Collagen type IV stimulates an increase in intracellular Ca^{2+} in pancreatic acinar cells via activation of phospholipase C

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Intracellular Ca^{2+} responses to extracellular matrix molecules were studied in suspensions of pancreatic acinar cells loaded with Fura-2. Collagen type I, laminin, fibrinogen and fibronectin were unable to raise cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), whereas collagen type IV, at concentrations from 5 to 50 $\mu\text{g}/\text{ml}$, significantly increased it. The effect of collagen type IV was not due to possible contamination with type-I transforming growth factor β or plasminogen, as neither of these agents was able to increase $[\text{Ca}^{2+}]_i$. Using highly specific mass assays, concentrations of inositol lipids, 1,2-diacylglycerol (DAG) and $\text{Ins}(1,4,5)\text{P}_3$ were measured in pancreatic acinar cells stimulated with collagen type IV. A decrease in the concentrations of $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}4\text{P}$ with a concomitant increase in the concentrations of DAG and InsP_3 mass were observed, showing that collagen type IV increases $[\text{Ca}^{2+}]_i$ by activation of phospholipase C. The observed $[\text{Ca}^{2+}]_i$ signals had two components, the first resulting from Ca^{2+} release from the intracellular stores, and the second resulting from Ca^{2+} flux from the extracellular medium through the verapamil-insensitive channels. A tyrosine kinase inhibitor

(tyrphostine) was able to block inositol lipid signalling caused by collagen type IV, which together with the insensitivity of this pathway to cholera toxin and pertussis toxin or to preactivation of protein kinase C, the longer duration of the increase in $[\text{Ca}^{2+}]_i$ and a longer lag period needed for observation of increases in DAG and InsP_3 concentration with collagen type IV than with carbachol (50 mM) suggest that activation of phospholipase C by collagen type IV is caused by tyrosine kinase activation. Inositol lipid signalling and increases in $[\text{Ca}^{2+}]_i$ were also observed with Arg-Gly-Asp (RGD)-containing peptide but not with Arg-Asp-Gly (RDG)-containing peptide. Collagen type IV and RGD-containing peptide, but not carbachol, competed in increasing $[\text{Ca}^{2+}]_i$ and DAG concentration, suggesting that the binding site of collagen type IV responsible for phospholipase C activation contains the RGD sequence. Together the present results suggest that, in pancreatic acinar cells, RGD sequence(s) within collagen type IV molecules cause activation of tyrosine kinase, probably through one of the integrin receptors, which then stimulates phospholipase C and increases $[\text{Ca}^{2+}]_i$.

INTRODUCTION

In multicellular organisms, basement membranes and extracellular matrix are found in most tissues. They are produced by epithelial, endothelial and mesenchymal cells and exert a strong influence on the adjacent cells. Although the molecular architecture and assembly of basement membranes are well documented (Yurchenco and Schittny, 1990; Paulsson, 1992), in recent years it has become evident that basement membranes not only act as passive selective molecular sieves between tissue compartments (Farquhar, 1981), but also play an important role in the passage of cells during inflammatory and metastatic processes, and act as solid-phase regulators of cell attachment, growth and differentiation (Barsky et al., 1984; Timpl, 1989). These functions are mainly dependent on the binding of cell-surface integrin receptors to extracellular matrix determinants (Hynes, 1992). The signal-transduction pathways by which integrin receptors respond to basement membrane and extracellular matrix components are only partially understood (Damsky and Werb, 1992; Juliano and Haskill, 1993). In particular, the role of the inositol lipid/ Ca^{2+} signalling pathway which controls many cellular functions, including cell growth, is unknown (Berridge and Irvine, 1989; Berridge, 1993). In inositol lipid/ Ca^{2+} signalling processes, there are two major receptor-mediated pathways for stimulating the formation of InsP_3 and diacylglycerol (DAG). In the first pathway, the receptor, which uses a GTP-binding protein, activates phospholipase C (PLC)- β 1,

whereas in the second, PLC- γ 1 is stimulated by a tyrosine kinase-linked receptor (Berridge, 1993).

The aim of the present study was to investigate the role of the inositol lipid/ Ca^{2+} signalling pathways in pancreatic acinar cells stimulated with different components of extracellular matrix. The results suggest that only collagen type IV, through its RGD sequence(s)-containing site(s) and as a result of activation of tyrosine kinases, stimulates PLC and increases $[\text{Ca}^{2+}]_i$.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: collagen type IV, laminin and FALRDGNP peptide were a generous gift from Dr. H. Kleinman, National Institutes of Health, Bethesda, MD, USA; type-I collagen and fibronectin from Collaborative Research, Bedford, MA, U.S.A.; GRGDSP peptide from Peninsula Laboratories, Belmont, CA, U.S.A.; collagenase type III from Worthington, Freehold, NJ, U.S.A.; cholera toxin and pertussis toxin from Gibco-BRL, Gaithersburg, MD, U.S.A.; Fura-2/AM from Molecular Probes, Eugene, OR, U.S.A.; tyrphostine (AG-115) was a generous gift from Dr. A. Levitzki, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, Jerusalem, Israel; $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ from Amersham International, Amersham, Bucks., U.K.; transforming growth factor β (TGF- β), phorbol 12-myristate 13-acetate (PMA), plasminogen, fibrinogen, chole-

Abbreviations used: DAG, 1,2-diacylglycerol; PLC, phospholipase C; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; TGF- β , type-I transforming growth factor β ; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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cystokinin octapeptide, carbachol, ionomycin and verapamil from Sigma, St. Louis, MO, U.S.A. All other chemicals were of analytical grade. All studies were performed on Wistar rats of both sexes aged 3–4 months.

Preparation of isolated cells

Isolated acinar cells from rat pancreas were prepared by two-step collagenase digestion exactly as described previously (Schnefeld et al., 1988).

Measurement of $[Ca^{2+}]_i$

Isolated pancreatic acinar cells were incubated in Hanks' physiological salt solution with 2 mM Fura-2/AM at 37 °C for 30 min, washed and resuspended in fresh solution. Cells (1.5×10^6) were transferred to a 3 ml cuvette for measurement of $[Ca^{2+}]_i$. The fluorescence was measured in a Perkin-Elmer LS50 spectrofluorimeter with excitation at 340 and 380 nm and emission at 510 nm. The cuvette was thermostatically controlled at 37 °C, and the cell suspension continuously stirred. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio, as described by Grynkiewicz et al. (1985). Maximum fluorescence was determined by the addition of 10 mM ionomycin, and the minimum fluorescence determined by the addition of 50 mM EGTA.

Mass assays of second messengers and inositol lipids

When second messengers and inositol lipids were to be measured, pancreatic acinar cells were incubated in Hanks' physiological salt solution at 37 °C. DAG was extracted from the cells using 0.75 ml of chloroform/methanol (1:2, v/v). When inositol lipids were to be measured, cells were extracted using 0.75 ml of chloroform/methanol/1 M HCl (80:160:1, by vol.). Further extraction was performed as described by Folch et al. (1957). Mass measurements of inositol lipids were made as described below, but for measurement of DAG concentration, the lipid extract was dissolved in 0.5 ml of chloroform and loaded on a silicic acid column (0.5 ml made in a Pasteur pipette), eluted with 1 ml of chloroform, dried again and the mass determined as described below. When $InsP_3$ concentration was to be measured, cells were extracted using 10% (w/v) trichloroacetic acid, which was removed by repeated washing with water-saturated diethyl ether (Palmer and Wakelam, 1990). The sample was evaporated to dryness in a Vac-Fuge, and the mass of $InsP_3$ was then determined as described below.

DAG

DAG kinase purification was achieved in a single step from rat brain on a DEAE-Sepharose column as described by Divecha and Irvine (1990). DAG mass was determined as follows. The dried lipid was dissolved in 20 μ l of CHAPS (9.2 mg/ml) and sonicated at room temperature for 15 s. After the addition of 80 μ l of buffer (50 mM Tris/acetate, 80 mM KCl, 10 mM magnesium acetate, 2 mM EGTA, pH 7.4), the reaction was started by the addition of 20 μ l of DAG kinase followed by 80 μ l of buffer containing 20 μ M ATP and 1 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After incubation for 1 h at room temperature, the reaction was stopped by the addition of 750 μ l of chloroform/methanol/HCl (80:160:1, by vol.). Phosphatidic acid was extracted as described by Folch et al. (1957) and chromatographed on oxalate (1%)-

sprayed t.l.c. plates using the following solvent system: chloroform/methanol/concentrated NH_3 /water (45:35:2:8, by vol.). After autoradiography, the spots corresponding to phosphatidic acid were scraped off and their ^{32}P content was determined by scintillation counting. DAG mass in each sample was adjusted to between 5 and 50 pmol, as the sensitivity of the mass assay is highest in this range (Divecha et al., 1991).

PtdIns P_2 and $InsP_3$

To the dried PtdIns P_2 , 150 μ l of monomethylamine reagent, prepared as described by Clarke and Dawson (1981), was added and the sample was incubated at 53 °C for 60 min. The sample was dried and 100 μ l of 50 mM sodium periodate was added. After 30 min at room temperature, 20 μ l of ethylene glycol (10% v/v) was added to stop the reaction, and after 15 min, 50 μ l of 1% (v/v) 1,1-dimethylhydrazine/formic acid (pH 4); the mixture was allowed to stand for a further 1 h. The pH of the sample was adjusted to 7 with 1 M Tris/HCl and the sample was evaporated to dryness. The preparation of binding protein and the procedure for the binding assay for $InsP_3$ were exactly as described by Palmer and Wakelam (1990). The PtdIns P_2 and $InsP_3$ content in each sample was adjusted to between 0.5 and 5 pmol, as the sensitivity of the mass assay is highest in this range (Divecha et al., 1991).

PtdInsP

This was performed using a highly purified bovine brain PtdInsP kinase as described by Divecha et al. (1991). The mass was measured as follows. Standard quantities of PtdInsP or lipid extract from cell membranes were dried by vacuum centrifugation and resuspended in 100 μ l of buffer (50 mM Tris, 10 mM MgSO_4 , 80 mM KCl, 2 mM EGTA, pH 7.4), followed by bath sonication. The reaction was started by the addition of 100 μ l of the above-mentioned buffer containing 20 μ l of enzyme, 10 μ M ATP and 1 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol). After 1 h at room temperature, the reaction was terminated by the addition of 750 μ l of chloroform/methanol/conc. HCl (80:160:1, by vol.), and the lipids were extracted as in Folch et al. (1957). After the separation of lipids by t.l.c. (as for the DAG assay), PtdIns P_2 spots were scraped off after identification by autoradiography and quantified by scintillation counting. The PtdInsP content in each sample was adjusted to between 2 and 16 pmol, as the sensitivity of the mass assay is highest in this range (Divecha et al., 1991).

PtdIns

The mass of PtdIns was measured using a coupled assay employing a mixture of PtdIns-specific PLC and the DAG kinase as described previously (Divecha et al., 1991). The dried lipid was dissolved by the addition of 50 μ l of buffer (80 mM Tris/acetate, 10 mM magnesium acetate, 2 mM EGTA, 10 mM KCl, 70 mM NaCl, pH 7.4) and sonicated for 15 s. Phosphatidylethanolamine (50 nmol) in 80 μ l of the above buffer was then added. The reaction was started by the addition of 20 μ l of enzyme mixture followed by 50 μ l of buffer containing 5 μ M ATP and 1 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction was continued for 1 h at room temperature and PtdOH was extracted, scraped off and quantified as for the DAG assay (above). The PtdIns content in each sample was adjusted to be between 5 and 50 pmol, as the sensitivity of the mass assay is highest in this range (Divecha et al., 1991).

Protein determination and statistical evaluation

Proteins were determined by the method of Bradford (1976). The

Figure 1 (a) Representative $[Ca^{2+}]_i$ response in a suspension of pancreatic acinar cells stimulated with collagen type IV (20 µg/ml) and (b)–(f) time courses of changes in InsP₃, DAG, PtdInsP₂, PtdInsP and PtdIns concentration respectively in pancreatic acinar cells stimulated with the same concentration of collagen type IV

In (a) cells were prepared, loaded with Fura-2 and incubated as described in the Materials and methods section. All other details are as described in the Materials and methods section. Each point represents the mean ± S.E.M. from four different experiments in duplicate. * Significant difference ($P < 0.05$, Student's *t* test) from controls.

data are shown as means ± S.E.M. For statistical analyses, Student's *t* test for unpaired samples at the level of significance of 0.05 was used.

RESULTS

Figure 1(a) shows a representative $[Ca^{2+}]_i$ response in a suspension of pancreatic acinar cells stimulated with collagen type IV. Resting $[Ca^{2+}]_i$ varied from 55 to 150 nM (mean value

103 ± 4 nM, number of cell preparations = 11) which is identical with the typical resting $[Ca^{2+}]_i$ obtained in several types of unstimulated cells including pancreatic acinar cells (Berridge and Irvine, 1989; Zhang and Muallem, 1992; Berridge, 1993). The average amplitude of the increase was 178 ± 28 ($n = 11$) with a minimum increase of 80 nM and a maximum increase of 400 nM. Within seconds of treatment of cells with collagen type IV, an increase in InsP₃ (Figure 1b) and DAG (Figure 1c) could be observed with a concomitant decrease in PtdInsP₂ (Figure 1d)

Figure 2 (a) Representative dose-response curves for $[Ca^{2+}]_i$ in a suspension of pancreatic acinar cells stimulated with different concentrations of collagen type IV ($\mu\text{g/ml}$) and (b) dose-response curve for collagen type IV-stimulated increase in DAG concentration in pancreatic acinar cells after 1 min of incubation

All details are as described in the Materials and methods section and the legend to Figure 1.

Figure 3 Representative $[Ca^{2+}]_i$ response in a suspension of pancreatic acinar cells stimulated with collagen type IV ($20 \mu\text{g/ml}$), in the presence of 1 mM verapamil or in medium without Ca^{2+} but containing 2 mM EGTA

All other details are as described in the Materials and methods section and the legend to Figure 1.

and PtdInsP (Figure 1e). The more sustained increase in DAG than in InsP_3 , together with the observation that the increase in DAG is greater than the loss of inositol lipids, suggests that some resynthesis of PtdInsP and PtdInsP₂ has also taken place; however, this would not produce an experimentally detectable

decrease in PtdIns, and a contribution to DAG from other lipids such as phosphatidylcholine may have also taken place, as has been demonstrated in some experimental models of stimulation of cell-surface receptors with agonist (Downes et al., 1989; Exton, 1990).

As it is known that purified collagen type IV could bind certain amounts of type-I TGF- β (Paralkar et al., 1991) and plasminogen (Stack et al., 1992), we checked whether these substances affected $[Ca^{2+}]_i$. Neither type-I TGF- β (500 pM) nor plasminogen (0.1 unit per ml) had any effect on $[Ca^{2+}]_i$ in pancreatic acinar cells (results not shown), demonstrating that collagen type IV, and not its potential contaminants, cause all the effects described above.

Figure 2 shows that collagen type IV at a concentration of $5 \mu\text{g/ml}$ produces a small increase in $[Ca^{2+}]_i$ and that a concentration of $10 \mu\text{g/ml}$ produces a maximal increase in both $[Ca^{2+}]_i$ and DAG. As shown in Figure 3, verapamil, an inhibitor of voltage-dependent plasma-membrane Ca^{2+} channels, did not influence the increase in $[Ca^{2+}]_i$ caused by collagen type IV. On the other hand, when Ca^{2+} was omitted from the medium, collagen type IV caused a smaller rise and shorter duration of $[Ca^{2+}]_i$ signal, demonstrating that extracellular Ca^{2+} is needed for a complete and sustained increase in $[Ca^{2+}]_i$.

To characterize the signalling system used by collagen type IV further, we performed experiments using carbachol, a well-known agonist for muscarinic cholinergic receptors in pancreatic acinar cells which are coupled to PLC via GTP-binding protein (Williams et al., 1989). As shown in Figure 4, carbachol induced an increase in $[Ca^{2+}]_i$, stimulating PLC-mediated inositol lipid breakdown and liberating Ca^{2+} from intracellular stores as has previously been described (Streb et al., 1983). Moreover, similar profiles of $[Ca^{2+}]_i$ mobilization and changes in concentrations of inositol lipids were observed when pancreatic acinar cells were stimulated with cholecystokinin (50 nM) (results not shown). Although it is known that preparation of pancreatic acinar cells by collagenase digestion yields about 90% pure acinar cell suspension (Streb et al., 1983; Schnefel et al., 1988), there still exists a small possibility that collagen type IV or the above-mentioned agonists in contaminating cells (e.g. duct cells, islets of Langerhans cells, blood cells etc.) could stimulate the inositol lipid/ Ca^{2+} signalling pathway. When the $[Ca^{2+}]_i$ signals obtained from carbachol- and collagen type IV-stimulated cells were compared, it was noticed that collagen type IV produced a less pronounced maximal rise in $[Ca^{2+}]_i$, but that the increase in $[Ca^{2+}]_i$ was more sustained, which, together with similar observations on the concentrations of InsP_3 and DAG (Figures 1 and 4), strongly suggests that collagen type IV and carbachol use the same, but in some way differently controlled, signalling pathway in pancreatic acinar cells. This was further delineated in experiments shown in Table 1. Pretreatment of cells with either cholera toxin or pertussis toxin had no influence on the carbachol- or collagen type IV-induced increase in DAG, suggesting that neither G_s nor G_i proteins are involved in signal transduction. Pretreatment of pancreatic acinar cells with the tumour-promoting phorbol ester, PMA, which binds and directly activates protein kinase C (PKC) (Nishizuka, 1986), completely blocked the carbachol-mediated increase in DAG only, indicating a negative-feedback control mechanism controlled by PKC in carbachol-mediated, but not collagen type IV-mediated, signal transduction. On the other hand, pretreatment of cells with tyrphostin, a tyrosine kinase blocker (Levitzki, 1992), inhibited only the collagen type IV-induced increase in DAG, further suggesting a difference between the carbachol- and collagen type IV-mediated pathways of PLC activation.

It is believed that the RGD sequences of many extracellular

Figure 4 (a) Representative $[Ca^{2+}]_i$ response in a suspension of pancreatic acinar cells stimulated with carbachol (50 μ M) and (b)–(f) time courses of changes in $InsP_3$, DAG, $PtdInsP_2$, $PtdInsP$ and $PtdIns$ concentration respectively in pancreatic acinar cells stimulated with the same concentration of carbachol

All other details are as described in the Materials and methods section and the legend to Figure 1.

matrix molecules are responsible for their binding to a family of cell-surface adhesion receptors called integrins, which are involved in adherence, spreading and migration of the cells and probably in signal transduction into the cell (Ruoslahti and Pierschbacher, 1986; Hynes, 1992; Damsky and Werb, 1992). As shown in Figure 5, GRGDSP, an RGD-containing peptide, was able to increase $[Ca^{2+}]_i$ and DAG concentration in a dose-dependent manner, whereas the RDG-containing peptide, FALRDGNP, was unable to change $[Ca^{2+}]_i$. Experiments were further designed to delineate whether or not the RGD-containing peptide and collagen type IV compete for the same receptor when activating PLC. As shown in Figure 6, addition of RGD peptide on the top of the signal developed by collagen type IV did

not produce any significant increase in DAG concentration (and vice versa). On the other hand, when RGD peptide or collagen type IV were applied on the top of the signal developed by carbachol, a significant increase in DAG concentration was observed, suggesting competition between RGD peptide and collagen type IV for the same receptor that is responsible for the development of the intracellular signal. It is important to note that RGD sequences can be found in other protein components of extracellular matrix (Timpl, 1989), but in our experimental model we were unable to observe any $[Ca^{2+}]_i$ changes when cells were stimulated with collagen type I (10 μ g/ml), laminin (10 μ g/ml), fibronectin (10 μ g/ml) or fibrinogen (20 μ g/ml) (results not shown).

Table 1 Effects of PMA, tyrphostine, cholera toxin and pertussis toxin on carbachol (50 μ M)- and collagen type IV (20 μ g/ml)-induced increase in DAG concentration in pancreatic acinar cells

Cells were pretreated for 15 min with PMA (10 μ M), for 1 h with tyrphostine (50 μ M), for 1 h with cholera toxin (40 μ g/ml) or for 6 h with pertussis toxin (5 μ g/ml) and then stimulated with carbachol or collagen type IV for 1 min. All other details are as described in the Materials and methods section. All data are from four different experiments and each measurement of DAG concentration was performed in duplicate. * Statistically significant difference ($P < 0.05$, Student's *t* test) from controls.

Pretreatment	DAG concentration (pmol/mg of protein)		
	Control	Carbachol	Collagen IV
None	62 $\pm$ 12	203 $\pm$ 17*	134 $\pm$ 14*
PMA	65 $\pm$ 13	71 $\pm$ 15	129 $\pm$ 13*
Tyrphostine	59 $\pm$ 10	191 $\pm$ 19*	62 $\pm$ 14
Cholera toxin	55 $\pm$ 4	178 $\pm$ 17*	125 $\pm$ 8*
Pertussis toxin	67 $\pm$ 7	180 $\pm$ 14*	137 $\pm$ 16*

Figure 5 (a) Representative dose-response curves for $[Ca^{2+}]_i$ in a suspension of pancreatic acinar cells stimulated with different concentrations of RGD peptide (1 μ g/ml), RDG peptide (10 μ g/ml) and collagen type IV (10 μ g/ml) and (b) time course of changes in DAG concentration in pancreatic acinar cells stimulated with the RGD peptide (10 μ g/ml)

All other details are as described in the Materials and methods section and the legend to Figure 1.

DISCUSSION

The signal-transduction pathways induced by protein components of the extracellular matrix are important in extracellular matrix cell interactions, but they are fragmentarily understood (Barsky et al., 1984; Yurchenco and Schittny, 1990; Paulsson, 1992). It is believed that, during transmission of information into the cell, integrin receptors, together with other cell surface receptors, are involved in a co-operative manner (Hynes, 1992; Damsky and Werb, 1992; Juliano and Haskill, 1993). Increased levels of $[^3H]$ arachidonylDAG were observed in glomerular epithelial cells when plated on type I or IV collagen-coated dishes (Cybulsky et al., 1990, 1993), while, in melanoma cells grown on coverslips, collagen type IV was capable of increasing $[Ca^{2+}]_i$ without stimulation of phosphoinositide hydrolysis (Savarese et al., 1992), suggesting that components of the extracellular matrix might directly activate the inositol lipid/ Ca^{2+} signalling pathway. In the present study, we have examined the mechanism by which single protein components of the extracellular matrix might activate intracellular signalling systems, and it was observed that, of all the proteins tested, only collagen type IV activates the inositol lipid/ Ca^{2+} signalling pathway. The results shown in Figure 1 corroborate the hypothesis (derived mainly from radiolabelling studies) that, in the plasma membrane of cells, DAG is initially produced in response to agonist as a result of PLC-mediated hydrolysis of inositol phospholipids, particularly $PtdInsP_2$. This DAG production is transient and temporally corresponds to the formation of $InsP_3$. It is followed by the second phase in which DAG formation results from the hydrolysis of other phospholipids, particularly phosphatidylcholine (Downes et al., 1989; Exton, 1990; Nishizuka, 1992).

In non-excitable cells, the response to Ca^{2+} -mobilizing agonists usually consists of rapid transient increases in $[Ca^{2+}]_i$ which is released from intracellular stores, followed by a sustained plateau caused by influx of extracellular Ca^{2+} (Von Tscharner et al., 1986; Berridge and Irvine, 1989). The present data also suggest that the initial part of the Ca^{2+} signal induced by collagen type IV appears to arise from the intracellular compartment, as depletion of extracellular Ca^{2+} fails to abolish completely the rise in $[Ca^{2+}]_i$. Although intracellular Ca^{2+} stores are clearly being mobilized, the data also reveal a major contribution to the Ca^{2+} signal of Ca^{2+} from extracellular stores by a, perhaps, capacitative Ca^{2+} entry. The lack of a significant effect of a Ca^{2+} -channel blocker on $[Ca^{2+}]_i$, together with hydrolysis of inositol lipids, clearly demonstrates that, in a suspension of pancreatic acinar cells, collagen type IV activates a 'classical' inositol lipid/ Ca^{2+} signalling pathway. Although Savarese et al. (1992) did not observe any inositol lipid hydrolysis on stimulation of melanoma cells with collagen type IV, a cell imaging technique showed that the majority of their cells (> 90%) displayed a $[Ca^{2+}]_i$ response to collagen type IV that was almost identical with ours (Figure 1). It is important to note that we used direct mass measurements of inositol lipids, whereas Savarese et al. (1992) prelabelled the cells with *myo*- $[^3H]$ inositol, and it is known that, if radiolabelling of inositides is very low, it is sometimes very difficult to observe any changes in inositol lipid breakdown (Divecha et al., 1991).

In the inositol lipid/ Ca^{2+} signalling process, there are two major receptor-mediated pathways for stimulating the formation of $InsP_3$ and DAG. In the first pathway, the receptors, which use a GTP-binding protein, activate members of the PLC- β family, whereas in the second, members of the PLC- γ family are stimulated by tyrosine kinase-linked receptors (Berridge, 1993). It is believed that activation of muscarinic cholinergic receptors by carbachol leads to activation of members of the PLC- β family

Figure 6 Competition between collagen type IV (20 μ g/ml), RGD peptide (20 μ g/ml) and carbachol (50 μ M) in stimulating the increase in DAG concentration in a suspension of pancreatic acinar cells

All other details are as described in the Materials and methods section and the legend to Figure 1.

via pertussis toxin-insensitive GTP-binding protein, probably a member of the Gq family (Williams et al., 1989; Cockcroft and Thomas, 1992), which is probably what we have observed in our experiments (Table 1). When $[Ca^{2+}]_i$, $InsP_3$ and DAG mobilization patterns in pancreatic acinar cells after stimulation with either carbachol or collagen type IV are compared (Figures 1 and 4), it can be observed that collagen type IV probably uses a different pathway for stimulation of PLC from that used by carbachol. This can be further demonstrated by pretreatment of cells with an activator of PKC (Table 1), as it is known that PLC- $\beta 1$ is phosphorylated by PKC whereas PKC-dependent inhibition of PLC- $\gamma 1$ is not always observed (Cockcroft and Thomas, 1992). Finally, a tyrosine kinase blocker inhibited only the collagen type IV-activated pathway for stimulation of PLC (Table 1), sug-

gesting involvement of tyrosine kinase-mediated activation of PLC. It is interesting that $\beta 1$ integrin, which binds collagen type IV (see below), causes protein tyrosine phosphorylation (Kornberg et al., 1991), but has no intrinsic tyrosine kinase activity (Hynes, 1992). On the other hand, it might stimulate one of the cytosolic tyrosine kinases which might in turn activate PLC- γ , as has been proved in lymphocytes (Deanin et al., 1991).

Considerable progress has been made in defining the recognition sites for extracellular matrix components on the cell surface. Certain members of the integrin receptor family are capable of recognizing RGD sequences in single protein components of extracellular matrix (Ruoslahti and Pierschbacher, 1986; Timpl, 1989; Hynes, 1992). Collagen type IV possesses four RGD sequences in the $\alpha 1$ chain and seven RGD sequences

in the $\alpha 2$ chain, which are potential binding sites for integrin receptors (Ruoslahti and Pierschbacher, 1986; Muthukumaran et al., 1989; Saus et al., 1989). The present results indicating that RGD- but not RDG-containing peptide elevates $[Ca^{2+}]_i$ and increases DAG concentration (Figure 5), together with the observation that RGD-containing peptide and collagen type IV but not carbachol compete when DAG concentration increases (Figure 6), strongly favour the possibility that one of the RGD sequences of collagen type IV is responsible for stimulation of PLC via activation of a member of the integrin receptor family. So far it has been demonstrated that of the various integrin receptors that recognize RGD sequences, only $\alpha 2\beta 1$ functions as a receptor for collagen type IV (Hynes, 1992). It is important to note that, using a cross-linking monoclonal antibody to the integrin β -subunit, Altieri et al. (1992) were able to observe an increase in $[Ca^{2+}]_i$ in leucocytes, whereas, in glomerular epithelial cells plated on to collagen gel, immunoprecipitation with anti- $(\beta 1$ -integrin subunit)-specific antibodies abolished phospholipase activation (Cybulsky et al., 1993). This, together with the present results, further suggests that integrin receptors are capable of stimulating intracellular Ca^{2+} signalling.

Another question that emerges from the present results is: why do other molecules of the extracellular matrix, especially collagen type I or fibronectin, which possess RGD sequences, not activate PLC and increase $[Ca^{2+}]_i$? Although collagen type I possesses several RGD sequences (Bernard et al., 1983), it is known that the $\alpha 2\beta 1$ integrin receptor, to which it binds, recognizes its $\alpha 1(I)$ -CB3 fragment which does not contain an RGD sequence (Staatz et al., 1991). Compared with collagen type I, collagen type IV contains more interruptions in the repetitive Gly-X-Y sequence, which makes it more flexible. Thus RGD sequences within the collagen type IV molecule have more conformational space, and can therefore be recognized by the integrin receptors (Schuppan et al., 1980). In other words, the conformational properties of the RGD motif in tertiary folds of extracellular matrix proteins may be crucial for their binding to cell surface receptors. Along this line of evidence is the recent observation that cyclic RGD oligopeptide is up to 100-fold more active than linear peptides, confirming the hypothesis that a constrained cyclic peptide can mimic the conformation necessary for receptor binding (Gurrath et al., 1992). Similar conclusions could also be deduced from the present results, as maximal increases in $[Ca^{2+}]_i$ and DAG were observed with the same doses of RGD peptide and collagen type IV (10 μ g/ml). This means that because of the different molecular masses of RGD peptide and collagen type IV, pancreatic acinar cells were stimulated with an approximately 1000-fold higher molar concentration of RGD peptide than collagen type IV. It can be predicted that only a small proportion of the flexible linear RGD peptide might adopt the necessary conformation for receptor binding (Gurrath et al., 1992). The importance of conformation for the interaction with a cell surface receptor can also be extended to the fibronectin molecule. This molecule was not capable of elevating $[Ca^{2+}]_i$, either in melanoma cells (Savarese et al., 1992) or in pancreatic cells (present study). The RGD sequence that is essential for fibronectin activity lies close to the C-terminus of the tenth type-III module of the molecule without stable conformation in water (Main et al., 1992), which may allow different orientations of the molecule without sufficient conformation required for PLC activation.

As it is known that, in multicellular organisms, the majority of collagen type IV is localized in the basement membrane, and that the epithelial cell layer is partially separated from the membrane (Yurchenco and Schittny, 1990), the physiological relevance of *in vitro* observations seems to be highly speculative. Nevertheless, the present results demonstrate that, in a suspension of pancreatic

acinar cells, RGD sequence(s) within collagen type IV molecules cause activation of tyrosine kinase, probably through one of the integrin receptors. In turn, tyrosine kinase stimulates PLC and increases $[Ca^{2+}]_i$.

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REFERENCES

- Altieri, D. C., Stamnes, S. J. and Gahmberg, C. G. (1992) *Biochem. J.* **288**, 465–473
- Barsky, S. H., Rao, C. N., Williams, J. E. and Liotta, L. A. (1984) *J. Clin. Invest.* **55**, 521–530
- Bernard, M. P., Myers, J. C., Chu, M. L., Ramirez, F., Eikenberry, E. F. and Prockop, D. J. (1983) *Biochemistry* **22**, 1139–1145
- Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- Berridge, M. J. and Irvine, R. F. (1989) *Nature (London)* **341**, 197–204
- Bradford, M. M. (1976) *Anal. Biochem.* **77**, 248–254
- Clarke, N. G. and Dawson, R. M. C. (1981) *Biochem. J.* **195**, 301–306
- Cockcroft, S. and Thomas, G. M. H. (1992) *Biochem. J.* **288**, 1–14
- Cybulsky, A. V., Bonventre, J. V., Quigg, R. J., Wolfe, L. S. and Salant, D. J. (1990) *Am. J. Physiol.* **259**, F326–F337
- Cybulsky, A. V., Carbonetto, S., Cyr, M. D., McTavish, A. J. and Huang, Q. (1993) *Am. J. Physiol.* **264**, C323–C332
- Damsky, C. H. and Werb, Z. (1992) *Curr. Opin. Cell Biol.* **4**, 772–781
- Deanin, G. G., Martinez, A. M., Pfeifer, J. R., Gardner, M. E. and Oliver, J. M. (1991) *Biochem. Biophys. Res. Commun.* **179**, 551–557
- Divecha, N. and Irvine, R. F. (1990) in *Methods in Inositide Research: Mass Measurement of Phosphatidylinositol 4-Phosphate and sn-1,2-Diacylglycerols* (Irvine, R. F., ed.), pp. 179–185, Raven Press, New York
- Divecha, N., Banfić, H. and Irvine, R. F. (1991) *EMBO J.* **10**, 3207–3214
- Downes, C. P., Hawkins, P. T. and Stephens, L. R. (1989) in *Inositol Lipids in Cell Signalling: Identification of the Stimulated Reaction in Intact Cells, its Substrate Supply and the Metabolism of Inositol Phosphates* (Michell, R. H., Drummond, A. H. and Downes, C. P., eds.), pp. 3–38, Academic Press, New York
- Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4
- Farquhar, M. G. (1981) in *Cell Biology of the Extracellular Matrix: The Glomerular Basement Membrane, a Selective Macromolecular Filter* (Hay, E. D., ed.), pp. 335–378, Plenum Press, New York
- Folch, J., Lees, M. M. and Sloane-Stanely, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Gurrath, M., Muller, G., Kessler, H., Aumailley, M. and Timpl, R. (1992) *Eur. J. Biochem.* **210**, 911–921
- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Juliano, R. L. and Haskill, S. (1993) *J. Cell Biol.* **120**, 577–585
- Kornberg, L. J., Shelton, E. H., Turner, C. E., Prockop, C. and Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8392–8396
- Levitzi, A. (1992) *FASEB J.* **6**, 3275–3282
- Main, A. L., Harvey, T. S., Baron, M., Boyd, J. and Campbell, I. D. (1992) *Cell* **71**, 671–678
- Muthukumaran, G., Blumberg, B. and Kurkinen, M. (1989) *J. Biol. Chem.* **264**, 6310–6317
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Nishizuka, Y. (1992) *Science* **258**, 607–614
- Palmer, S. and Wakelam, M. J. O. (1990) in *Methods in Inositide Research: Mass Measurement of Inositol 1,4,5-Trisphosphate Using a Specific Binding Assay* (Irvine, R. F., ed.), pp. 127–135, Raven Press, New York
- Paralkar, V. M., Vukičević, S. and Reddi, A. H. (1991) *Dev. Biol.* **143**, 303–308
- Paulsson, M. (1992) *Crit. Rev. Biochem. Mol. Biol.* **27**, 93–127
- Ruoslahti, E. and Pierschbacher, M. D. (1986) *Cell* **44**, 517–518
- Saus, J., Quinones, S. and MacKrell, A. (1989) *J. Biol. Chem.* **264**, 6318–6324
- Savarese, D. M. F., Russell, J. T., Fatatis, A. and Liotta, L. A. (1992) *J. Biol. Chem.* **267**, 21928–21935
- Schnefeld, S., Banfić, H., Eckhardt, L., Schultz, G. and Schulz, I. (1988) *FEBS Lett.* **230**, 125–130
- Schuppan, D., Timpl, R. and Givanville, R. W. (1980) *FEBS Lett.* **115**, 297–300
- Staatz, W. D., Fok, K. F., Zutter, M. M., Adams, S. P., Rodriguez, B. A. and Santoro, S. A. (1991) *J. Biol. Chem.* **266**, 7363–7367
- Stack, M. S., Moser, T. L. and Pizzo, S. V. (1992) *Biochem. J.* **284**, 103–108
- Streb, H., Irvine, R. F., Berridge, M. J. and Schulz, I. (1983) *Nature (London)* **306**, 67–69
- Timpl, R. (1989) *Eur. J. Biochem.* **180**, 487–502

- Von Tscharner, V., Prud'hom, B., Baggiolini, M. and Reuter, H. (1986) *Nature* (London) **324**, 369–372
- Williams, J. A., Burnham, D. B. and Hootman, S. R. (1989) in *Handbook of Physiology. Gastrointestinal Physiology. Salivary, Gastric, Pancreatic, and Hepatobiliary Secretion:*

- Hormonal Regulation of Exocrine Pancreas*, section 6, vol. 3, chapter 21, pp. 419–441, American Physiology Society, Bethesda
- Yurchenco, P. D. and Schittny, J. C. (1990) *FASEB J.* **4**, 1577–1590
- Zhang, B. X. and Muallem, S. (1992) *J. Biol. Chem.* **267**, 24387–24393

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