport on the net H^+ efflux (Urcelay *et al.*, 1993a) and the stimulation of respiration to meet the energy cost of gluconeogenesis. As in livers from fed rats, colchicine prevented the sustained release of Ca^{2+} to the effluent perfusate, and produced a significant increase in the portal pressure in livers from starved rats (Figure 1).

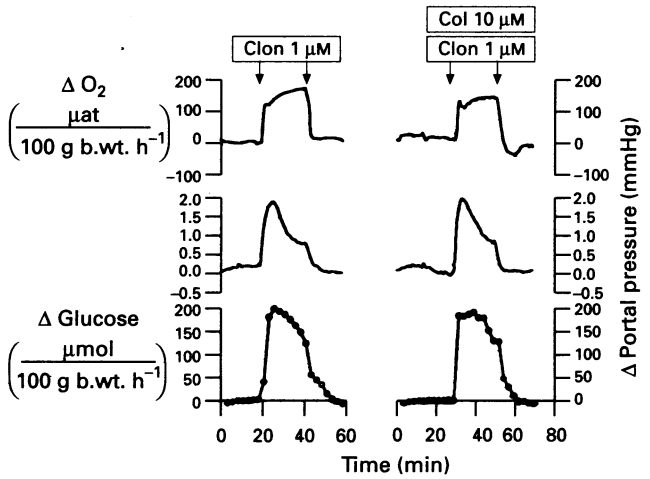

Figure 2 Effect of colchicine on α_2-adrenoceptor-induced hepatic responses in livers from fed rats. Livers from fed rats were allowed to equilibrate until steady rates of oxygen consumption were attained in the presence of prazosin (0.1 nM). Colchicine (Col) dissolved in KRB was then infused into the portal vein. After 20 min of perfusion clonidine (Clon, 1 μM) was added to the perfusion medium for the period of time indicated by the arrows. Details on data collection and processing are described in Methods. The experiment was repeated three times obtaining reproducible results and data from a representative experiment are shown.

The simultaneous addition of colchicine and the α_1-agonist did not produce significant effects on the α_1-adrenoceptor responses, while the addition of colchicine 10 min before the α_1-agonist was fully inhibitory (results not shown). Therefore, the

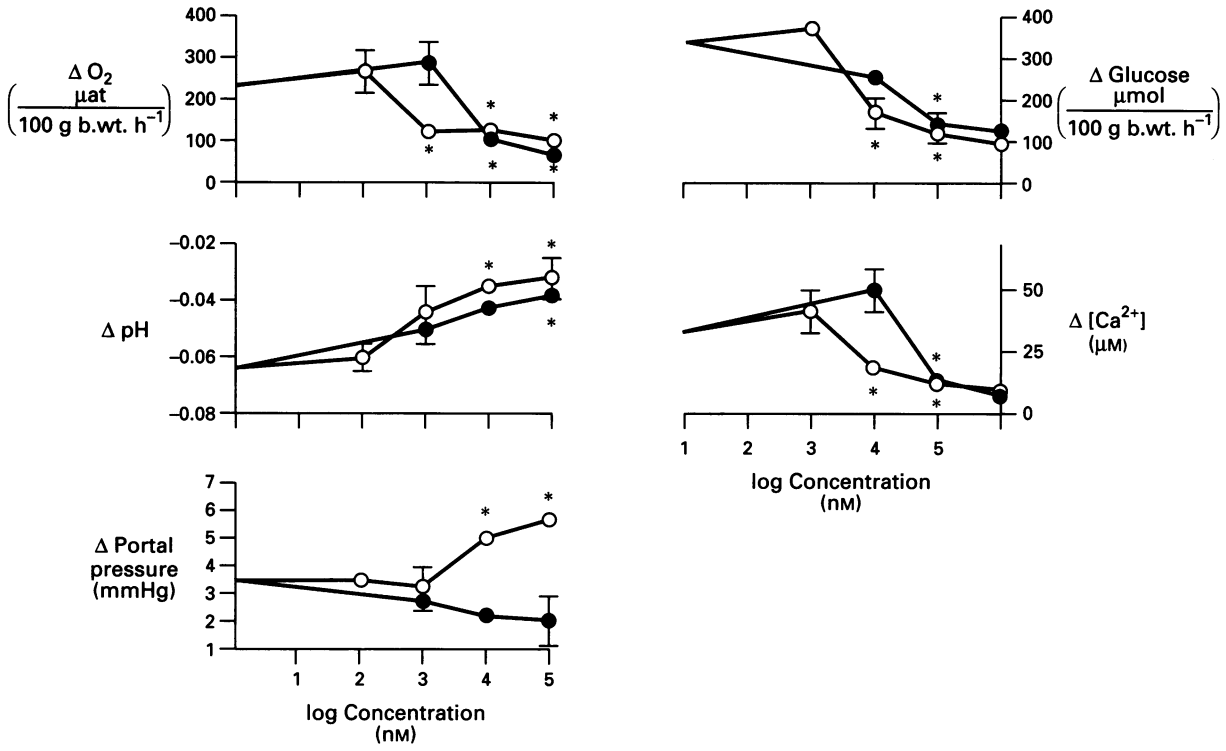

Figure 3 Effect of different concentrations of colchicine (○) and β-lumicolchicine (●) on the α_1-adrenoceptor-induced responses of livers from fed rats. Livers from rats fed *ad libitum* were allowed to equilibrate until steady rates of oxygen consumption were attained. Colchicine or β-lumicolchicine dissolved in KRB, or KRB alone, were then infused into the portal vein. The indicated concentrations were attained by delivering appropriate dilutions of stock solutions of each drug at the same rate of infusion (70 μl min^{-1}). Only one concentration of each drug was tested in each liver. After 20 min of perfusion phenylephrine (1 μM) was added to the perfusion medium. The responses were measured 10 min after addition of phenylephrine, during the sustained phase of the response. Data points represent mean values ± s.e.mean of, at least, three experiments. The asterisk denotes significance ($P<0.05$) by paired t test, compared to the responses in the absence of colchicine or β-lumicolchicine. The basal average absolute values ± s.e. in the absence and in the presence of colchicine (10 μM) are described in the legend to Figure 1. In the presence of β-lumicolchicine (10 μM) these values were: oxygen uptake: 1010 ± 65 μatom 100 g^{-1} body wt. h^{-1}; pH: 7.407 ± 0.072 units; [Ca^{2+}]: 1.000 ± 0.005 mM; glucose production: 343 ± 29 μmol 100 g^{-1} body wt. h^{-1}.

effectiveness of colchicine in preventing the α_1-adrenoceptor effects is a function of the time of hepatic exposure to the inhibitor.

Figure 3 shows the effect of different colchicine and β-lumicolchicine concentrations on the hepatic α_1-adrenoceptor stimulation by 1 μM phenylephrine. The lowest effective concentration of colchicine was 1 μM. All hepatic α_1-adrenoceptor-mediated responses show a similar sensitivity to colchicine but the increase in portal pressure requires a 10 fold higher concentration. The isomer β-lumicolchicine prevented all the α_1-adrenoceptor-mediated responses but the increase in vascular resistance. This observation indicates that the action of colchicine cannot be merely ascribed to its effects in depolymerizing tubulin.

The possibility that colchicine acted to decrease the α_1-adrenoceptor ligand affinity could be ruled out by the determination of the colchicine effect on the binding of [^{3}H]-prazosin to liver membranes. The B_{max} of the binding of prazosin to liver membranes was 768 ± 50 fmol mg^{-1} of protein and the K_d 227 ± 17 pM (mean ± s.e.mean of 4 determinations). Neither the binding, nor its displacement by phenylephrine was altered by colchicine (Figure 4).

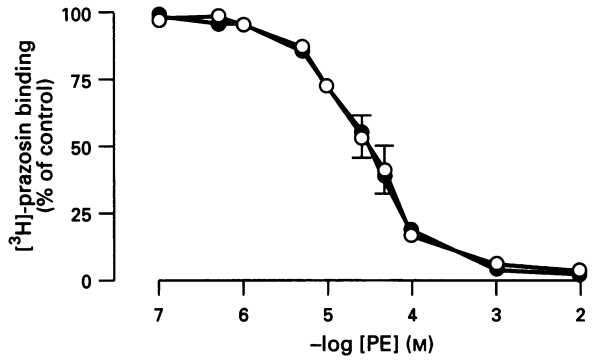

Figure 4 Effect of colchicine on phenylephrine displacement of bound [^{3}H]-prazosin to hepatic plasma membranes. Concentration-dependent phenylephrine displacements of bound 1 nM [^{3}H]-prazosin to plasma membranes were performed after 20 min of preincubation at room temperature of 80 μg of membranes in the absence (○) or in the presence of colchicine (10 μM) (●). The reaction mix (500 μl of final volume) was incubated for 30 min at 25°C. Non-specific binding was determined in the presence of prazosin (1 mM). The results are expressed as percentage values of the binding of [^{3}H]-prazosin in the absence of phenylephrine, which was 6940 ± 385 and 7022 ± 408 c.p.m. for control and colchicine-treated membranes, respectively. The values represent means of five separate experiments ± s.e.

Effect of colchicine on the vasopressin receptor-mediated stimulation of hepatic functions

Unlike the α_1-agonists, the Ca^{2+} mobilizing vasoactive peptide, vasopressin, produced only acute and transient increases in respiration and Ca^{2+} release (Figure 5 left panels) and a more prolonged increase in glucose mobilization that lasted as long as the agonist was infused. As previously reported (Butta *et al.*, 1993), vasopressin did not alter portal pressure (result not shown) and the extracellular acidification could be accounted for by the H^+ accompanying lactate release. Colchicine inhibited the glycogenolytic action of vasopressin and this effect was accompanied by near proportional changes in H^+ output.

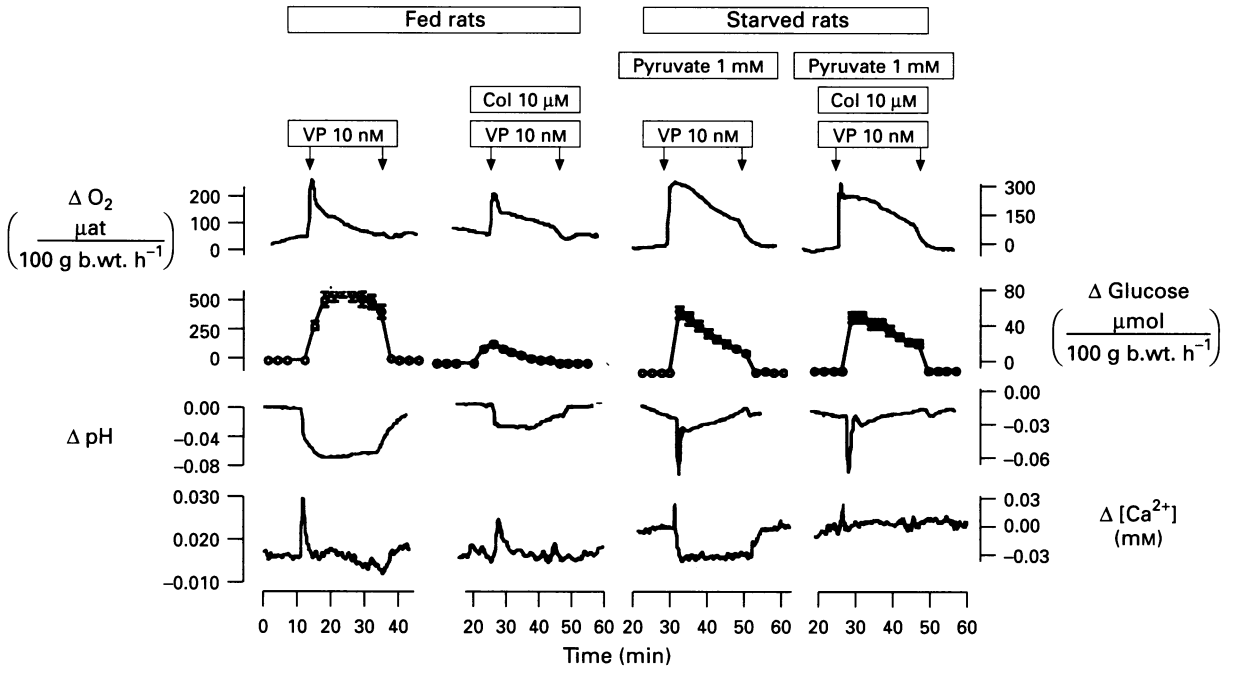

Figure 5 Effect of colchicine on the vasopressin-induced responses of livers from fed or starved rats. Livers from rats fed *ad libitum* were allowed to equilibrate until steady rates of oxygen consumption were attained. Livers from 48 h starved rats were allowed to equilibrate for ≅30 min and then pyruvate (1 mM) was added to the perfusion medium. When steady rates of oxygen consumption were attained colchicine was infused into the portal vein. When indicated, vasopressin (VP) was added to the perfusion medium. The final concentrations used are indicated. Each experiment was repeated five times obtaining reproducible results and representative experiments are presented. The basal average absolute values ± s.e. obtained in the absence and in the presence of colchicine (Col, 10 μM) are described in the legend to Figure 1.

Vasopressin produces a stimulation of gluconeogenesis and a concomitant increase in respiration. This effect is accounted for by the energy cost of the increased rate of glucose production, in livers from starved rats perfused with pyruvate as the carbon source (Figure 5). Colchicine does not inhibit the vasopressin stimulation of gluconeogenesis. In livers from starved rats, vasopressin elicits a net inflow of Ca^{2+}, that lasts as long as it is administered (Altin & Bygrave, 1985; Butta *et al.*, 1993). This effect was prevented by colchicine (Figure 5, lower right panels).

Effect of colchicine on α_1-adrenoceptor-induced rise in cytosolic free Ca^{2+}, intracellular pH, and protein kinase C activity

The action of colchicine in perturbing the α_1-adrenoceptor or vasopressin-induced changes in Ca^{2+} fluxes (Figures 1, 3, and 5) prompted us to study whether the receptor-induced changes in cytosolic free Ca^{2+} were also impaired. Experiments carried out in isolated liver cells demonstrated that colchicine does not prevent either the α_1-adrenoceptor or the vasopressin receptor-induced rise of cytosolic free Ca^{2+} concentration (Figure 6). Moreover, colchicine enhanced both the α_1-adrenoceptor as well as the vasopressin receptor-induced mobilization of Ca^{2+}.

We have recently reported that an essential feature of the hepatic α_1-adrenoceptor activation is the stimulation of the Na^+/H^+ neutral antiporter, resulting in a sustained H^+ release and intracellular alkalosis (Urcelay *et al.*, 1993b). In view of this antecedent we found it of interest to determine whether colchicine alters the α_1-adrenoceptor-induced intracellular alkalosis. Figure 6 shows that colchicine reduced by about 50% the α_1-adrenoceptor-induced intracellular alkalinization. Since colchicine also decreases the α_1-agonist-induced extracellular acidification (Figure 1), it seems plausible to conclude that colchicine impedes the α_1-agonist-induced activation of the Na^+/H^+ exchanger.

The α_1-agonist-induced stimulation of the hepatic Na^+/H^+ exchanger is associated to the activation of protein kinase C (Urcelay *et al.*, 1994). Therefore we examined whether colchicine had any effect on the α_1-agonist-mediated activation of protein kinase C. Figure 7 shows that phenylephrine induces the translocation of protein kinase C from the cytosol to the plasma membrane in isolated hepatocytes. Colchicine prevented the translocation of protein kinase C to the plasma membrane. The structural analogue β-lumicolchicine, which is ineffective in depolymerizing microtubules, has the same effect as colchicine in preventing the receptor-induced protein kinase C translocation. Neither colchicine nor β-lumicolchicine altered the basal activity of protein kinase C. The maximal effect of α_1-adrenoceptor stimulation on protein kinase C translocation was attained at 5 min in perfused livers (results not shown). This timing coincides with that of the other hepatic sustained responses (Figure 1). We have also studied the time course of the effect of colchicine on the α_1-adrenoceptor-induced protein kinase C translocation in perfused livers. Figure 8 shows that 20 min infusion of colchicine dissolved in KRB, but not KRB alone, abolished the α_1-agonist-induced protein kinase C translocation. Treatment with colchicine for only 5 min was sufficient to detect its inhibitory action. The time course of colchicine effectiveness coincides with that of the other hepatic responses (results not shown).

Effect of colchicine on α_1-agonist-induced activation of glycogen phosphorylase activity

Colchicine did not alter either the basal or the α_1-adrenoceptor activation of phosphorylase activity in perfused liver (Figure 9). Although this observation is consistent with the lack of effect of colchicine in preventing Ca^{2+} mobilization, it seems to be in conflict with its action in decreasing the α_1-adrenoceptor-induced stimulation of glycogenolysis (Figure 1). This observation may indicate that colchicine acts to prevent the accessibility of glycogen phosphorylase to its site of action.

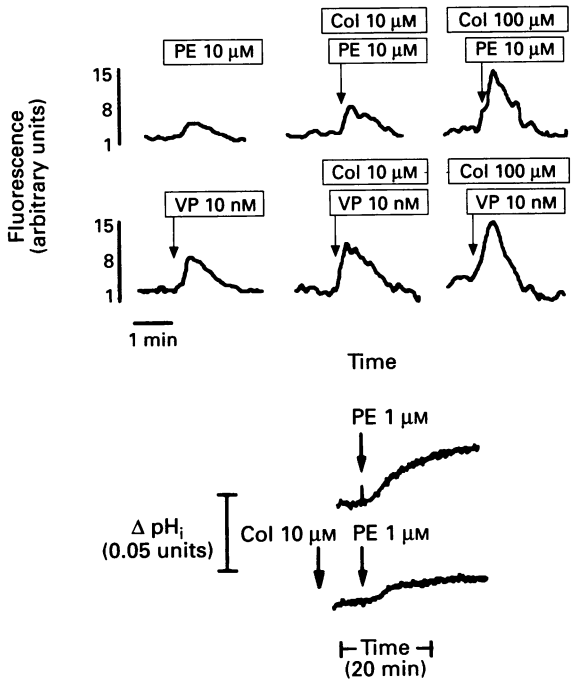

Figure 6 Effect of colchicine on the α_1-adrenoceptor or vasopressin-induced changes in intracellular free cytosolic Ca^{2+} and on the α_1-adrenoceptor-induced intracellular alkalosis. For the measurement of intracellular free cytosolic $[Ca^{2+}]$, portions of Fura-2-loaded hepatocyte suspensions were incubated at 36.5°C and the intracellular fluorescence of the indicator was continuously monitored as described in the Methods section. Each experiment was repeated at least three times and representative tracings are shown. The results are expressed as arbitrary fluorescence units. The average basal $[Ca^{2+}]$ values ± s.e. were 327 ± 31; 327 ± 32 and 327 ± 32 nM in the absence and in the presence of 10 or 100 μM colchicine (Col), respectively. For the measurement of intracellular pH, aliquots of BCECF-loaded hepatocytes were incubated for 15 min in HEPES, pH 7.4, at 37°C, in the absence or in the presence of colchicine (10 μM) and then phenylephrine (PE, 1 μM) was added. Each trace is representative of observations of at least five different cell preparations. The cytosolic basal pH was 7.04 ± 0.04 ($n = 5$). The mean ± s.e. increase in cytosolic pH induced by phenylephrine was 0.067 ± 0.001 ($n = 5$). Similar results were obtained when the experiments were performed in a bicarbonate buffered medium.

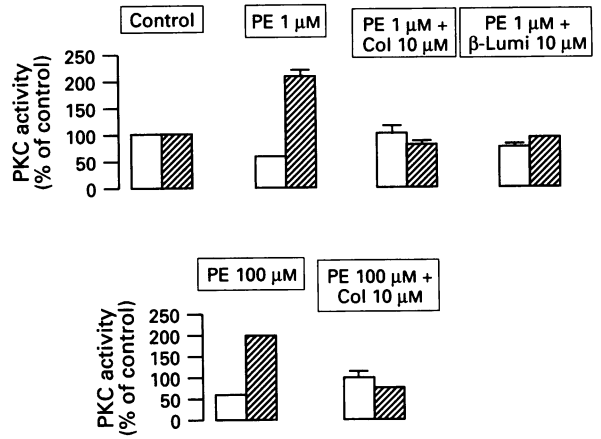

Figure 7 Effect of colchicine (Col) and β-lumicolchicine (β-Lum) on the α_1-adrenoceptor-induced stimulation of protein kinase C. Isolated hepatocytes (100 mg wet wt. ml^{-1}) were preincubated for 20 min in the absence (control) or in the presence of colchicine (10 μM) or β-lumicolchicine. Then, 1 μM or 100 μM phenylephrine (PE) was added for 10 min. Protein kinase C was assayed, as described in Methods, in cytosol (open columns) and in solubilized plasma membranes (hatched columns). Values are presented as the percentage of the basal activities of protein kinase C in cytosolic and in membrane fractions, which were, respectively, 233 ± 32 and 115 ± 26 pmol $min^{-1} mg^{-1}$ of protein. After treatment with colchicine or β-lumicolchicine the protein kinase C activity (pmol $min^{-1} mg^{-1}$ of protein) was 190 ± 17 and 214 ± 15 in cytosol and 90 ± 12 and 98 ± 20 in membranes, respectively. Values shown are the mean ± s.e. of 5 experiments carried out in triplicate.

Discussion

The transmission of signal(s) from a ligand-activated α_1-adrenoceptor is coupled to guanine nucleotide regulatory proteins in rat liver (Im & Graham, 1990; Butta *et al.*, 1993). Receptor-coupled G proteins and tubulin are oligomeric proteins showing significant structural homology and functional similarities (Sternlicht *et al.*, 1987; Rasenick *et al.*, 1989; Mandelkow *et al.*, 1985). Moreover, interactions between signal transducing G proteins and tubulin are well characterized (Higashi & Ishibashi, 1985; Wang *et al.*, 1990). Recent work has also demonstrated that a functional relationship seems to exist between the cytoskeleton and ion channels (Nakamura & Rodbell, 1990) or G-protein-regulated membrane phospholipase C (Vaziri & Downes, 1992). In the light of these observations, the inhibitory effects of colchicine on the hepatic α_1-adrenoceptor responses could be related to either disorganization of the cytoskeleton or perturbation of protein-protein interactions, or both.

Colchicine binds irreversibly to tubulin resulting in the time- and concentration-dependent depolymerization of microtubules (Wilson *et al.*, 1974). The formation of the colchicine-tubulin complex is a slow process that may take several hours to reach an equilibrium (Wilson *et al.*, 1974). The action of colchicine in perturbing the α_1-adrenoceptor responses was observed 10 or less minutes after its addition (results not

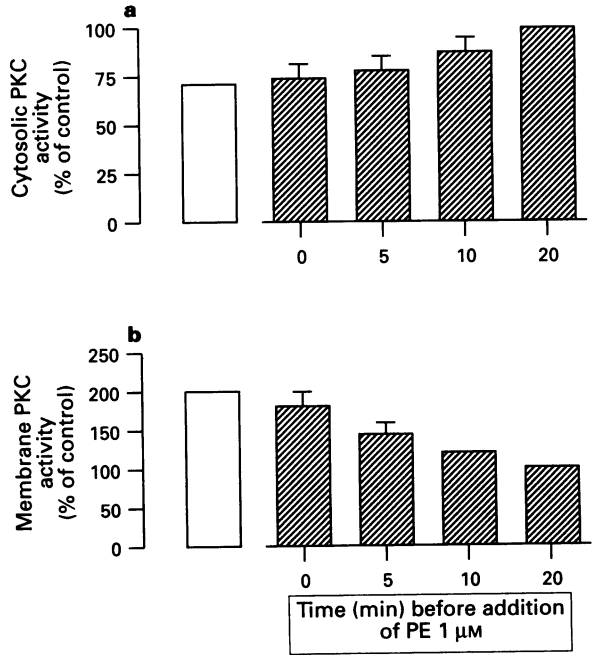

Figure 8 Time course of the effect of colchicine on the α_1-adrenoceptor-induced stimulation of protein kinase C in perfused livers from fed rats. Livers from rats fed *ad libitum* were allowed to equilibrate until steady rates of oxygen consumption were attained and then colchicine was infused into the portal vein. At the indicated times thereafter, phenylephrine (1 μM) was administered diluted in the medium (hatched columns). After 10 min of perfusion with phenylephrine, one liver biopsy was taken. The open columns represent the effect of phenylephrine (1 μM) added after 20 min of perfusion in the absence of colchicine. Protein kinase C was assayed, as described in Methods, in cytosol (a) and in solubilized plasma membranes (b). Values are presented as the percentage of the activity of protein kinase C in cytosolic and in membrane fractions at time zero, that is, just before the addition of colchicine, which were, respectively, 61.3 ± 2.5 and 30 ± 2.9 pmol $min^{-1} mg^{-1}$ of protein. Values shown are the mean ± s.e. of 5 experiments carried out in triplicate.

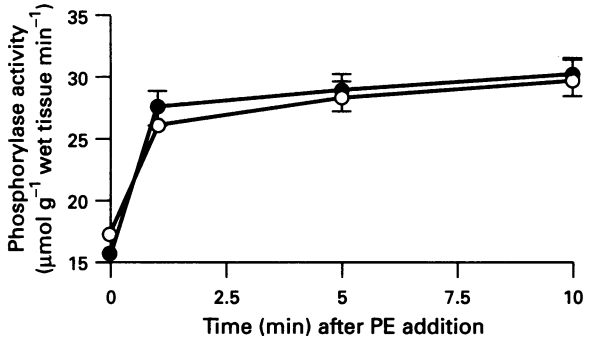

Figure 9 Effect of colchicine on the α_1-adrenoceptor-induced stimulation of glycogen phosphorylase in perfused livers. Livers isolated from rats fed *ad libitum* were perfused in a non-recirculating system as described in Methods. The time course of the phenylephrine action after a 20 min period of perfusion either in the absence of (○) or with colchicine (10 μM final concentration) (●) is represented. When steady rates of O_2 consumption were attained, two liver biopsies were taken. The first one was taken from a small lobule, immediately before the addition of phenylephrine (PE, 1 μM). The second biopsy was taken at one of the indicated times. Values shown are the mean ± s.e. of 4 experiments.

shown), indicating that colchicine alters the α_1-adrenoceptor-mediated responses by means other than merely disrupting the tubulin assembly. Moreover, β-lumicolchicine is able to reproduce most of the effects of colchicine (Figures 3 and 7) even though it is not effective in depolymerizing microtubules. This interpretation agrees with previous work indicating that colchicine could elicit responses apparently unrelated to its ability to disassemble the microtubules in mammalian cells (Mizel & Wilson, 1972; Ravindra & Grosvenor, 1990). Among these actions is the ability of colchicine to modify the behaviour of membrane proteins (Wunderlich *et al.*, 1973). In earlier work in which colchicine was used to study adrenal medullary secretion evoked by activation of the acetylcholine receptor, the proposal was advanced that its action was exerted at an early step of the stimulus-secretion coupling (Douglas & Sorimachi, 1972). Recent work on this subject has shown that colchicine specifically inhibits the acetylcholine receptor-mediated stimulation of G protein GTPase activity (Ravindra & Aronstam, 1990). On these grounds, it seems likely that colchicine acts on the α_1-adrenoceptor signalling pathway by interacting with receptor-coupled regulatory elements either directly or through its binding to associated tubulin. We have recently shown that a pertussis toxin-sensitive G protein controls the α_1-adrenoceptor ligand affinity (Butta *et al.*, 1993). The possibility that colchicine may also act to decrease the receptor ligand affinity seems improbable since colchicine does not alter the binding of α_1-agonist to isolated plasma membranes or its displacement by phenylephrine (Figure 4).

In contrast to pertussis toxin that inhibited all the α_1-adrenoceptor-induced responses, colchicine did not inhibit gluconeogenesis (Figure 1) and enhanced intracellular Ca^{2+} mobilization (Figure 6) and increase in portal pressure (Figure 1). This difference most probably reflects interaction with specific regulatory elements. The heterogeneity of the G proteins α subunit (Birnbaumer, 1992) might offer the molecular basis to postulate the existence of populations of α_1-receptor subtypes differing only in the functional properties of their associated regulatory elements. Therefore, an alternative explanation to the colchicine effect in dissociating the α_1-agonist-induced responses might be that colchicine interacted exclusively with some but not all α_1-receptor subtypes. The differential effects of colchicine on the α_1-adrenoceptor responses of hepatocytes and in the portal blood pressure, that depend on other cell types such as vascular smooth muscle or sinusoidal cells, may also be explained by the existence of distinct patterns of receptor-associated proteins in each tissue. However, the lack of enhancing effect of β-lumicolchicine on the α_1-agonist-mediated increase in portal pressure suggests that colchicine acts on the vascular smooth muscle or sinusoidal cells by a different mechanism from that in parenchymal cells. It should also be considered that colchicine may interact with intracellular pathways involved in vascular contraction induced by α_1-agonists or interfere with the release and/or metabolism of regulatory endogenous products, such as nitric oxide (Ignarro, 1990) or peptides (Yanagisawa *et al.*, 1988), in the endothelial cells.

The hepatic actions of the Ca^{2+} mobilizing agonists have been intimately associated with their ability to increase the intracellular concentration of free cytosolic Ca^{2+} and the subsequent activation of Ca^{2+}-dependent processes (Williamson *et al.*, 1985; Exton 1988). However, work showing that α_1-agonists may activate hepatic functions under Ca^{2+} loading conditions that precluded any further significant increase in the cytosolic free Ca^{2+} concentration (Buxton *et al.*, 1982; García Sainz & Hernández-Sotomayor, 1985; Saz *et al.*, 1989), supported the operation of a free cytosolic Ca^{2+}-independent branch of the signalling pathway. In the present work, the use of colchicine has achieved for the first time a clear dissociation between free cytosolic Ca^{2+}-dependent and independent α_1-adrenoceptor-induced responses in the intact liver. According to our observations, a correlation exists between the receptor-mediated elevation of cytosolic free Ca^{2+} and the stimulation of gluconeogenesis or increase in portal pressure (Figures 1 and 6); however, the elevation of cytosolic free Ca^{2+} is not a sufficient signal to permit maximal stimulation of energy production, glucose mobilization, extracellular acidification or Ca^{2+} release (Figures 1 and 3). These observations suggest that, unlike gluconeogenesis or portal pressure, the rate control of these parameters is governed by a colchicine-sensitive/free cytosolic Ca^{2+}-independent mechanism. We have recently reported that α_1-agonists-induced sustained stimulation of hepatic respiration, and H^+ and Ca^{2+} release, are interrelated through a coupling of plasma membrane Na^+/H^+ and Na^+/Ca^{2+} exchangers, that ultimately rely on the activation of protein kinase C (Urcelay *et al.*, 1993b). On these grounds, it was reasonable to assume that colchicine could interact with the mechanism(s) involved in the α_1-adrenoceptor-mediated activation of protein kinase C. This assumption appears to be correct in view of the effects of either colchicine or β-lumicolchicine in preventing the α_1-agonist-induced translocation of protein kinase C to the plasma membrane (Figure 7). Moreover, the effects of colchicine on protein kinase C activation and protein kinase C-mediated responses were not reversed by increasing the α_1-agonist concentration (Figure 7). Thus, the mechanism(s) of colchicine inhibition of the α_1-adrenoceptor responses is related to the receptor-mediated activation of protein kinase C.

Colchicine enhances the α_1-adrenoceptor-induced acute intracellular mobilization of Ca^{2+} (see Figure 6). The acute phase of release of Ca^{2+} did not seem to be perturbed by colchicine (Figure 1); therefore, the augmented cytosolic Ca^{2+} signal seems to be a consequence of an enhanced mobilization from intracellular stores and/or enhanced plasma membrane inflow. In contrast, colchicine inhibited the sustained phase of release of Ca^{2+} to the effluent perfusate (Figures 1 and 3). This finding corroborates the idea that the α_1-adrenoceptor-induced sustained release of Ca^{2+} to the effluent perfusate, a distinct feature of the α_1-adrenoceptor stimulation, is independent of the mechanism(s) controlling its acute mobilization and most probably is a consequence of the limited stimulation of the Na^+/H^+ exchanger.

In addition to its effect in enhancing the α_1-adrenoceptor-induced acute intracellular Ca^{2+} mobilization, colchicine also enhances the vasopressin-induced rise of cytosolic free Ca^{2+} in isolated cells (Figure 6) and inhibits the vasopressin-induced sustained entry of Ca^{2+} from the extracellular medium (Figure 5). These observations are consonant with the idea that a signal-transducing G protein may be associated with more than one Ca^{2+}-mobilizing receptor (Dasso & Taylor, 1992). It

also agrees with the finding that a pertussis toxin-sensitive G regulatory protein controls α_1-adrenoceptor and vasopressin receptor-induced Ca^{2+} fluxes (Butta *et al.*, 1993; Berven *et al.*, 1994).

In agreement with the normal Ca^{2+} mobilizing response, colchicine did not alter the α_1-adrenoceptor stimulation of glycogen phosphorylase (Figure 9); however, the rate of glycogen breakdown was considerably reduced (Figure 1). The latter observation agrees with previous work in which colchicine was observed to perturb the carbohydrate metabolism (Tomomura *et al.*, 1980; Al-Habori *et al.*, 1991). The finding that phosphorylase 'a' activation is not sufficient to elicit a normal glycogenolytic response suggests that the active enzyme must be relocated in a different cell compartment and this process is impeded by colchicine. This observation again opens the debate on whether the compartmentation of glycogen phosphorylase plays a role in the regulation of glycogenolysis (Law & Tidball, 1992).

In conclusion, the present study reveals that colchicine prevents the α_1-adrenoceptor-induced activation of protein kinase C without altering the receptor-mediated intracellular Ca^{2+}-mobilization. To our knowledge, this is the first time that a clear dissociation of the α_1-adrenoceptor-induced free cytosolic Ca^{2+}-dependent and independent responses has been reported. Our data support the existence of at least two major signalling pathways associated with the hepatic α_1-adrenoceptor activation. The first is colchicine-sensitive and protein kinase C-dependent; while in contrast the second one is colchicine-insensitive and free cytosolic Ca^{2+}-dependent. Processes that are characteristically stimulated by α_1-adrenoceptor activation, such as sustained H^+ and Ca^{2+} release to the extracellular medium are associated with the protein kinase C-dependent pathway. Stimulation of gluconeogenesis occurs in the absence of protein kinase C activation, therefore, this process may be regulated by the free cytosolic Ca^{2+}-dependent branch of the signalling pathway. Although the usefulness of colchicine as an inhibitor may be questioned, based on its multiple sites of interaction, it seems to be a valid tool for studying the functional organization of Ca^{2+}-mobilizing receptors.

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