Activation of alpha-2-adrenoceptors results in an increase in F-actin formation in HIT-T15 pancreatic B-cells

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1. Alpha-2-adrenoceptor agonists, such as noradrenaline, are potent inhibitors of insulin secretion, and it has been suggested that they control a late step in the pathway of exocytosis. We have investigated whether this could be related to a change in the extent of actin polymerization in the pancreatic B-cell, since actin microfilaments are implicated in regulating the access of secretory granules to the plasma membrane prior to exocytosis. 2. Cultured HIT-T15 pancreatic B-cells responded to noradrenaline with an increase in F-actin content, as judged by a rise in the fluorescence output after probing of the cells with phalloidin (a toxin which binds specifically to F-actin) conjugated to rhodamine. The response to noradrenaline was rapid, dose-dependent and sustained and could be reproduced by the highly selective alpha-2-agonist UK14,304. Examination of HIT-T15 cells by fluorescence microscopy after treatment with rhodamine-phalloidin, revealed a significant localization of F-actin immediately adjacent to the plasma membrane. The pattern of F-actin distribution in the cells was not altered dramatically by noradrenaline, although the

intensity of staining close to the plasma membrane appeared to be slightly reduced. 3. The increase in F-actin content induced by noradrenaline and UK14,304 was inhibited significantly by the alpha-2-antagonist idazoxan but not by the alpha-1-selective antagonist prazosin. Pretreatment of HIT-T15 cells with pertussis toxin did not lead to any direct alteration in F-actin content, although the toxin significantly modified the responses induced by noradrenaline and UK14,304. In each case, cells incubated for 24 h with pertussis toxin responded to the alpha-2-agonist with an enhanced fluorescence output, indicating that F-actin levels had increased still further. This did not correlate with any gross change in the distribution of F-actin as judged by fluorescence microscopy. 4. The results demonstrate that alpha-2-adrenoceptors are coupled to control of actin polymerization in HIT-T15 cells. They suggest that regulation of F-actin formation could be a component of the mechanism by which alpha-2-agonists mediate inhibition of insulin secretion.

INTRODUCTION

The secretory activity of the pancreatic B-cell is regulated by a wide variety of nutrients, hormones and neurotransmitters which can either increase or decrease the rate of insulin secretion (reviewed in [1,2]). Among these various agents, the catecholamines adrenaline and noradrenaline are particularly effective since they completely inhibit the secretory response to all physiological stimuli [3,4]. This implies that they control a critical step in the exocytotic pathway which lies distal to the point at which all signalling mechanisms converge.

The inhibitory action of noradrenaline and adrenaline on insulin secretion is mediated by members of one particular subclass of adrenoceptor, the alpha-2-adrenoceptors [3–7], which are expressed on the surface of pancreatic B-cells [4,8–10]. Activation of islet alpha-2-adrenoceptors has been correlated with a variety of intracellular responses including inhibition of adenylate cyclase and reduction of cyclic AMP [9,11,12], activation of a membrane potassium channel [13], closure of voltage-sensitive calcium channels [14], alterations in glucose metabolism [15] and changes in cyclic GMP levels [16]. However, the ability of adrenergic agonists to inhibit insulin secretion cannot be explained fully, by any of these responses. Thus, studies performed with permeabilized B-cell systems have revealed that alpha-2-agonists can still mediate inhibition of insulin secretion under conditions where intracellular messenger generation is inconsequential, where the effects of membrane permeability changes are negated and where glucose metabolism cannot proceed [9,17,18]. On this basis, it has been argued that

islet alpha-2-adrenoceptors must be coupled to an effector system(s) that controls one of the more distal components of the exocytotic pathway [3,9,18].

Since alpha-2-adrenoceptors are localized at the cell surface and a primary role for intracellular messengers has been excluded, it seems likely that the effector system involved in adrenergic inhibition of insulin secretion must be directly associated with the plasma membrane. If this is true, then it follows that the number of candidate effector systems is small since the late stages of secretion are thought to be regulated primarily by elements of the cytoskeleton (particularly contractile microfilaments which play a role in secretory granule margination) and by biochemical changes to the plasma and granule membranes which render them fusogenic [19]. In the present work, we have investigated whether activation of pancreatic B-cell alpha-2-adrenoceptors can elicit changes to the polymerization of actin microfilaments. Cultured HIT-T15 cells were used for these studies since they are responsive to alpha-2-agonists [14] and can be grown in sufficient numbers for measurement of F-actin using a fluorescent probe. This could not be achieved readily with rat islet cells, due to the limiting availability of tissue.

EXPERIMENTAL

HIT-T15 cells (passages 65–85) were obtained from the American Tissue Culture Collection and were grown in culture medium RPMI-1640 supplemented with glutamine (2 mM), fetal calf

Abbreviation used: PT, pertussis toxin.

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serum (10%, v/v), penicillin G (400 IU/ml) and streptomycin (200 µg/ml). The cells were grown in 150 cm³ tissue-culture flasks (GIBCO-BRL) at 37 °C in a humidified atmosphere of air/CO₂ (95:5) and were passaged weekly.

For individual experiments, cells were detached from the substratum with trypsin-EGTA and seeded into 24-well tissue-culture plates at a density of 10⁵ cells/cm². They were cultured for a further 24 h then washed with bicarbonate-buffered physiological saline solution, pH 7.4 [20], containing 4 mM glucose and BSA (1 mg/ml). They were then incubated in this buffer for 30 min at 37 °C prior to the start of each experiment.

Insulin secretion studies

For measurement of insulin secretion, cultured HIT-T15 cells were incubated in 1 ml of bicarbonate-buffered physiological saline solution, pH 7.4, supplemented with 4 mM glucose and 1 mg/ml BSA plus test reagents for 1 h at 37 °C. After this time, the incubation medium was sampled for measurement of insulin levels by radioimmunoassay.

Fluorescence measurements of F-actin formation

For determination of F-actin content after incubation, test reagents were added to the cell incubation buffer and incubation continued for various periods of time (as defined for individual experiments) after which the medium was removed. The F-actin content of the cells was then determined by a method based on that described by Wu et al. [21] for chromaffin cells. HIT-T15 cells were fixed by addition of 0.3 ml of 3.7% formaldehyde in PBS (138 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) and incubated for 15 min at room temperature. An aliquot (0.3 ml) of Triton X-100 (0.2% in PBS) was then added to permeabilize the cells, after which 0.3 ml of rhodamine-phalloidin (1.5 µM in PBS) was added and left for 5 min. The cells were washed with PBS (3 × 1 ml) and dissolved in 1 ml of 0.1 M NaOH. Fluorescence of the samples was measured in a Perkin-Elmer fluorimeter using excitation and emission wavelengths of 485 nm and 520 nm respectively.

Fluorescence microscopy

HIT-T15 cells were grown on glass coverslips previously coated with fibronectin by incubation for 18 h in a sterile solution of fibronectin (50 µg/ml) in PBS. After culture for 24–48 h in RPMI-1640 medium, the medium was replaced with bicarbonate-buffered physiological saline solution, pH 7.4 [20]. Cells were pre-incubated for 15 min prior to treatment with test reagents. After incubation, the buffer was removed and the cells fixed in 300 µl of 3.7% formaldehyde in PBS for 15 min. The cells were then permeabilized with 0.2% Triton X-100 in PBS prior to blocking with a solution of 10 mg/ml BSA in PBS for 30 min. This was then removed and replaced with 0.15 µM rhodamine-phalloidin in PBS. The solution was aspirated and the cells washed three times with PBS. Finally, the cells were examined under a binocular microscope with fluorescence illumination.

Statistical analysis

The data presented represent mean values obtained in a single experiment in each case but all experiments were repeated on a minimum of two separate occasions, with similar results. Statistical analyses were performed using Student's *t* test for unpaired data.

Materials

Rhodamine-phalloidin, penicillin G, streptomycin, cytochalasin A, pertussis toxin (PT) and noradrenaline were purchased from Sigma Chemicals. Idazoxan was a gift from Reckitt and Colman Products, UK14,304 was provided by Pfizer and prazosin obtained from Ciba-Geigy. Tissue-culture media and fetal calf serum were purchased from GIBCO-BRL. ¹²⁵I for labelling of insulin was obtained from DuPont-NEN. All other chemicals were of analytical reagent quality.

RESULTS

Phalloidin is a toxin isolated from the toadstool *Amanita phalloides* which binds specifically to polymerized (F-) actin but not to monomeric G-actin [22]. When coupled to a fluorescent dye such as rhodamine, the conjugate (rhodamine-phalloidin) provides a sensitive and convenient assay reagent which can be used to obtain qualitative information about changes in the F-actin content of cells, after fixation and permeabilization [21].

In initial experiments the concentration of rhodamine-phalloidin bound by permeabilized HIT-T15 cells was varied between 0.1 and 3 µM to determine a suitable concentration which yielded close to maximal fluorescence. A concentration of 1.5 µM rhodamine-phalloidin was found to be appropriate and was used in all subsequent studies. It was then confirmed that fluorescence output was linear with cell number, and a seeding density of 10⁵ cells/cm² was chosen and used routinely in further experiments. In order to ensure that changes in actin polymerization could be detected readily, cells were first incubated with the microfilament-disrupting agent, cytochalasin. As expected, this compound caused an immediate, dose-dependent and sustained decrease in F-actin content, as judged by a decrease in rhodamine-phalloidin fluorescence (Figure 1).

Figure 1 Effect of cytochalasin A on F-actin in HIT cells

HIT cells were incubated in the presence of 1 µg/ml (●) or 5 µg/ml (○) cytochalasin A. F-actin levels were measured with rhodamine-phalloidin after various time periods of incubation. The fluorescence emission from control cells incubated with vehicle alone is shown by open squares. Each point represents the mean value from duplicate determinations in a single experiment which was repeated twice with similar results.

Figure 2 Effect of noradrenaline on F-actin in HIT cells

Cultured HIT cells were treated with either vehicle (○) or 10 μ M noradrenaline (●). F-actin levels were measured using rhodamine-phalloidin at the time points shown. Each value is the mean $\pm$ S.E.M from triplicate determinations. * $P < 0.001$ relative to fluorescence at $t = 0$.

In contrast to the actions of cytochalasin, addition of noradrenaline to cultured HIT cells resulted in a significant increase in rhodamine-phalloidin fluorescence within 30 s (Figure 2). This increase was sustained for at least 15 min after addition of 10 μ M noradrenaline. The distribution of F-actin in control and noradrenaline-treated cells was examined using fluorescence microscopy, after staining with rhodamine-phalloidin (Figure 3). In control cells, the most intense staining was seen in a region immediately adjacent to the plasma membrane (Figure 3; upper panel), consistent with the presence of F-actin in the cell web. Despite the overall increase in F-actin content induced by noradrenaline (Figure 2) this was not associated with any marked change in the intensity of labelling of the cell web in HIT cells (Figure 3; lower panel). Rather, the overall staining pattern of the cells appeared to become more diffuse after noradrenaline treatment (Figure 3; lower panel).

The increase in actin polymerization induced by noradrenaline was dose-dependent (Figure 4). The response exhibited an ED_{50} of approximately 2 μ M with the maximal effect being elicited by 5 μ M noradrenaline. This dose-response relationship suggests that the change in actin polymerization induced by noradrenaline requires higher concentrations of agonist than are necessary to inhibit insulin secretion. Thus, in parallel studies, insulin secretion induced by membrane depolarization was inhibited more than 50% by 0.1 μ M noradrenaline (Table 1) despite the fact that, at this concentration, noradrenaline did not cause a measureable change in F-actin fluorescence (Figure 4).

In order to determine which subtype of adrenoceptor was responsible for mediating the response to noradrenaline, the effects of the selective α -1-antagonist prazosin and the α -2-antagonist idazoxan were tested. Neither compound caused a significant alteration in rhodamine-phalloidin fluorescence when added to HIT cells in the absence of noradrenaline (Table 2) and prazosin failed to modify the increase induced by noradrenaline. By contrast, idazoxan significantly reduced the response to

Figure 3 Distribution of F-actin in cultured HIT-T15 cells

Cultured HIT-T15 cells were grown on fibronectin-coated glass coverslips and then incubated under either control conditions (upper panel) or in the presence of 10 μ M noradrenaline (lower panel) for 5 min. The cells were fixed, permeabilized and incubated with rhodamine-phalloidin to stain F-actin, prior to microscopic examination under fluorescence illumination.

noradrenaline ($P < 0.001$), suggesting that it was mediated by an adrenoceptor of the α -2 subtype. In confirmation of this result, the highly selective α -2-agonist UK14,304 also promoted an increase in rhodamine-phalloidin fluorescence in HIT cells, which was significantly inhibited by idazoxan (Table 3).

In a final series of experiments, HIT cells were incubated in the presence of PT for 24 h prior to measurement of the effects of α agonists on actin polymerization. As observed previously, treatment of the cells with either noradrenaline or UK14,304 caused an increase in rhodamine-phalloidin fluorescence. PT did not alter the fluorescence output measured in control cells (Table 4), suggesting that it did not modify the basal extent of actin polymerization. However, pretreatment of the cells with PT did lead to significant modulation of the responses to noradrenaline and UK14,304 (Table 4). Surprisingly, PT did not cause inhibition of the effects of the agonists; rather, a significant enhancement of the response to each compound was evident (Table 4). Examination of PT-treated cells by fluorescence microscopy after incubation with noradrenaline and staining with rhodamine-phalloidin, did not reveal any obvious changes in F-actin distribution by comparison with non-treated controls (results not shown).

Figure 4 Dose-response relationship for stimulation of F-actin formation by noradrenaline in HIT cells

HIT-T15 cells were incubated with increasing concentrations of noradrenaline for 5 min prior to measurement of F-actin content with rhodamine-phalloidin. Control fluorescence from cells incubated in the absence of noradrenaline was not significantly different from that measured in the presence of 0.1 μ M noradrenaline. Values represent the mean fluorescence $\pm$ S.E.M. ($n = 4$).

Table 1 Inhibition of insulin secretion from cultured HIT-T15 cells by noradrenaline

Cultured HIT cells were incubated in the presence of 30 mM KCl to depolarize the plasma membrane. Increasing concentrations of noradrenaline were included as shown. After incubation for 1 h at 37 °C, samples of medium were withdrawn for assay of insulin secretion by radioimmunoassay. Results are expressed as the mean rate of insulin release relative to basal (100%) $\pm$ S.E.M. ($n = 10$). Statistical significance: * $P < 0.001$ relative to KCl alone.

Noradrenaline concentration (μ M)	Insulin secretion (% of basal)
0	452 $\pm$ 63
0.1	232 $\pm$ 29*
0.5	213 $\pm$ 27*
1.0	187 $\pm$ 28*

Table 2 Effect of alpha-adrenoceptor antagonists on the increase in F-actin formation mediated by noradrenaline

HIT-T15 cells were incubated with test reagents as shown for 5 min. The cells were then fixed and permeabilized prior to incubation with rhodamine-phalloidin. Values shown represent mean fluorescence $\pm$ S.E.M. ($n = 3$). Statistical significance: * $P < 0.001$ relative to control; ** $P < 0.001$ relative to noradrenaline alone.

Incubation conditions	Fluorescence (arbitrary units)
Control	64.8 $\pm$ 2.2
Prazosin (10 μ M)	64.9 $\pm$ 1.6
Idazoxan (10 μ M)	64.1 $\pm$ 0.9
Noradrenaline (10 μ M)	75.5 $\pm$ 0.6*
Noradrenaline + prazosin	73.1 $\pm$ 0.2
Noradrenaline + idazoxan	68.7 $\pm$ 0.4**

Table 3 Effect of the selective alpha-2-agonist UK14,304 on F-actin formation in HIT-T15 cells

Cultured HIT-T15 cells were incubated for 5 min in the presence of test reagents, after which time the cells were fixed, permeabilized and treated with rhodamine-phalloidin to measure F-actin levels. Results are presented as arbitrary fluorescence units $\pm$ S.E.M. ($n = 4$). Statistical significance: * $P < 0.001$ relative to no addition; ** $P < 0.001$ relative to UK14,304 alone.

Incubation conditions	Rhodamine-phalloidin fluorescence (units)
No addition	62 $\pm$ 2
UK14,304 (10 μ M)	72 $\pm$ 3*
UK14,304 (10 μ M) + Idazoxan (10 μ M)	61 $\pm$ 1**

Table 4 Effects of pertussis toxin on the increase in F-actin formation caused by alpha-2-agonists in HIT-T15 cells

HIT cells were cultured either in the absence or presence of pertussis toxin (200 ng/ml) for 18 h. After this time they were washed and exposed to alpha-2-agonists for 5 min. The cells were then fixed and permeabilized and F-actin formation monitored by rhodamine-phalloidin fluorescence. Each value represents the mean $\pm$ S.E.M. ($n = 3$). Statistical significance: * $P < 0.001$ relative to agonist in the absence of pertussis toxin.

Incubation conditions	Fluorescence (arbitrary units)	
	— Pertussis toxin	+ Pertussis toxin (200 ng/ml)
Control	54.8 $\pm$ 1.8	55.2 $\pm$ 1.0
Noradrenaline (10 μ M)	61.8 $\pm$ 0.6	68.6 $\pm$ 1.4*
UK14,304 (10 μ M)	58.4 $\pm$ 1.0	71.0 $\pm$ 4.0*

DISCUSSION

Alpha-2-agonists are potent inhibitors of insulin secretion which can exert an influence on several diverse intracellular processes in the pancreatic B-cell. These include effects on cyclic nucleotide levels [11,12,16], ion permeability [13,14] and nutrient metabolism [15]. In the present work evidence that alpha-2-agonists can also promote F-actin formation in clonal pancreatic B-cells is provided. This response appears to be mediated by alpha-2-adrenoceptors since it was elicited by noradrenaline (Figures 2 and 4; Tables 2 and 4) and by the highly selective alpha-2-agonist UK14,304 (Tables 3 and 4) and, in each case, the response was inhibited by the alpha-2-selective antagonist idazoxan (Tables 2 and 3). To our knowledge, this represents the first demonstration that activation of alpha-2-adrenoceptors can lead to increased F-actin formation, although control of actin polymerization has been described in a number of other cells following activation of receptors for growth factors [23–25], cholinergic agonists [21], cytokines [26] and chemoattractants [27,28].

Actin microfilaments form part of a structure defined as the 'cell web' in pancreatic B-cells, which lies beneath the plasma membrane and is thought to play a role in regulating the access of secretory granules to the plasma membrane during exocytosis [19,29–31]. This structure exists in a dynamic equilibrium controlled by the relative rates of polymerization of G-actin monomers and depolymerization of F-actin. As such, it may either permit or inhibit the access of secretory granules to their sites of fusion at the plasma membrane. The precise mechanisms by which this leads to alterations in the rate of exocytosis are still controversial, since both increases and decreases in F-actin have been correlated with enhancement of insulin secretion. For

example Orci et al. demonstrated that disruption of the cell web with cytochalasin resulted in potentiation of both phases of the insulin secretory response to glucose [29]. They suggested that actin microfilaments form a physical barrier to secretory granule access and that this barrier must be overcome by depolymerization of the filaments during stimulation of insulin secretion. In support of this, evidence has been presented that islets isolated from animals having a decreased secretory responsiveness to glucose (e.g. after starvation) exhibit an increase in F-actin content [32]. However, treatment of electrically permeabilized islets with phalloidin (to prevent depolymerization of actin filaments) results in stimulation of insulin secretion [33]. When coupled with the finding that insulin secretory granules can interact with F-actin *in vitro* [34], these results support the view that an increase in F-actin content of the cell web can lead to modulation of insulin secretion. A similar model has also been derived for control of catecholamine secretion from chromaffin cells [21,35].

However, the evidence is not unequivocal since some reports suggest that islet F-actin levels may actually increase during stimulation of insulin secretion [19,36,37], and that disruption of actin microfilaments (e.g. with clostridial toxins) can lead to inhibition of insulin secretion [38]. Thus, the precise mechanisms by which a change in actin polymerization is involved in control of secretion still remain unclear, although there is a firm consensus that alterations in the extent of actin polymerization are important for regulation of insulin secretion.

On this basis, the present finding that alpha-2-agonists can modulate the extent of actin polymerization in pancreatic B-cells suggests that this response could be a component of the mechanism by which these agents inhibit insulin secretion. Indeed, it is noteworthy that, of all of the responses currently attributed to alpha-2-adrenoceptor activation in the pancreatic B-cell, control of F-actin formation is the only one which might remain functionally intact in permeabilized B-cells. Furthermore, since there is evidence for direct coupling between plasma membrane components and actin microfilaments in a variety of cell types [39,40], it is not necessary to postulate the involvement of intracellular messengers to explain how cell-surface receptors might influence microfilament formation in permeabilized cells. Despite these considerations, however, it should also be noted that the dose-response data obtained in the present work (Figure 4) suggest that measureable stimulation of F-actin formation in HIT cells is only seen at relatively high concentrations of catecholamine compared with those required for inhibition of insulin secretion (Table 1). This may imply that changes in actin polymerization are not central to adrenergic inhibition of insulin secretion. Alternatively, it could be that very small changes are sufficient to mediate inhibition, and that these are below the limits of detection of the method employed herein.

The mechanism by which HIT cell alpha-2-adrenoceptors are coupled to the control of actin polymerization remains to be established, although one of the most surprising aspects revealed by the present work is the finding that the effect was not inhibited following exposure of the cells to PT (Table 4). Indeed, prior exposure to PT actually enhanced the response to noradrenaline or UK14,304 (Table 4). These results suggest that the increase in F-actin formation mediated by alpha-2-adrenoceptors does not result from activation of a member of the G_i or G_o family of G-proteins. As such, it is at variance with data obtained for control of actin polymerization in neutrophils [41] and in differentiating U937 cells [42]. Moreover, at first sight, this finding seems to effectively exclude the possibility that alpha-2-adrenoceptor-induced F-actin formation might be associated with inhibition of insulin secretion, since it is well established that exposure of islet

cells (including cultured HIT cells) to PT prevents adrenergic inhibition [4,9,11,12,43]. At present, there is no adequate resolution for this apparent anomaly although, as outlined above, no direct correlation has yet been established between changes in the direction of actin polymerization and alterations in insulin secretion. In this context, we undertook a visual examination of the distribution of F-actin in HIT-T15 cells using fluorescence microscopy (Figure 3) and were unable to detect any gross changes in distribution in response to any of the agents tested. This suggests that the whole-cell fluorescence measurements were responding to relatively subtle alterations in actin polymerization and that large-scale redistribution does not occur in response to alpha-2-adrenoceptor activation.

Pretreatment of HIT cells with PT did not alter the basal rhodamine-phalloidin fluorescence (Table 4), suggesting that PT did not, itself, modify the F-actin content of unstimulated cells. This result is similar to observations made with neutrophils (where PT only affects F-actin formation mediated by receptor agonists [41]) but differs from the situation in chromaffin cells where PT promotes a direct reduction in F-actin [21]. Despite these differences, it is clear that PT can affect actin polymerization in several secretory cells, which supports the suggestion that a G-protein may be involved in regulation of F-actin levels [24,28,42,44,45]. This molecule has not yet been identified, but could be related to the putative G_c thought to be responsible for regulation of certain distal events in exocytosis [18]. In this context, it may be significant that low-molecular-mass (20–25 kDa) GTP-binding proteins of the *rac* and *rho* families have been implicated in receptor-mediated regulation of cytoskeletal organization [24,46]. Low-molecular-mass GTP-binding proteins have been identified in cells of the endocrine pancreas [47–49] and, in preliminary studies, we have observed that islet cells contain a low-molecular-mass PT substrate (A. El-Mansoury and N. G. Morgan, unpublished work). Further work will be required to establish whether there is a functional relationship between any of these proteins and alpha-2-adrenoceptors in islet cells.

In conclusion, we have demonstrated that activation of alpha-2-adrenoceptors in cultured HIT-T15 cells is associated with a rapid, dose-dependent increase in F-actin formation. These observations raise the possibility that control of actin polymerization may form part of the elusive distal mechanism by which alpha-2-agonists regulate insulin secretion.

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