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Transfection of insulin-producing cells with a transforming c-Ha-*ras* oncogene stimulates phospholipase C activity

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Pancreatic islet β-cells and insulin-producing RINm5F cells were electroporated in the presence of the c-Ha-*ras* oncogene, to assess the possible involvement of the encoded product in coupling extracellular receptors to phospholipase C. After two days the c-Ha-*ras*-transfected cells increased their expression of c-Ha-*ras* mRNA. These cells were also found to contain more [^{3}H]InsP_3, suggesting an increased basal (non-ligand-activated) phospholipase C activity. In addition, the transfected cells were unable to respond to ligand (bombesin) activation of phospholipase C. The *ras*-transfected insulin-producing cells showed enhanced phosphorylation of a 200 kDa substrate crossreacting with an antibody to an 80 kDa protein kinase C substrate. The phorbol ester 12-*O*-tetradecanoyl 13-acetate and bombesin also induced phosphorylation of the 200 kDa substrate. All of these changes occurred without changes in the rates of [^{3}H]thymidine incorporation. The results suggest that the mutated c-Ha-*ras* oncogene directly or indirectly stimulates the basal phospholipase C activity of these cells.

INTRODUCTION

The recent discovery of oncogenes and their normal cellular counterparts, the proto-oncogenes, has significantly increased our understanding of many cellular processes, such as cell replication, gene expression and ionic fluxes [1–3]. Despite this, the exact mechanisms by which the products of oncogenes or proto-oncogenes operate remain elusive. An example is given by the *ras* group of proteins which have been found to bind and hydrolyse GTP [4,5]. These proteins are thought to serve as signal transducers at the plasma membrane [3], although their putative targets have not yet been clearly identified. It has been suggested that the *ras* proteins regulate the activity of phospholipase C [6], an enzyme involved in the regulation of many cellular processes, including cell proliferation [2]. This suggestion is supported by the findings that phospholipase C is regulated by a GTP-binding protein [7], that cells transfected with N-*ras* respond to bombesin with increased InsP_3 production [6], and that a phorbol ester mimics the effects of *ras* on gene expression [8]. Other studies have failed to demonstrate consistent stimulatory effects of *ras* on phospholipase C [9,10]. However, such discrepancies could reflect differences between various cell types or compensatory mechanisms which inhibit this enzyme.

We have recently described a system for studying the effects of oncogene constructs transfected into insulin-producing pancreatic β-cells by means of electroporation [11]. It was observed that certain oncogenes (*src* and *myc* plus *ras*) stimulated [^{3}H]thymidine incorporation, and that the *src* oncogene was expressed at elevated levels in a high proportion (> 50%) of the islet cells three days after the transfection. Since these studies were performed on primary cells three days after electroporation, effects secondary to clonal selection and transformation are not likely to be observed.

Although the exact physiological role of phospholipase C in insulin secretion is unknown, this enzyme can be activated by stimulating insulin-producing cells with various agonists. So far, no G-protein has been identified that might couple the actual receptors to phospholipase C. By investigating the effects of the G-protein encoded by the mutated c-Ha-*ras* oncogene [12], we have now attempted to obtain more detailed information concerning the mechanisms whereby phospholipase C is regulated in insulin-producing cells. The results suggest that the *ras* protein stimulates the phospholipase C activity of these cells in the absence of ligand activation.

EXPERIMENTAL

Materials

[^{32}P]Orthophosphate, [*methyl*-^{3}H]thymidine, [α-^{32}P]-deoxycytidine triphosphate, *myo*-[2-^{3}H]inositol, an oligonucleotide labelling kit and Hyperfilm were from Amersham International, Amersham, Bucks., U.K. Biolyte ampholytes were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Genescreen was purchased from New England Nuclear, Boston, MA, U.S.A. Ficoll 400 and protein A–Sepharose were obtained from Pharmacia, Uppsala, Sweden. pSP64 was from Promega Biotech, Madison, WI, U.S.A. Nu-serum was obtained from Collaborative Research Inc., Bedford MA, U.S.A.

Cell preparation

Pancreatic islets were isolated from male NMRI mice (Anticimex, Stockholm, Sweden) by collagenase digestion [13]. The crude digests were partially purified on Ficoll gradients [14], and isolated islets were transferred manually to dishes for culture in 5% CO_2 in RPMI 1640 (11 mM-glucose) plus 10% bovine serum and antibiotics [15]. For the determinations of islet phospholipase C

Abbreviation used: TPA, 12-*O*-tetradecanoyl 13-acetate.

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activity, 2000–3000 islets were dispersed into cellular aggregates 12 h before electroporation as described in detail by Welsh *et al.* [11]. RINm5F cells [16] at a passage number of about 70 were kept in culture in RPMI 1640 plus 10% fetal bovine serum in 5% CO_2. Before electroporation, such cells were detached by gentle trypsin/EDTA treatment and washed in Hanks' solution.

Transfection by means of electroporation

The electroporation was carried out exactly as described [11] using pSP64 or pRI-7 as control and pUCEJ6.6RI as the plasmid containing mutated c-H-*ras* sequences. This plasmid also contains sequences from the rat insulin II promoter [11].

Phospholipase C activity

Electroporated islet and RINm5F cells were cultured for 2 or 1 days, respectively, before replacing the media with [2-^{3}H]inositol-containing media [17]. After 24 h, the labelling media were discarded, the cells were washed and incubated for 30 min in a Hepes-buffered medium with Cl^- as the sole anion [18], and containing 11 mM-glucose, before termination of the incubation by the addition of trichloroacetic acid to a final concentration of 10%. In some experiments bombesin was added to a final concentration of 0.1 μM five minutes before the addition of trichloroacetic acid. Phosphoinositide-specific phospholipase C activity was determined as the contents of [^{3}H]InsP_3 as described [17] assuming no differences in the specific activities of relevant [^{3}H]inositol phospholipid pools after the 24 h equilibration period and constant rates of InsP_3 breakdown. A 3 h labelling period has previously been reported to be sufficient to obtain steady state levels of [^{3}H]inositol phospholipids in islet cells [19]. For the DNA determinations, the contents of the culture dishes were sonicated for 15 s after the addition of trichloroacetic acid, and aliquots of the suspensions were taken for further analysis [20].

[^{3}H]Thymidine incorporation

Adult islet cells were electroporated in the presence of pSP64 or pSV2.26RI [11], and the incorporation of [^{3}H]thymidine was determined during the fourth day of culture in RPMI 1640 plus 10% fetal bovine serum with or without the addition of 10 μM-carbamylcholine to the media during the last two days. Alternatively, RINm5F cells were electroporated in the presence of pSP64 or pUCEJ6.6RI. After two days, the cells were incubated for 4 h in 0.5 μCi of [^{3}H]thymidine/ml as described [11].

Immunoprecipitation of ^{32}P-labelled proteins

RINm5F cells were in some cases electroporated and maintained for two days in culture. The cells were labelled with ^{32}P (1 mCi/ml) for 3 h in a Hepes-buffered Krebs-Ringer bicarbonate salt solution (KRBH) (0.01 mM-phosphate) containing 17 mM-glucose and 1% fetal calf serum at 37 °C in 5% CO_2. The cells were then washed with ice-cold KRBH, scraped off the dishes and lysed in 100 μl of 150 mM-NaCl/50 mM-Tris, pH 7.4/0.5% NP-40/0.1% SDS/5 mM-EDTA/0.02% NaN_3/100 ku of trasylol/ml/10 mM-benzamidine. Nuclei were pelleted and the supernatants were pre-cleaned with 20 μl of 50% (v/v) protein A–Sepharose in the lysis buffer described above with the further addition of 2.5% bovine serum albumin. After pelleting the Sepharose, the supernatants were immunoprecipitated as described [21] with an antibody directed against a protein kinase C substrate [21]. The immunoprecipitated products were subjected to two-dimensional gel electrophoresis as described [22] before autoradiography and densitometric analysis.

Determination of c-Ha-*ras* mRNA

RINm5F cells were electroporated and then kept in culture for two days. Total cellular RNA was isolated according to Chirgwin *et al.* [23]. Approx. 15 μg of RNA was denatured with glyoxal and subsequently subjected to agarose (1.2%) gel electrophoresis according to Thomas [24]. After transfer of the RNA to Genescreen transfer membranes, the blots were hybridized in 50% deionized formamide to a [^{32}P]oligonucleotide-labelled 0.6 kb *Pst* I–*Sst* I fragment of the human c-Ha-*ras* gene (commercially available from Oncor Inc., Gaithersburg, MD, U.S.A.) as described [24]. The blots were washed three times for 30 min each at 50 °C in the 15 mM-NaCl/1.5 mM-sodium citrate/0.1% SDS before autoradiography.

Measurements of cytoplasmic free Ca^{2+} concentration

Following electroporation in the presence of pSP64 or pUCEJ6.6RI, the RINm5F cells were cultured for two days in RPMI 1640 medium containing 10% NU-serum. After the culture period the cells were detached from the culture flask by mild trypsinization. The cell suspensions were then incubated at 37 °C, pH 7.4, for 45 min in a similarly composed culture medium supplemented with 5 μM-quin 2/AM, which gave a quin 2 loading of about 0.40 nmol per 10^6 cells. This value was obtained from calculations based on fluorescence maximum and extracellular quin 2 values at the beginning of each experiment, assuming that 1 mg of dry weight corresponds to 3.6×10^6 cells [25]. To avoid attachment of the cells to the culture flask during incubation, the suspensions were shaken gently. After the loading period the cell suspensions were washed twice at 37 °C in a Hepes buffer, pH 7.4, physiologically balanced in cations with Cl^- as the sole anion [18] containing 11 mM-glucose, 0.1% (w/v) bovine serum albumin and 1.28 mM-Ca^{2+}. The cells were then resuspended in 1.5 ml of a similar Hepes buffer in 1 cm polystyrene cuvettes, and measurements were performed at 37 °C in an Aminco–Bowman spectrofluorimeter, slightly modified to allow constant stirring. The excitation and emission wavelengths were 340 nm and 490 nm, respectively. Calibration was done essentially as described by Hesketh *et al.* [26]. In each experiment, 50 μM-D-600 was added to block possible Ca^{2+} channels. Additions of bombesin were made from a 200 × concentrated stock solution.

RESULTS

Expression of the c-Ha-*ras*-genes

To evaluate the efficiency of the electroporation procedure, c-Ha-*ras* mRNA was determined two days after electroporation of RINm5F cells in the presence of pUCEJ6.6RI (*ras*) or pSP64 (control). Densitometry of Northern blots revealed a 52% increase in the amounts of c-Ha-*ras* mRNA, using a probe detecting both normal and mutated Ha-*ras* mRNA, after transfection with pUCEJ6.6RI (*ras*) as compared to controls (Fig. 1).

Phospholipase C activity

When islet cell phospholipase C activity (measured as the [^{3}H]-InsP_3 content/μg of DNA) was determined, *ras*-

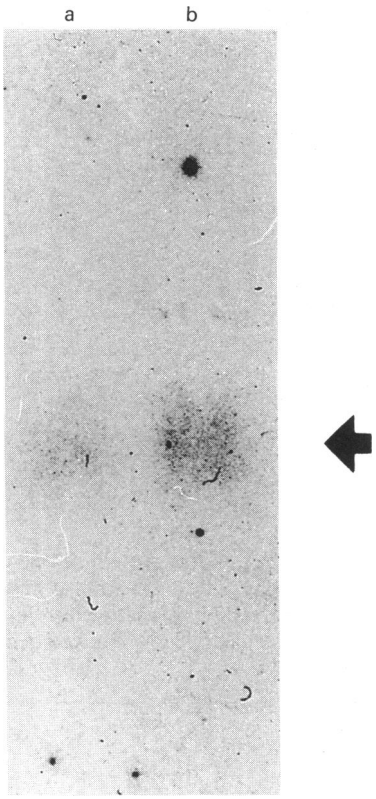

Fig. 1. Effect of electroporation of RINm5F cells in the presence of pUCEJ 6.6RI (*ras*) on the expression of the c-Ha-*ras* genes

RNA was isolated two days after electroporation with pSP64 (control, lane a) or pUCEJ 6.6RI (*ras*, lane b), glyoxylated, electrophoresed on a 1.2% agarose gel, transferred to Genescreen filters and hybridized to a c-Ha-*ras* probe. The position of a 1.4 kb DNA marker is indicated by the arrow.

transfection (pUCEJ6.6RI) increased the activity of the enzyme (Table 1). Also in the RINm5F cells, *ras*-transfection produced a significant increase in phospholipase C activity (Table 1). When 0.1 μM-bombesin was added to control transfected RINm5F cells, there was an increase in the production of [^{3}H]InsP_3 (Table 2). Furthermore, the *ras*-transfected RINm5F cells again showed an increased basal phospholipase C activity, whereas the addition of bombesin exerted no further effect.

Protein phosphorylation

Constitutive activation of phospholipase C would be expected to elevate the release of diacylglycerol in the plasma membrane with an increase in the activity of protein kinase C as a consequence [2]. To test this hypothesis, RINm5F cells electroporated with *ras* (pUCEJ6.6RI) or control (pSP64) were labelled with ^{32}P and immunoprecipitated with a serum directed against a widely distributed substrate for protein kinase C [21]. Figs. 2(*a*) and 2(*b*) show autoradiographs of two-dimensional gels after this procedure. In control transfected cells, phosphorylation of a 80 kDa, pI = 4 protein is consistent (arrow 1). Futhermore, other phosphorylated products are present of which a faint 200 kDa, pI = 6 (arrow 2) product is reproducibly immunoprecipitated.

Table 1. Effect of pUCEJ6.6RI (*ras*) on [^{3}H]InsP_3 contents of insulin-producing cells

Phospholipase C activity was measured as d.p.m. of [^{3}H]InsP_3/μg of cellular DNA and was determined in adult mouse islet cells three days after transfection with pSP64 or pUCEJ6.6RI. Means ± S.E.M. are given, as well as the average percent increase with *ras*. Similar experiments were performed using RINm5F cells two days after electroporation. Means ± S.E.M. are given. ** denotes $P < 0.01$ using a paired Student's *t*-test. The percentage increase with *ras* for each cell line is given in parentheses.

	Phospholipase C activity (d.p.m. of [^{3}H]InsP_3/μg of cellular DNA)	
Transfection conditions	Islet cells ($n = 4$)	RINm5F cells ($n = 9$)
Control (pSP64)	317 ± 96	589 ± 164
Ras (pUCEJ6.6RI)	346 ± 108** (10 ± 1)	805 ± 274** (42 ± 12)

Table 2. Effects of electroporation in the presence of pUCEJ6.6RI (*ras*) on RINm5F cell [^{3}H]InsP_3 contents with or without the addition of bombesin

The activity of phosphoinositide-specific phospholipase C was determined in 4–5 separate experiments two days after electroporation of RINm5F cells in the presence of pSP64 or pUCEJ6.6RI as d.p.m. of [^{3}H]InsP_3/μg of cellular DNA. Means ± S.E.M. are given. * Denotes $P < 0.05$ when tested against basal control with a paired *t*-test. The bombesin effect as a percentage of basal [^{3}H]InsP_3 content is also shown in parentheses for each cell line.

	Phospholipase C activity (d.p.m. of [^{3}H]InsP_3/μg of cellular DNA)	
Transfection conditions	Basal	+0.1 μM bombesin
pSP64 (control)	361 ± 148	501 ± 285* (132 ± 10%)
pUCEJ6.6RI (*ras*)	452 ± 156*	404 ± 202 (97 ± 8%)

Ras-transfected RINm5F cells (Fig. 2*b*) showed decreased phosphorylation (46 ± 33%, $n = 2$) of the 80 kDa substrate (arrow 3), as did the control cells. However, the 200 kDa substrate was phosphorylated to a greater extent (arrow 4) than in the control group (584 ± 216%, $n = 2$). Other ^{32}P-labelled proteins showed no difference in their labelling in the two groups. RINm5F cells treated with the phorbol ester TPA (0.1 μM) for 10 min after the ^{32}P-labelling period showed enhanced phosphorylation of both the 80 and 200 kDa substrates (324 ± 138% and 337 ± 49% of control, $n = 3$; Fig. 3*b* versus Fig. 3*a*) whereas other ^{32}P-labelled proteins were not affected. Similarly, 0.1 μM-bombesin stimulated the phosphorylation of both the 80 kDa and 200 kDa substrates under these conditions (results not shown). Neither of the 80 or 200 kDa substrates was precipitated by normal rabbit serum (results not shown), suggesting that the 200 kDa protein is an antigenically

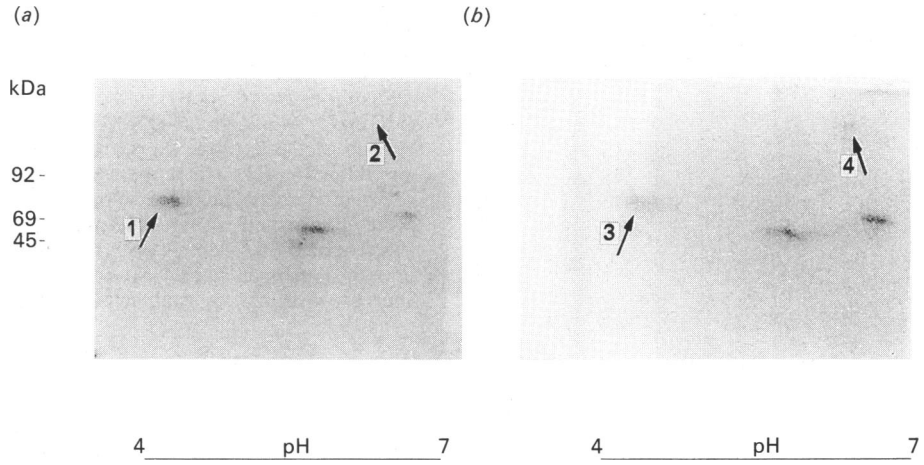

Fig. 2. Effects of electroporation of RINm5F cells in the presence of pSP64 (control, *a*) or pUCEJ6.6RI (*ras b*) on protein phosphorylation two days later

Electroporated RINm5F cells were labelled with ^{32}P and immunoprecipitated with an antibody for a protein kinase C substrate [21]. The products were subjected to two-dimensional gel electrophoresis and autoradiography. The left arrows (1 and 3) indicate the positions of the 80 kDa substrate and the right arrows (2 and 4) indicate the positions of a 200 kDa phosphorylated product which is immunoprecipitated with the 80 kDa substrate antibody. The positions of molecular weight markers (in kDa) and isoelectric points are indicated.

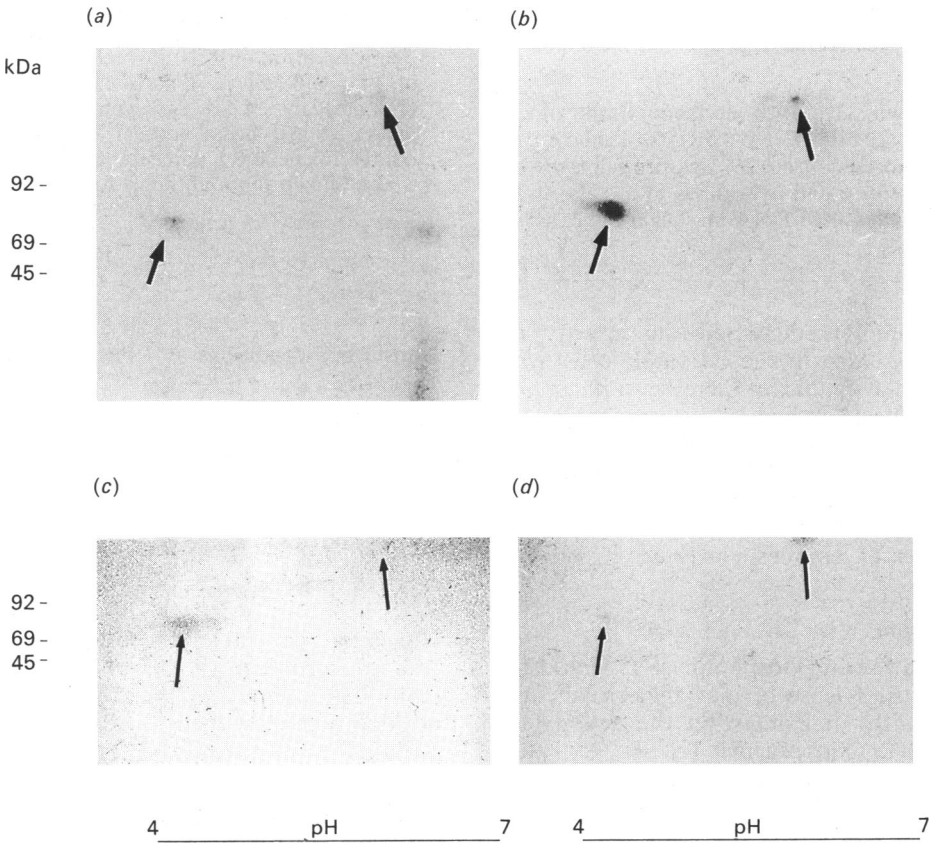

Fig. 3. Effects of TPA on protein phosphorylation in RINm5F cells

RINm5F cells were labelled with ^{32}P after which the cells were control- (*a*) or TPA- (*b*) incubated for 10 min, after which the proteins were immunoprecipitated and electrophoresed as in Fig. 2. In (*d*), the RINm5F cells were exposed to 0.1 μM-TPA for 24 h before ^{32}P labelling, during which TPA was also present. The corresponding control is shown in (*c*). The arrows indicate the same positions as in Fig. 2. Note that (*c*) and (*d*) have been electrophoresed for a shorter time in the second dimension than (*a*) and (*b*). The positions of molecular weight markers (in kDa) and isoelectric points are given.

related form of the 80 kDa protein kinase C substrate [21] which also serves as a substrate for protein kinase C. Pretreatment of RINm5F cells with 0.1 μM-TPA 24 h before the ^{32}P-labelling period resulted in a phosphorylation pattern very similar to that observed after *ras*-transfection, i.e. the phosphorylation of the 80 kDa substrate was slightly decreased (48 ± 21 %, $n = 2$) whereas the phosphorylation of the 200 kDa substrate was enhanced (Figs. 3*c* and 3*d*) (570 ± 180 %, $n = 2$) compared with the control-transfected cells. The inhibition of phosphorylation of the 80 kDa substrate after long-term activation of protein kinase C is probably due to down-regulation of this substrate, as has been observed in other cells [27].

Cytoplasmic free Ca^{2+} concentration

As evident from Fig. 4(*a*) there was a rapid and transient increase in cytoplasmic free Ca^{2+} concentration when 0.5 μM-bombesin was added to RINm5F cells electroporated in the presence of pSP64 (control). However, RINm5F cells transfected with the c-Ha-*ras* oncogene (pUCEJ6.6RI) displayed no increase in free cytoplasmic Ca^{2+} when exposed to bombesin (Fig. 4*b*), indicating that RINm5F cells transfected with the mutated c-Ha-*ras* oncogene lose their ability to activate phospholipase C in response to ligands. No difference in the basal free cytoplasmic Ca^{2+} concentration was observed after transfection with pUCEJ6.6RI. However, this does not contradict a role of the c-Ha-*ras* oncogene in activation of phospholipase C, since the intracellular release of Ca^{2+} in response to InsP_3 is only transient in islet cells [17].

[^{3}H]Thymidine incorporation

When the incorporation of [^{3}H]thymidine was determined in adult islet cells, the presence of 10 μM-carbamylcholine during days three and four after electroporation resulted in no effects on [^{3}H]thymidine incorporation when added to control (pSP64) electroporated cells in three experiments (106 ± 12 %). Similarly, *myc* (pSV2.26RI) exerted no effect (105 ± 12 % of control). However, the addition of carbamylcholine to the *myc*-electroporated cells significantly ($P < 0.05$) stimulated [^{3}H]thymidine incorporation (212 ± 26 % of control electroporated cells) to a degree very similar to that of pUCEJ6.6RI (*ras*) when transfected together with *myc* into islet cells [11]. The [^{3}H]thymidine incorporation of RINm5F cells transfected with pUCEJ6.6RI was 91 ± 29 % of control electroporated cells (pSP64) in three experiments.

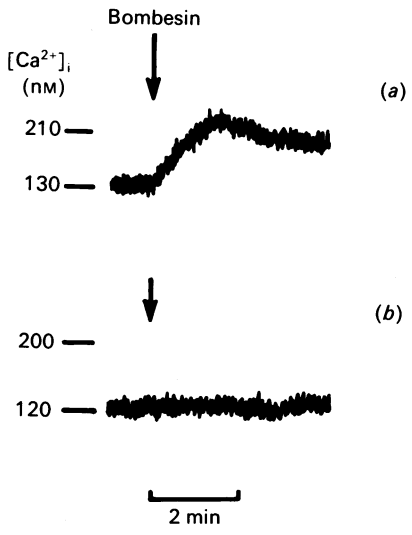

Fig. 4. Effects of transfection of RINm5F cells with pUCEJ 6.6RI (*ras*) on free cytoplasmic Ca^{2+} in response to bombesin

RINm5F cells were electroporated in the presence of pUCEJ6.6RI (*ras*, *b*) or pSP64 (control, *a*). Free cytoplasmic Ca^{2+} was determined two days later. The arrow indicates the time of addition of bombesin. This experiment is a representative of three separate experiments.

DISCUSSION

The *ras* family of proteins consists of very similar proteins with GTP-binding and GTP-hydrolysing properties [4,5]. These proteins are of importance for cell proliferation, since cells microinjected with *ras* antibodies cease to divide [28]. Furthermore, certain point mutations in the *ras* genes render these highly oncogenic, with the ability to transform cells [12]. Such mutated *ras*-proteins have been implicated in oncogenesis in tumours from various tissues [12,29,30]. Point mutations in the *ras* proteins cause changed GTP-binding and hydrolysing properties [31–33]. Although it is difficult to see a simple pattern of how these mutations affect the properties of *ras* proteins, it is clear that many *ras* mutants show decreased GTP-binding and GTP-hydrolysing activities without a loss of the transforming capability.

GTP-binding proteins have been implicated as signal transducers in many systems. Thus a search for a target of the *ras*-proteins in mammalian cells has focused on the regulation of phospholipase C [34], since this enzyme is controlled by a GTP-binding protein [7] and is also closely related to cellular growth among other processes [2]. Many reports indicate that the *ras* proteins exert a positive control of phospholipase C. For example, this was suggested by the experiments demonstrating that cells transfected with N-*ras* responded to bombesin with increased production of InsP_3 [6], that *ras* and TPA exerted similar effects on gene expression [8], that certain cells display elevated phospholipase C activity after transfection with activated Ha-*ras* [35], and that *ras*-transformed fibroblasts showed elevated levels of diacylglycerol [36,37]. However, other studies have obtained contradictory results as to whether *ras* proteins stimulate phospholipase C [9,10,38,39]. Many of these discrepancies might be explained by differences between different cells. Furthermore, one could hypothesize that constitutive activation of phospholipase C induces compensatory down-regulatory mechanisms which in turn make it difficult to detect effects on phospholipase C activity. An example of such a compensatory down-regulatory mechanism could be TPA-stimulated phosphorylation of the c-Ki-*ras* product [40], which via activation of protein kinase C would phosphorylate and control the *ras* proteins, the hypothesized regulators of phospholipase C. Furthermore, it has been reported that addition of TPA decreases the sensitivity in the response of phospholipase C to stimulators [41].

The pancreatic β-cells as well as the clonal insulin-producing RINm5F cells can serve as cells in which signal transmission involves receptors coupled to phospholipase C. However, the extent to which the *ras*

proteins serve as the general coupling factors between extracellular receptors and phospholipase C is at the moment not known. We have defined a system for transfecting primary β-cells with oncogenes and then studying cellular responses 2–4 days later. The gene products were efficiently expressed in a high number of the cells [11]. Transfection of islet cells with a mutated c-Ha-*ras* oncogene isolated from a human bladder carcinoma [12] alone resulted in no effects on islet cell replication, whereas the combination *myc*- plus *ras*-stimulated islet cell replication [11]. Subsequent to transfection with the c-Ha-*ras* oncogene we were presently able to detect an increased phospholipase C activity both in β-cell-rich islets, and in the insulin-producing RINm5F cells. The observed stimulation of phospholipase C was further supported by the observation of increased phosphorylation *in vivo* of a 200 kDa substrate which was also phosphorylated in response to the protein kinase C activator TPA. This substrate was also immunoprecipitated with an antiserum against a protein kinase C substrate, suggesting a structural resemblance between these proteins which makes them both substrates for protein kinase C. Constitutive stimulation of phospholipase C would result in activation of protein kinase C, since one of the products of phospholipase C action is diacylglycerol, the major physiological stimulator of protein kinase C [2]. Down-regulation of the 80 kDa substrate after long-term activation of protein kinase C has been observed in other cells [27] making the phosphorylation of this substrate unsuitable as a marker for protein kinase C activity in RINm5F cells. The effects of *ras* on phospholipase C occurred without a concomitant increase in cell replication, suggesting that such an activation in the insulin-producing cells is not secondary to increased DNA replication. Moreover, since these effects occurred 2–3 days after transfection, it is unlikely that they reflect clonal outgrowth of a subpopulation of cells.

Despite the *ras* stimulation of basal β-cell and RINm5F cell phospholipase C activity, bombesin was unable to activate phospholipase C. Thus, it appears that ligand coupling is not generally enhanced by *ras*-transfection, contrary to a previous report [6], in which ligand-activated phospholipase C was stimulated by N-*ras* despite the lack of effects of N-*ras* on basal phospholipase C activity. Such a discrepancy might be explained by the use of a mutated c-Ha-*ras* oncogene in the present study. However, we cannot exclude that in some specific cases the c-Ha-*ras* oncogene promotes the ligand coupling to phospholipase C in insulin-producing cells. Curiously, *ras* increases the activity of phospholipase C in cells in which phospholipase C, indirectly or directly, primarily appears to play a role for the regulation of exocytotic processes. In agreement with this, culture of islet cells in the presence of carbamylcholine alone failed to affect [^{3}H]thymidine incorporation rates. Nevertheless, islet cells transfected with *myc* plus *ras* [11] markedly increased their [^{3}H]thymidine incorporation rates, as did the *myc*-transfected cells cultured in the presence of carbamylcholine, suggesting a role of islet phospholipase C for cell replication when combined with another growth-promoting agent or condition.

The possibility exists that our observed effects of a mutated c-Ha-*ras* oncogene on insulin-producing cell phospholipase C activity are secondary to other changes occurring in these cells. Alternatively, our observed effects of *ras* on phospholipase C may reflect 'promiscuous' behaviour of the product of the mutated Ha-*ras* oncogene, in these cells, and thus the main target(s) for *ras* action could be other enzymes involved in phospholipid metabolism ultimately causing activation of protein kinase C. In support of this, others have observed elevated levels of diacylglycerol after *ras* transformation [36,37] without an increase in $InsP_3$. Nevertheless, our observed increase in phospholipase C activity may be a significant component of the action of the mutated Ha-*ras* oncogene in insulin-producing cells.

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