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# Multiple effector pathways regulate the insulin secretory response to the imidazoline RX871024 in isolated rat pancreatic islets

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**1** When isolated rat islets were cultured for 18 h prior to use, the putative imidazoline binding site ligand, RX871024 caused a dose-dependent increase in insulin secretion at both 6 mM and 20 mM glucose. By contrast, a second ligand, efaroxan, was ineffective at 20 mM glucose whereas it did stimulate insulin secretion in response to 6 mM glucose.

**2** Exposure of islets to RX871024 (50 $\mu$M) for 18 h, resulted in loss of responsiveness to this reagent upon subsequent re-exposure. However, islets that were unresponsive to RX871024 still responded normally to efaroxan.

**3** The imidazoline antagonist, KU14R, blocked the insulin secretory response to efaroxan, but failed to prevent the stimulatory response to RX871024.

**4** By contrast with its effects in cultured islets, RX871024 inhibited glucose-induced insulin release from freshly isolated islets. Efaroxan did not inhibit insulin secretion under any conditions studied.

**5** In freshly isolated islets, the effects of RX871024 on insulin secretion could be converted from inhibitory to stimulatory, by starvation of the animals.

**6** Inhibition of insulin secretion by RX871024 in freshly isolated islets was prevented by the cyclo-oxygenase inhibitors indomethacin or flurbiprofen. Consistent with this, RX871024 caused a marked increase in islet $PGE_2$ formation. Efaroxan did not alter islet $PGE_2$ levels.

**7** The results suggest that RX871024 exerts multiple effects in the pancreatic $\beta$-cell and that its effects on insulin secretion cannot be ascribed only to interaction with a putative imidazoline binding site.

**Keywords:** Islets of Langerhans; insulin secretion; pancreatic $\beta$-cell; efaroxan; RX871024; imidazoline; imidazoline binding site; prostaglandin $E_2$

**Abbreviations:** BSA, bovine serum albumin; CDS, clonidine displacing substance; DMSO, dimethylsulphoxide; ELISA, enzyme linked immunoassay; $K_{ATP}$, ATP-sensitive potassium channel; $PGE_2$, prostaglandin $E_2$

## Introduction

A wide range of imidazoline derivatives have been identified over recent years which possess the capacity to stimulate insulin secretion from pancreatic $\beta$-cells (reviewed by Chan, 1993; Molderings, 1997; Eglen *et al*., 1998). Some of these have attracted interest as possible therapeutic agents for use in type II diabetes, and the group includes well-characterized molecules such as phentolamine and efaroxan (Schulz & Hasselblatt, 1988; Chan & Morgan, 1990; Berdeu *et al*., 1994; Jonas *et al*., 1992) as well as newer compounds, e.g. RX871024 (Zaitsev *et al*., 1996; Efanov *et al*., 1998; Efanova *et al*., 1988a; Mourtada *et al*., 1998) S21633 (Wang *et al*., 1996; Le Brigand *et al*., 1997 and S22068 (Pele-Tounian *et al*., 1998). An important component of the insulin secretory response to all of these molecules involves the closure of $\beta$-cell ATP-sensitive ($K_{ATP}$) potassium channels (Chan *et al*., 1991; Dunne, 1991; Jonas *et al*., 1992) an effect which probably results from interaction of the ligands with an imidazoline binding site associated with the channel (Proks & Ashcroft, 1997). The relationship between this site (recently designated $I_3$; Eglen *et al*., 1998) and other components of the $K_{ATP}$ channel complex has still to be established, but there is evidence that it may be associated with the pore-forming subunit, Kir6.2 (Proks & Ashcroft, 1997; Monks *et al*., 1999).

Until recently, it had been assumed that all imidazoline insulin secretagogues exert their influence in a similar manner and that binding to a single site (the putative $I_3$ site) could account for their effects. However, this view has come under challenge for a number of reasons. Firstly, in addition to actions localized within the $K_{ATP}$ channel complex, it has recently been proposed that RX871024 may also regulate more distal components of the exocytotic pathway in $\beta$-cells (Zaitsev *et al*., 1996; Efanov *et al*., 1998). While it is conceivable that a single binding site (associated with the $K_{ATP}$ channel) could mediate both of these responses, a more parsimonious explanation would be that two or more target sites of RX871024, exist. (It is noteworthy that there is currently no firm evidence that Kir6.2 participates in exocytosis except by regulation of membrane potassium permeability). Secondly, it has become evident that stimulation of insulin secretion by efaroxan and RX871024 is accompanied by activation of separate intracellular effector mechanisms (Mourtada *et al*., 1998). This differential response is not easily reconciled with a single, common binding site for the two agents. Thirdly, it has also been argued that certain features of the secretory response to the novel imidazoline, S21663, are inconsistent with an action solely at the site activated by efaroxan (Le Brigand *et al*., 1997).

Thus, there is accumulating evidence that imidazolines may exert effects in the pancreatic $\beta$-cell which cannot be attributed to their interaction with a single imidazoline binding site. In order to investigate this issue further, we have studied the responses of pancreatic $\beta$-cells to the imidazoline, RX871024, in more detail. The results suggest that this compound may

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exert mutliple effects in the $\beta$-cell which are not all attributable to an interaction with imidazoline binding sites.

## Methods

### *Materials*

Collagenase (typeXI), dimethyl sulphoxide (DMSO), yohimbine, diazoxide, efaroxan, flurbiprofen and indomethacin were purchased from Sigma Chemical Co. (Dorset, U.K.). RX871024 was provided by Reckitt and Colman (U.K.). KU14R was synthesized as described previously (Chan *et al*., 1998) and is available from Tocris (Bristol, U.K.). Prostaglandin $E_2$ ($PGE_2$) ELISA system was from Amersham (U.K.). All other reagents were of analytical reagent grade. Drugs were prepared as stock solutions either in water or DMSO. The final DMSO concentration did not exceed 1% and, at this concentration, it did not affect insulin secretion.

### *Isolation and incubation of islets of Langerhans*

Islets were isolated from male Wistar rats (body weight 180–250 g) by collagenase digestion of excised pancreata. In most experiments the animals were allowed free access to food and water prior to islet isolation, although in some studies food was removed at either 18 or 24 h prior to sacrifice. The islets were selected by hand under a binocular dissecting microscope and were either used immediately ('fresh' islets) or were cultured in RPMI-1640 (supplemented with 200 $\mu$g ml$^{-1}$ streptomycin, 400 IU ml$^{-1}$ penicillin, 10% (v v$^{-1}$) foetal calf serum and L-glutamine (2 mM)) for 18–24 h, before assessment of their secretory function. The methods used have been described previously (Brown *et al*., 1993; Chan *et al*., 1993).

For measurements of insulin release, incubations were performed in 96 well plates. Group of three isolated islets were incubated in 200 $\mu$l of a bicarbonate-buffered physiological saline solution gassed with $O_2:CO_2$ (95:5) to pH 7.4, containing bovine serum albumin (BSA; 1 mg ml$^{-1}$) and test reagents. Following incubation in a humidified air : $CO_2$ (95 : 5) atmosphere at 37°C for appropriate periods of time, samples of the incubation medium were removed for measurement of insulin secretion by radioimmunoassay.

### *Measurement of islet $PGE_2$*

Groups of 50 freshly isolated islets were incubated for 20 min at 37°C in 0.5 ml of incubation medium supplemented with 20 mM glucose, BSA (1 mg ml$^{-1}$) and appropriate concentrations of test agents. After incubation, the tubes were centrifuged, the supernatant was discarded and the pellet resuspended in 200 $\mu$l assay buffer (provided in the kit) supplemented with flurbiprofen (200 $\mu$M). The samples were sonicated for 5 s (low power) then frozen by immersion in liquid nitrogen prior to storage at −80°C. Samples were assayed for $PGE_2$ using a commercial ELISA kit, according to the manufacturer's instructions.

## Results

### *Role of putative imidazoline binding site in the stimulation of insulin release from cultured rat islets by RX871024*

Recently we have reported a number of differences between the intracellular signalling pathways activated in rat islets treated with efaroxan and RX871024 (Mourtada *et al*., 1998). This raises the possibility that these two imidazoline drugs may bind to different sites in the $\beta$-cell and, in the present work, we have investigated this proposal. Initially, this was achieved by exploiting the observation that treatment of islets with an imidazoline for a prolonged period (~18 h) leads to desensitization upon subsequent re-exposure (Chan *et al*., 1994; 1997a–c; Chan, 1998). This response displays features characteristic of 'homologous' desensitization since exposure of islets to efaroxan is accompanied by loss of responses to structurally related imidazoline ligands (including phentolamine, midaglizole, RX871024; Chan *et al*., 1994) and to the putative endogenous ligand, clonidine-displacing substance (CDS; Chan, 1998) but not to 'non-imidazoline' secretagogues (e.g. glibenclamide and $\sigma$-agonists; Chan *et al*., 1994; 1997c).

The data presented in Table 1 reveal that, following culture for 18 h under control conditions, rat islets were fully responsive to either efaroxan (100 $\mu$M) or RX871024 (100 $\mu$M). Each compound potentiated the insulin secretory response from islets incubated in the presence of 6 mM glucose although, as observed previously (Mourtada *et al*., 1998) an important difference was noted in their actions at 20 mM glucose. Under these conditions, RX871024 increased insulin secretion from islets incubated in the presence of 20 mM glucose, whereas efaroxan did not (see also Chan & Morgan, 1990). Both imidazolines antagonized the inhibitory action of diazoxide (200 $\mu$M) on glucose-induced insulin secretion (Table 1). Islets which had been cultured in the presence of RX871024 (50 $\mu$M) for 18 h displayed a different profile of responses

**Table 1** Effect of culture with RX871024 on islet secretory responses to efaroxan and RX871024

| *[Glucose]* (mM) | *Efaroxan* (100 $\mu$M) | *RX871024* (100 $\mu$M) | *Diazoxide* (200 $\mu$M) | *Control islets* (ng islet$^{-1}$ h$^{-1}$) | *Desensitized islets* (ng islet$^{-1}$ h$^{-1}$) |
|---|---|---|---|---|---|
| 6 | − | − | − | 2.6±0.36 | 2.1±0.38 |
| 6 | + | − | − | 4.3±0.40* | 3.9±0.40* |
| 6 | − | + | − | 4.0±0.48* | 2.2±0.36 |
| 20 | − | − | − | 4.2±0.40 | 4.0±0.50 |
| 20 | + | − | − | 4.3±0.48 | 3.9±0.41 |
| 20 | − | + | − | 5.9±0.41** | 3.9±0.30 |
| 20 | − | − | + | 2.4±0.3 | 2.3±0.32 |
| 20 | + | − | + | 5.0±0.39*** | 3.8±0.38*** |
| 20 | − | + | + | 4.8±0.39*** | 3.6±0.35*** |

Groups of isolated rat islets were cultured for 18 h in either the absence (control islets) or presence of 50 $\mu$M RX871024 (desensitized islets) then washed and incubated with test reagents. The level of insulin secretion was measured by radioimmunoassay. Results represent mean rates of insulin secretion±s.e.mean ($n=24$) from three separate islet preparations. *$P<0.01$ relative to the absence of imidazoline; **$P<0.01$ relative to the 20 mM glucose alone; ***$P<0.001$ relative to 20 mM glucose + diazoxide.

(Table 1). In such islets re-exposure to RX871024 failed to elicit any potentiation of insulin secretion in the presence of 6 mM or 20 mM glucose, whereas these islets were fully responsive to efaroxan. Surprisingly, despite its failure to potentiate insulin secretion, RX871024 was still able to antagonize the inhibitory effect of diazoxide in 'desensitized' islets (Table 1).

These results clearly suggest that efaroxan and RX871024 do not utilize a single, common pathway to exert their effects on insulin release since the responses were regulated differentially by prior agonist exposure. In order to examine this further, experiments were performed using the newly available analogue of efaroxan, KU14R, which acts as a functional antagonist to the insulin secretory responses mediated by efaroxan and several other imidazolines (Chan *et al*., 1997a–c; 1998). As shown previously (Chan *et al*., 1997a–c; 1998) KU14R failed to exert any direct affect on insulin secretion from rat islets incubated in the presence of either 6 mM or 20 mM glucose (Table 2) whereas it completely antagonized the increase in insulin secretion induced by efaroxan (Table 2). By contrast, the potentiating effect of RX871024 on insulin secretion was unaffected by KU14R irrespective of whether the experiment was performed in the presence of 6 mM or 20 mM glucose (Table 2).

### *Effects of RX871024 on insulin secretion from freshly isolated rat islets*

In the studies reported above, islets were maintained in organ culture for 18 h prior to assessment of their secretory function, in order to facilitate comparison with earlier work (Zaitsev *et al*., 1996; Efanov *et al*., 1998; Efanova *et al*., 1998a). However, it was important to establish that freshly isolated islets also respond to RX871024, in order to eliminate the possibility that the culture conditions *per se* might modify their responses (although there is no evidence that this happens in the case of efaroxan). Thus, we compared the effects of RX871024 on insulin secretion from freshly isolated islets (i.e. islets which have not been exposed to tissue culture medium and were used within 2–3 h of isolation) with those from islets maintained in organ culture for 18 h. As expected (Zaitsev *et al*., 1996; Mourtada *et al*., 1998) RX871024 potentiated the rate of glucose (20 mM)-induced insulin secretion, in a dose-dependent manner, from cultured islets (Figure 1A). However, a completely opposite response was observed in freshly isolated islets (Figure 1B). In this case, rather than increasing insulin secretion, addition of RX871024 was associated with a substantial, dose-dependent inhibition of glucose (20 mM)-induced insulin secretion.

**Table 2** Effects of KU14R on insulin secretory responses to efaroxan and RX871024

| *Glucose* (mM) | *RX871024* (100 $\mu$M) | *Efaroxan* (100 $\mu$M) | *KU14R* (100 $\mu$M) | *Insulin secretion* (ng islet$^{-1}$ h$^{-1}$) |
|---|---|---|---|---|
| 6 | – | – | – | 1.1±0.24 |
| 6 | – | + | – | 2.0±0.12* |
| 6 | + | – | – | 2.0±0.40* |
| 6 | – | – | + | 1.2±0.11 |
| 6 | – | + | + | 1.3±0.14** |
| 6 | + | – | + | 2.2±0.21 |
| 20 | – | – | – | 2.3±0.22 |
| 20 | + | – | – | 3.5±0.30*** |
| 20 | – | – | + | 2.4±0.30 |
| 20 | + | – | + | 3.2±0.30*** |

Cultured rat islets were treated with test reagents and insulin secretion measured after 1 h. *$P<0.01$ relative to 6 mM glucose alone; **$P<0.02$ relative to efaroxan in the absence of KU14R; ***$P<0.005$ relative to 20 mM glucose alone.

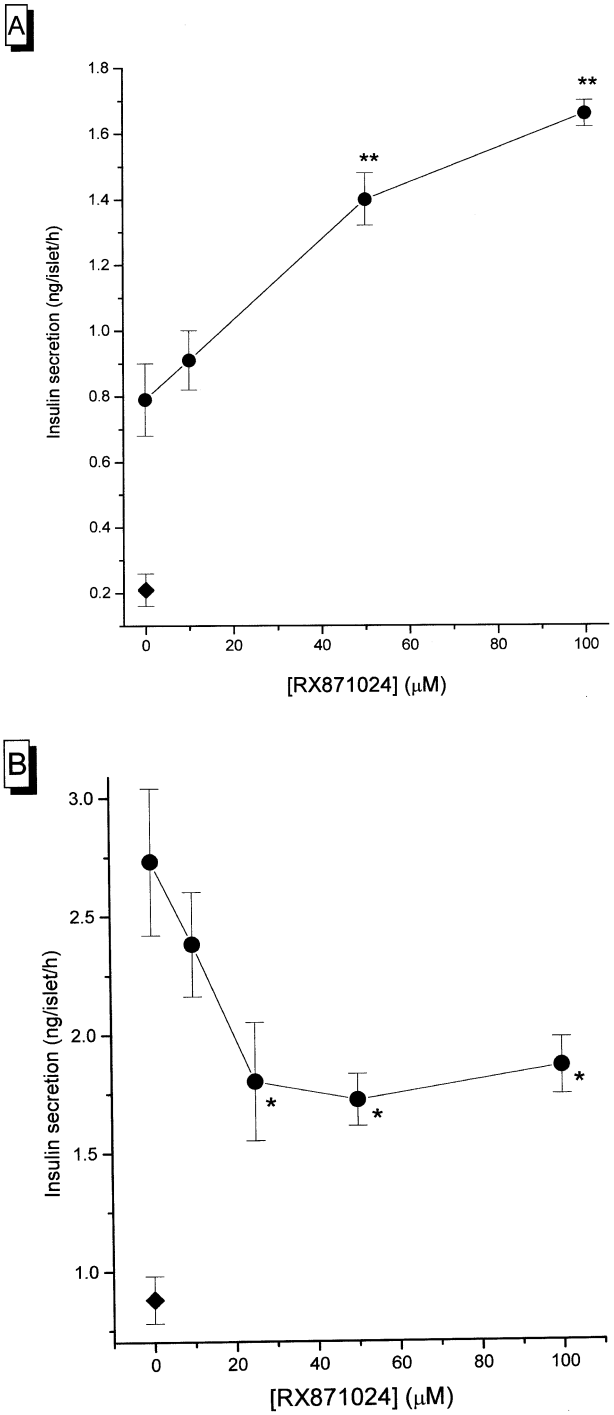


**Figure 1** Effects of RX871024 on glucose-induced insulin secretion in freshly isolated and cultured rat islets. Groups of rat islets were isolated by collagenase digestion and either cultured for 18 h (A) or used within 2 h of isolation (B). Islets were exposed to either 6 mM glucose (closed diamonds) or 20 mM glucose (closed circles); increasing concentrations of RX871024 were included, as shown. Following incubation for 1 h at 37°C, samples of incubation medium were removed and insulin secretion measured by radioimmunoassay. Results represent mean rates of insulin secretion±s.e.mean ($n=24$) from three separate islet preparations in each case. *Significant inhibition relative to 20 mM glucose alone ($P<0.001$); **Significant stimulation relative to 20 mM glucose alone ($P<0.001$).

One major difference between cultured and freshly isolated islets is that cultured islets are exposed to a stimulatory glucose concentration (11 mM) during the culture period. In order to investigate whether this may have contributed to the conflicting effects of RX871024 in fresh and cultured islets, parallel experiments were performed in which isolated islets were maintained in culture media containing either 11 mM or 6 mM glucose, for 18 h. Insulin secretory responses were subsequently determined in static incubations. The results revealed that the stimulatory response to RX871024 (100 $\mu$M) was maintained in the cultured islets irrespective of the glucose concentration of the culture medium (results not presented).

Many of the imidazolines which control insulin secretion are also ligands at $\alpha_2$-adrenoceptors (Schulz & Hasselblatt, 1988; Chan & Morgan, 1990; Berdeu *et al*., 1994; Plant & Henquin, 1990; Mourtada *et al*., 1997), thus it was important to establish whether the inhibitory response to RX871024 seen in fresh islets might reflect some residual $\alpha_2$-agonist activity. However, co-incubation with either efaroxan or yohimbine (two structurally unrelated $\alpha_2$-antagonists) failed to prevent the inhibitory effect of RX871024 (Table 3) suggesting that $\alpha_2$-agonism is not involved.

In order to gain a more complete understanding of the secretory responses of freshly isolated islets to RX871024, experiments were performed to establish the glucose-dependence of the responses. The results are shown in Figure 2 and reveal a complex pattern. Indeed, it appears that the inhibitory effect of the compound is observed at elevated glucose concentrations (8 mM and above) and that incubation of islets with lower glucose concentrations is associated with either a net lack of effect upon addition of RX871024 (at 4–6 mM glucose; see also Figure 3A) or is actually stimulatory (at sub-threshold glucose concentrations). Thus, it seems likely that RX871024 activates competing processes in islets, which lead to either stimulation or inhibition of insulin secretion according to the prevailing glucose concentration. This is quite different from any effects previously observed with a wide range of other imidazolines.

### *Effects of RX871024 on insulin secretion from islets isolated from fasted rats*

In considering the possible differences between fresh and cultured islets which might account for the differential responses to RX871024, we began to focus on the possible involvement of paracrine regulators of insulin secretion. This is because the inhibitory effects of RX871024 are not seen in the isolated perfused rat pancreas (Zaitsev *et al*., 1996; Efanova *et al*., 1998b) nor in freshly isolated, perifused, islets (Chan & Morgan; unpublished observations) where paracrine influences are minimized by the flow of perfusate. One important paracrine inhibitor of insulin secretion is prostaglandin $E_2$ ($PGE_2$; Robertson, 1986; 1988; Laychock & Bilgin, 1989; Metz, 1998; 1991; Metz *et al*., 1991; Morgan & Montague, 1992) and it has been reported that the levels of this agent are markedly reduced in islets during conditions of fasting (Tadayyon *et al*., 1990). Thus, we investigated the ability of RX871024 to regulate insulin secretion from islets of animals deprived of food for increasing periods of time. The islets were not maintained in culture but were used as freshly isolated tissue and the results are presented in Figure 3. Islets harvested from control animals (allowed free access to food and water) displayed responses to RX871024 and efaroxan consistent with those seen earlier (Figure 3A). In these islets, efaroxan enhanced insulin secretion at 6 mM glucose but not at 20 mM glucose, whereas RX871024 failed to affect secretion at 6 mM glucose and inhibited the response to 20 mM glucose.

When islets were isolated from animals deprived of food for 18 h, the insulin secretory responses to glucose were reduced but efaroxan was still able to potentiate insulin secretion at 6 mM glucose, whereas RX871024 did not. More significantly, the ability of RX871024 to inhibit insulin secretion from islets treated with 20 mM glucose was impaired (though still statistically significant) under these

**Table 3** Effects of $\alpha_2$-adrenoceptor antagonists on inhibition of glucose-induced insulin secretion by RX871024 from freshly isolated rat islets

| *RX871024* (100 $\mu$M) | *Efaroxan* (100 $\mu$M) | *Yohimbine* (100 $\mu$M) | *Insulin secretion* (ng islet$^{-1}$ h$^{-1}$) |
|---|---|---|---|
| − | − | − | 5.40±0.41 |
| + | − | − | 3.00±0.26* |
| + | + | − | 2.20±0.36* |
| + | − | + | 2.56±0.29* |

Islets were incubated in the presence of 20 mM glucose plus test reagents and insulin release then measured by radioimmunassay. Results represent mean rates of insulin secretion±s.e.mean ($n=24$) from three islet preparations. *$P<0.005$ relative to 20 mM glucose in the absence of RX871024.

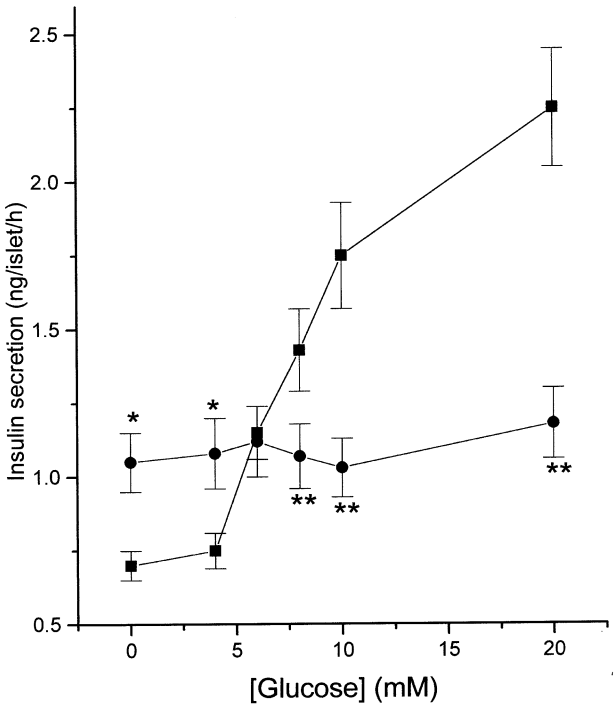


**Figure 2** Glucose-dependence of the effects of RX871024 in freshly isolated rat islets. Groups of freshly isolated rat islets were incubated for 1 h in the presence of increasing concentrations of glucose in either the absence (squares) or presence (circles) of 100 $\mu$M RX871024. Following incubation, samples of the medium were removed and insulin secretion measured by radioimmunoassay. Each point represents the mean value±s.e.mean ($n=22$) obtained from three separate islet preparations. *Significant stimulation relative to the equivalent glucose concentration in the absence of RX871024 ($P<0.01$). **Significant inhibition relative to the equivalent glucose concentration in the absence of RX871024 ($P<0.01$).

conditions (Figure 3B). Finally, in islets obtained from animals deprived of food for 24 h, efaroxan failed to alter insulin secretion and the inhibitory effect of RX871024 was lost (Figure 3C). Rather, in these islets, RX871024 was observed to stimulate insulin secretion (at both 6 and 20 mM glucose). Thus, food deprivation of the donor animals was able to markedly alter the secretory responses of freshly isolated islets to RX871024.

*Investigation of the role of $PGE_2$ in the inhibitory effects of RX871024 on glucose-induced insulin secretion from fresh islets*

In view of the evidence that food deprivation leads to lowered islet $PGE_2$ levels (Tadayyon *et al*., 1990) and can also alter the secretory effects of RX871024, we investigated whether the inhibitory effect of RX871024 in islets freshly prepared from

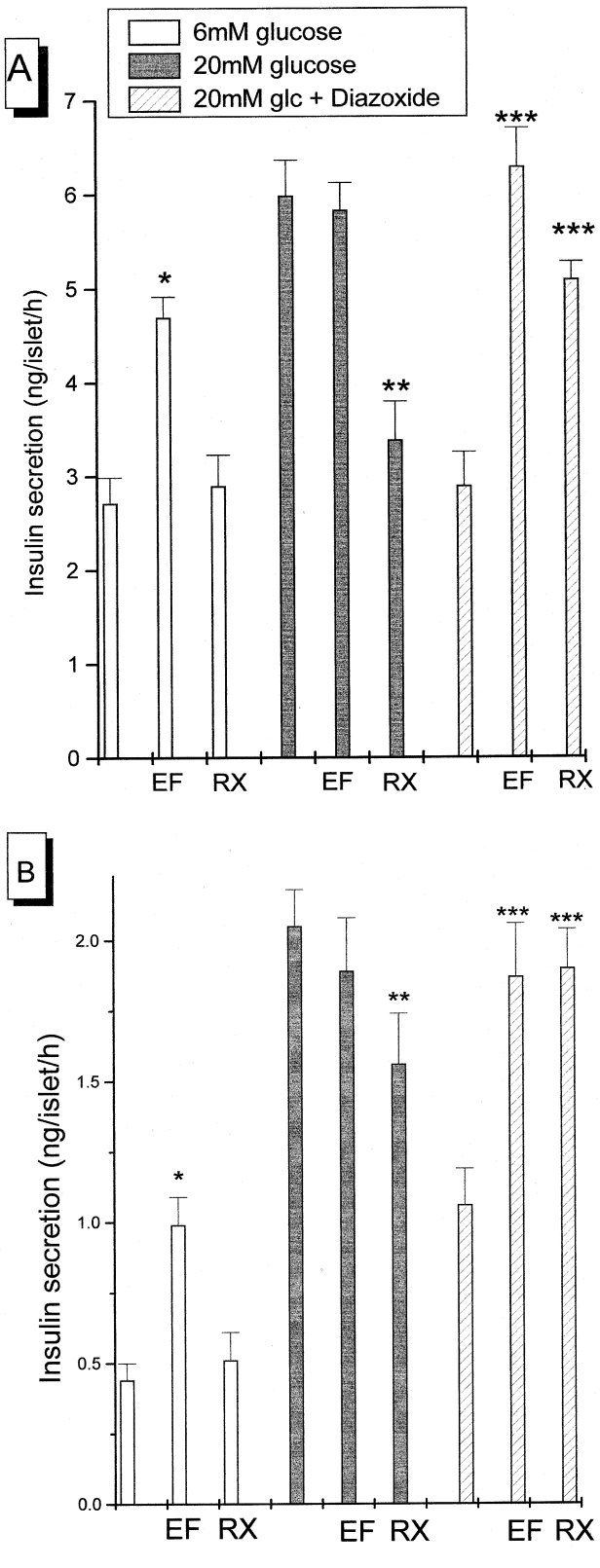


**Figure 3** Effects of RX871024 on insulin secretion from islets isolated from either fed or fasted rats. Islets of Langerhans were isolated from rats allowed free access to food (A) or from animals which had been fasted for either 18 h (B) or 24 h (C). All animals were provided with free access to water. Following isolation, the islets were incubated in the presence of 6 mM glucose (open bars) 20 mM glucose (grey bars) or 20 mM glucose plus 200 $\mu$M diazoxide (hatched bars) with either efaroxan (100 $\mu$M; Ef) or RX871024 (100 $\mu$M; RX). Following incubation for 1 h, the medium was sampled and assayed for insulin secretion by radioimmunoassay. Results are presented as mean rates of insulin secretion $\pm$ s.e.mean ($n=24$) from three separate islet preparations in each case. *Significant stimulation relative to 6 mM glucose alone ($P<0.01$); **significant inhibition relative to 20 mM alone ($P<0.01$); ***significant stimulation relative to diazoxide in the absence of imidazoline ($P<0.001$); ****significant stimulation relative to 20 mM glucose alone ($P<0.001$).

fed animals could be influenced by inhibitors of islet cyclo-oxygenase activity. In agreement with previous studies (Metz *et al*., 1981), initial experiments revealed that high concentrations (100 $\mu$M) of either of two inhibitors (indomethacin and flurbiprofen) led to stimulation of insulin secretion (not shown) but that no effects were observed when the inhibitor concentration was 50 $\mu$M or less. The data presented in Figures 4 and 5 reveal that both indomethacin and flurbiprofen were able to reverse the inhibitory effects of RX871024 (100 $\mu$M) on glucose-induced insulin secretion. The effect of each drug was dose-dependent: the $EC_{50}$ for indomethacin was approximately 5 $\mu$M (Figure 4) and the $EC_{50}$ for flurbiprofen was approximately 1 $\mu$M. Neither drug allowed the development of a stimulatory response to RX871024, even at concentrations up to 50 $\mu$M (Figures 4 and 5).

Since the use of cyclo-oxygenase inhibitors antagonized the inhibitory effects of RX871024 on insulin secretion from fresh islets, it was important to investigate whether RX871024 directly altered the levels of $PGE_2$ in islet cells. In these experiments, the phospholipase A2 activator, melittin, was used as positive control. Melittin is known to promote the release of arachidonic acid from membrane phospholipids in islet cells which, in turn, is metabolized *via* the cyclo-oxygenase and lipoxygenase pathways to yield products which include $PGE_2$ (Morgan *et al*., 1985; Metz, 1988). As expected, melittin (2 $\mu$g ml$^{-1}$) significantly increased the islet content of $PGE_2$ relative to control (Table 4). RX871024 (100 $\mu$M) also enhanced islet $PGE_2$ levels, which achieved levels greater than those observed with mellitin. By contrast, efaroxan (100 $\mu$M) did not alter islet $PGE_2$ levels (Table 4).

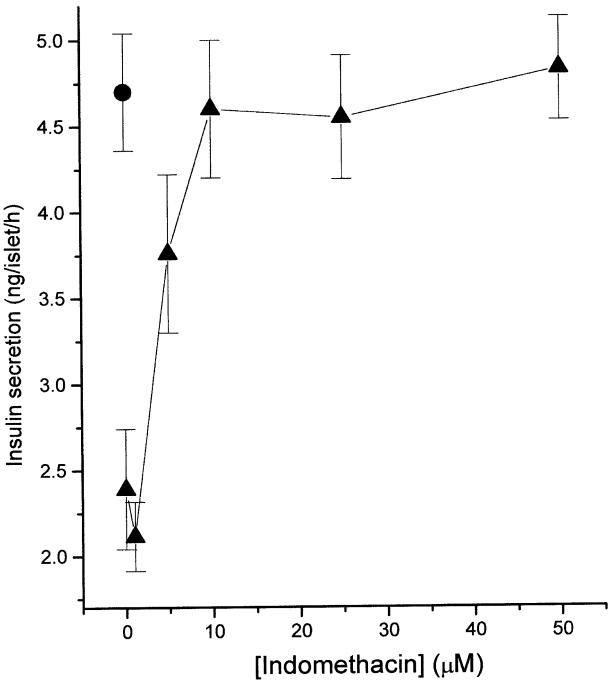


**Figure 4** Effect of indomethacin on inhibition of glucose-induced insulin secretion in response to RX871024. Groups of freshly isolated rat islets were incubated with 20 mM alone (circle) or 20 mM glucose plus 100 $\mu$M RX871024 (triangles) in the presence of increasing concentrations of indomethacin. Following incubation for 1 h at 37°C, samples of medium were removed and the level of insulin secretion measured by radioimmunoassay. Results are mean values ± s.e.mean ($n = 16$) from two separate islet preparations.

## Discussion

Ample evidence has accumulated over recent years to support the view that pancreatic $\beta$-cells are equipped with some type of imidazoline binding site involved in control of insulin secretion (reviewed by Morgan *et al*., 1995; Molderings, 1997; Eglen *et al*., 1998). However, it has proved very difficult to identify this site by classical pharmacological methods due to the relatively low affinity of the available ligands (Chan *et al*., 1994; Ishida-Takahashi *et al*., 1996; Rustenbeck *et al*., 1997). Despite this, there is considerable functional evidence which is consistent with the existence of a $\beta$-cell imidazoline binding site (Eglen *et al*., 1998).

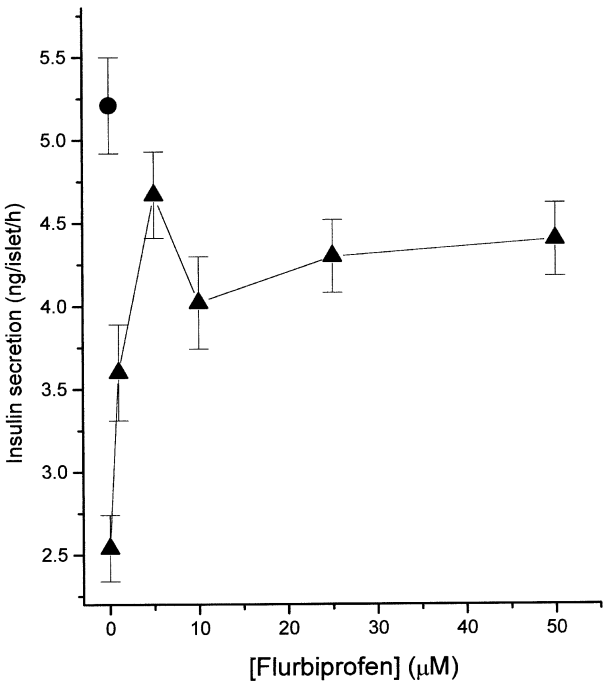


**Figure 5** Effect of flurbiprofen on inhibition of glucose-induced insulin secretion in response to RX871024. Groups of freshly isolated rat islets were incubated with 20 mM glucose alone (circle) or 20 mM glucose plus 100 $\mu$M RX871024 (triangles) in the presence of increasing concentrations of flurbiprofen. Following incubation for 1 h at 37°C, samples of medium were removed and the level of insulin secretion measured by radioimmunoassay. Results are mean values ± s.e.mean ($n = 16$) from two separate islet preparations.

**Table 4** Effects of RX871024 and efaroxan on prostaglandin $E_2$ levels in isolated rat islets

| *Test reagent* | *Prostaglandin $E_2$* (pg. 50 islets$^{-1}$) |
|---|---|
| Control | 47.3 ± 4.4 |
| Melittin (2 $\mu$g ml$^{-1}$) | 60.0 ± 3.3* |
| Efaroxan (100 $\mu$M) | 46.6 ± 4.4 |
| RX871024 (100 $\mu$M) | 75.7 ± 8.5** |

Groups of 50 rat islets were incubated in the presence of 20 mM glucose alone (control) or 20 mM glucose plus reagents. The islets were harvested and prostaglandin $E_2$ levels measured by ELISA. Data represent the mean levels of prostaglandin $E_2$ ± s.e.mean from three separate preparations of islets. *$P < 0.01$ relative to control; **$P < 0.001$ relative to control.

Among the most persuasive pieces of evidence have been the findings that the $\beta$-cell imidazoline binding site shows stereospecificity with respect to agonist potency (Chan *et al*, 1993; Mourtada *et al*., 1997) and that it displays the property of agonist-induced desensitization (Chan *et al*., 1994; 1997b, c; Chan, 1998). In addition, recent work has resulted in the synthesis of an imidazole analogue of efaroxan (KU14R) which exhibits the characteristics of an antagonist, in functional studies (Chan *et al*., 1997a–c; 1998; Eglen *et al*., 1998).

Recently, several new imidazoline insulin secretagogues have been synthesized and tested which display certain properties that do not readily accord with the suggestion that their actions are entirely attributable to imidazoline binding site agonism (Zaitsev *et al*., 1996; Efanov *et al*., 1998; Le Brigand *et al*., 1997). The present work provides direct evidence of this in the case of RX871024. The insulin secretagogue activity of this reagent was initially reported by Zaitsev *et al*. (1996) who proposed that it may be a useful therapeutic molecule for use in patients with type II diabetes. They confirmed that RX871024 shares with other, better characterized, imidazolines the ability to influence the gating of $K_{ATP}$ channels but also reported a novel action involving the direct stimulation of exocytosis in permeabilized islets.

$K_{ATP}$ channels are likely to be the major target for imidazolines acting on the pancreatic $\beta$-cell (Plant & Henquin, 1990; Chan *et al*., 1991; Dunne, 1991; Jonas *et al*., 1992; Proks & Ashcroft, 1997) and there is increasing evidence that imidazolines may interact with a channel component (Proks & Ashcroft, 1997; Monks *et al*., 1999). Thus, the finding that RX871024 can influence insulin secretion by a mechanism which is independent of the membrane potassium permeability raises a crucial issue: namely, whether the $K_{ATP}$ channel can control secretion by multiple mechanisms (only some of which involve regulation of membrane potential) or whether a second (more important?) site is involved. In this context, it may be significant that another new imidazoline insulin secretagogue, S21663, has recently been proposed to stimulate insulin secretion by interacting with a target which is separate from the efaroxan binding site (Le Brigand *et al*., 1997).

The present results extend earlier findings that RX871024 and efaroxan elicit different intracellular responses in rat pancreatic islets (Mourtada *et al*., 1998) and suggest that they do not stimulate insulin secretion by interacting with a common binding site. For example, when islets were exposed to RX871024 for 18 h they became desensitized to this reagent but remained fully responsive to efaroxan. This contrasts markedly with previous results demonstrating that desensitization induced by efaroxan leads to loss of responsiveness to a range of structurally related molecules (Chan *et al*., 1994). Furthermore, the imidazole analogue of efaroxan, KU14R, which acts antagonistically against a range of imidazolines (including efaroxan, phentolamine, RX821002 and the putative endogenous ligand, CDS; Chan *et al*., 1997a–c; 1998) failed to block responses to RX871024 in islets.

These considerations suggest that RX871024 may interact at a different site from that activated by efaroxan in $\beta$-cells. There is no direct evidence that this site is a 'receptor' and the data obtained here indicate that multiple mechanisms may contribute. In earlier work, Zaitsev and colleagues suggested that changes in protein kinase activity and redistribution of intracellular calcium may contribute to the secretory effects of RX871024 (Zaitsev *et al*., 1996; Efanova *et al*., 1998a). The latter conclusion was also reached very recently by Louchami *et al*. (1998) on the basis of isotopic flux measurements in islets treated with RX871024. We have previously confirmed the involvement of protein kinase A in the secretory response to RX871024 and have shown that, unlike efaroxan, this compound can elevate islet cyclic AMP levels (Mourtada *et al*., 1998). We now demonstrate that RX871024 can also increase islet prostaglandin production; an effect not reproduced by efaroxan. The mechanism by which islet PG synthesis is enhanced in RX871024-treated islets has not been disclosed, although it is known that some imidazole-containing drugs can divert arachidonic acid metabolism towards prostaglandin production (by inhibition of thromboxane synthesis; Robertson, 1986). Thus, it is possible that this mechanism could underlie the ability of RX871024 to increase $PGE_2$ formation in islets.

A further novel finding arising from this work is that islet PG production in response to RX871024, may influence the insulin secretory response to this agent. This conclusion emerged from studies in which the observation was made that RX871024 preferentially increases glucose-induced insulin secretion in islets isolated from fasted animals, whereas it is inhibitory in islets from fed rats. Taddayon *et al*. (1990) have shown that one major difference between islets from fed and fasted rats is that the levels of islet prostaglandins are markedly reduced in the latter. Thus, it appears that under conditions where RX871024 is able to cause significant production of islet prostaglandins, it acts to inhibit glucose-induced insulin release. By contrast, when this inhibitory effect is minimized (by reduced PG production) then a second, stimulatory, response becomes evident. On this basis it might be expected that blockade of islet PG synthesis by indomethacin or flurbiprofen would reveal a stimulatory effect of RX871024 in fresh islets. However, this was not the case; the effects of the inhibitors being restricted to abolition of the reduction in insulin secretion caused by RX871024.

An additional complication arises from consideration of the glucose-dependency of the actions of RX871024 in freshly isolated islets (Figure 4). It was evident from such studies that the inhibitory effect of the imidazoline was most evident at elevated glucose concentrations. Moreover, in the presence of sub-threshold glucose concentrations ($<4$ mM) RX871024 actually increased insulin release. This represents another important difference between RX871024 and efaroxan since the secretory response to the latter compound is only seen under stimulatory conditions (Chan & Morgan, 1990; Chan *et al*., 1991). Indeed, in the context of development of an anti-hyperglycaemic agent, the finding that RX871024 can promote insulin secretion when glucose concentrations are low, suggests that this agent may not exhibit an ideal profile of activity.

Overall, when considered in the context of previously published information on the insulin secretory effects of RX871024, the results presented here suggest that this agent exerts multiple actions in the pancreatic $\beta$-cell, not all of which can be attributed to imidazoline receptor agonism.

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