ELSEVIER

European Journal of Pharmacology 350 (1998) 251–258

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Effector systems involved in the insulin secretory responses to efaroxan and RX871024 in rat islets of Langerhans

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Received 18 December 1997; revised 23 March 1998; accepted 25 March 1998

Abstract

One component of the mechanism by which imidazoline compounds promote insulin secretion involves closure of ATP-sensitive K^+ channels in the β-cell plasma membrane. Recently, however, it has also been proposed that these compounds may exert important effects on more distal effector systems. In the present work, we have investigated the contribution played by protein kinases A and C to the insulin secretory responses of isolated rat islets of Langerhans treated with efaroxan and RX871024 (1-phenyl-2-(imidazolin-2-yl) benzimidazole). Removal of extracellular Ca^{2+} or blockade of voltage-sensitive Ca^{2+} channels prevented stimulation of insulin secretion by efaroxan, confirming a critical role for increased Ca^{2+} influx in the secretory response. By contrast, inhibition of protein kinases A or C failed to alter efaroxan-induced insulin secretion. RX871024 dose-dependently increased insulin secretion from cultured islets incubated with 20 mM glucose. This effect was unaffected by modulation of protein kinase C, but was significantly attenuated by a selective inhibitor of protein kinase A (Rp-cAMPs). Measurements of cAMP revealed that RX871024 increased the islet cAMP content by more than 3-fold; reaching values similar in magnitude to those elicited by 50 μM 3-isobutyl-1-methyl xanthine. The results reveal that neither protein kinase A nor protein kinase C is obligatory for stimulation of insulin secretion by imidazolines. However, they suggest that a rise in cAMP may contribute to the amplified secretory response observed when cultured islets are incubated with RX871024 in the presence of a stimulatory glucose concentration.

Keywords: Insulin secretion; Islet of Langerhans; Imidazoline receptor; K^+ channel; Protein kinase; cAMP

1. Introduction

It is now well-established that certain compounds bearing an imidazoline moiety act as secretagogues in the pancreatic β-cell (reviewed by Chan, 1993; Morgan et al., 1995; Molderings, 1997). These include imidazoline α_2-adrenoceptor antagonists (such as efaroxan (Berdeu et al., 1994; Chan and Morgan, 1990; Chan et al., 1991) and phentolamine (Schulz and Hasselblatt, 1988) as well as non-adrenergic drugs (e.g., antazoline (Berdeu et al., 1994, 1997) and RX871024 (1-phenyl-2-(imidazolin-2-yl) benzimidazole; Zaitsev et al., 1996)). The mechanisms underlying the response to these agents are still the subject of debate, although it is clear that a major component of their action involves the closure of β-cell ATP-sensitive K^+ (K_{ATP}) channels (Chan et al., 1991; Dunne, 1991; Ishida-Takahashi et al., 1996; Jonas et al., 1992; Plant and Henquin, 1990; Proks and Ashcroft, 1997; Zaitsev et al., 1996). It has also been suggested, however, that more distal components of the stimulus–secretion coupling pathway could be targets for imidazolines (Zaitsev et al., 1996).

Since much of the evidence obtained to date has implicated K_{ATP} channel blockade as the principal mode of action of imidazolines, it has been assumed that their ability to stimulate insulin secretion results from an increase in Ca^{2+} influx and the subsequent activation of Ca^{2+}-dependent pathways controlling exocytosis. Recently, however, results obtained with the imidazoline RX871024 have suggested that this may be an oversimplification and that other messenger systems are also activated in islets exposed to imidazolines. Thus, although RX871024 was reported to block K_{ATP} channels and stimulate Ca^{2+} influx into pancreatic β-cells, its effects on insulin secretion were attenuated by inhibitors of protein

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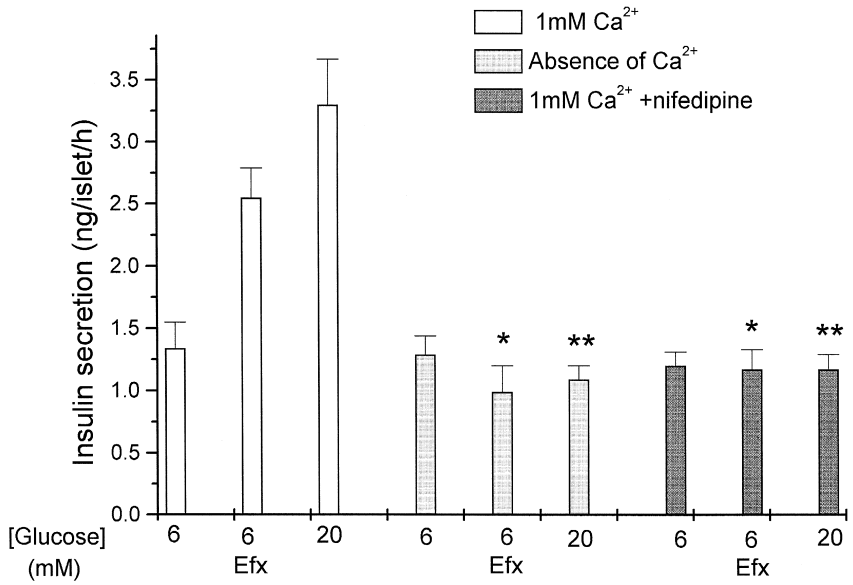

Fig. 1. Effects of Ca^{2+} depletion or nifedipine on efaroxan-induced insulin secretion from isolated rat islets of Langerhans. Groups of isolated rat islets were incubated with 6 mM glucose and efaroxan (100 μM) in the presence or absence of Ca^{2+}, or in the presence or absence of nifedipine (1 μM). After incubation at 37°C, samples of the medium were removed and assayed for insulin content. Data are presented as mean rates of insulin secretion ± S.E.M. ($n = 16$). $*$ $P < 0.01$ relative to efaroxan in the presence of 6 mM glucose and 1 mM Ca^{2+}; $**$ $P < 0.01$ relative to 20 mM in the presence of 1 mM Ca^{2+}.

kinase A and protein kinase C (Zaitsev et al., 1996). On the basis of these findings, it was proposed that activation of protein kinases A and C may form an essential component of the secretory response to RX871024. Surprisingly, activation of protein kinase A by RX871024 was not accompanied by a measureable rise in cAMP, prompting the suggestion that a more indirect mechanism of kinase activation is involved (Zaitsev et al., 1996).

In the present work, we have investigated whether the ability of the well-characterised imidazoline, efaroxan, to stimulate insulin secretion can be attributed to activation of second messenger systems which are additional to Ca^{2+} influx. We have also compared the secretory responses of islets treated with efaroxan to those mediated by RX871024 to establish whether significant differences exist in the mechanism of action of these two agents.

2. Materials and methods

2.1. Materials

4-β-Phorbol-myristate-acetate (TPA), dimethyl sulphoxide (DMSO), 3-isobutyl-1-methyl xanthine (IBMX), trichloroacetic acid, EGTA, and diazoxide were purchased

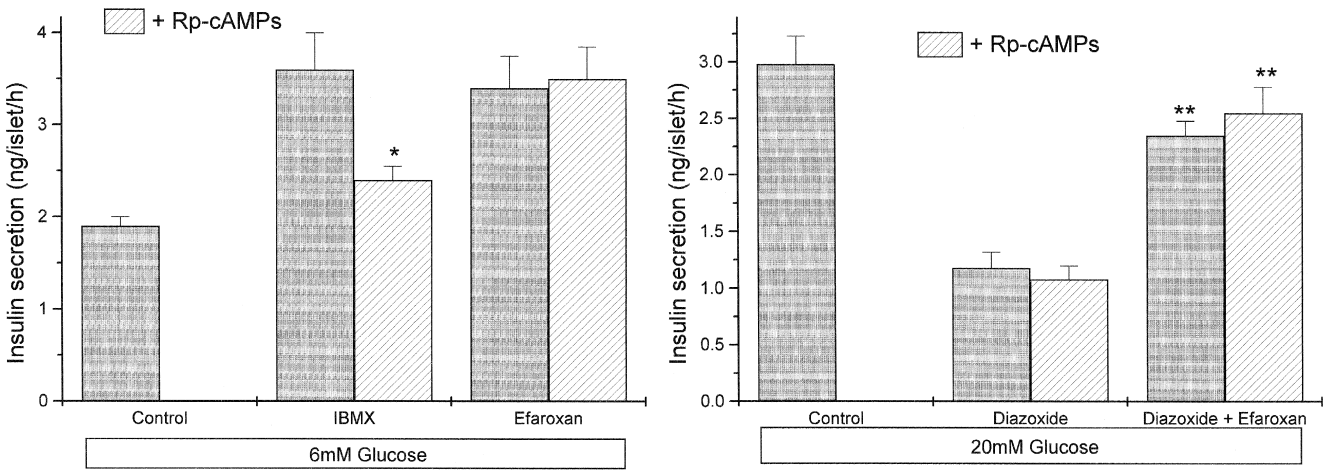

Fig. 2. Effect of Rp-cAMPs on insulin secretory responses to efaroxan. Groups of freshly isolated rat islets were preincubated for 30 min in the absence or presence of 200 μM Rp-cAMPs (hatched bars). They were then incubated for a further 1 h at 37°C in the presence of 6 mM glucose and either IBMX (50 μM) or efaroxan (100 μM) (panel A) or in the presence of 20 mM glucose, diazoxide (200 μM) and efaroxan (100 μM; Efx) (panel B). Insulin secretion was determined by RIA. Results represent mean values ± S.E.M. ($n = 16$). $*$ $P < 0.01$ relative to IBMX in the absence of Rp-cAMPs; $**$ $P < 0.001$ relative to diazoxide in the absence of efaroxan.

from Sigma (UK). The imidazolines efaroxan and RX871024 were provided by Reckitt and Colman (UK). Adenosine-3′,5′-cyclic phosphorothioate-Rp (Rp-cAMPs), Go6976, KT5720 and Ro31-8220 were purchased from Calbiochem (UK). Cyclic AMP (cAMP) ELISA system was from Amersham (UK). All other reagents were of analytical grade.

2.2. Isolation of islets of Langerhans

Islets of Langerhans were isolated by collagenase digestion (Montague and Taylor, 1968) from the pancreata of male Wistar rats (body weight 180–250 g), allowed free access to food and water. The isolation medium was a bicarbonate-buffered physiological saline solution (Gey and Gey, 1936) gassed with O_2:CO_2 (95:5) to pH 7.4 and containing 4 mM glucose and 1 mM $CaCl_2$. Isolated islets were selected by hand under a binocular dissecting microscope and were used within 2 h of isolation. In some experiments, islets were cultured in RPMI-1640 medium supplemented with 200 μg/ml streptomycin, 400 IU/ml penicillin, 10% (v/v) foetal calf serum and L-glutamine (2 mM). They were incubated in a humidified atmosphere of CO_2:air (5:95) for 18–24 h.

2.3. Insulin secretion experiments

Incubations were performed in 96-well plates. Group of three isolated islets were incubated in 200 μl of bicarbonate buffered physiological saline solution (pH 7.4) for 75 min in a humidified air: CO_2 (95:5) atmosphere at 37°C, in the presence of test reagents. Samples of the medium were removed and assayed for insulin content by radioimmunoassay. When protein kinase inhibitors were used, islets were preincubated for 30 min at 37°C in 100 μl of incubation medium supplemented with the required con-

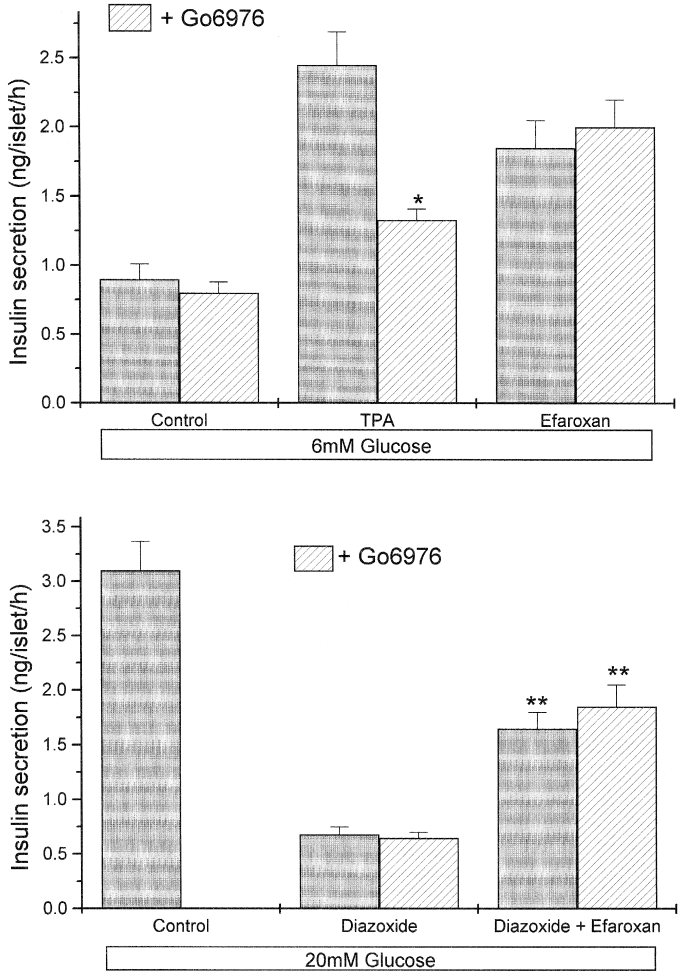

Fig. 3. Effect of Go6976 on efaroxan-induced insulin release. Groups of three isolated rat islets were preincubated for 30 min at 37°C in the absence or presence of Go6976 (1 μM; hatched bars). After this time, islets were further incubated for 1 h following the addition of appropriate glucose concentrations and test reagents. Samples of the medium were assayed for their insulin content. Results are means ± S.E.M. for 28–30 observations. Panel A: Islets were incubated in 6 mM glucose with either efaroxan (100 μM; Efx) or TPA (500 nM). Panel B: Islets were incubated in 20 mM glucose with the inclusion of 200 μM diazoxide and 100 μM efaroxan. $* P < 0.01$ relative to TPA in the absence of Go6976; $** P < 0.001$ relative to diazoxide in the absence of efaroxan.

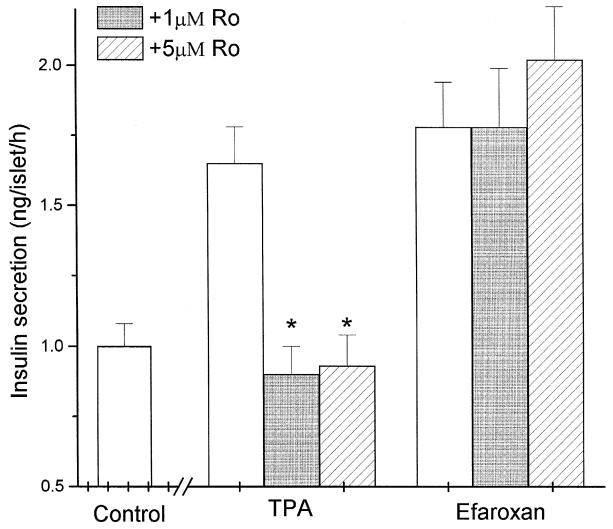

Fig. 4. Effect of Ro31-8220 on the stimulation of insulin secretion by efaroxan. Islets were preincubated in the absence or presence of Ro31-8220 (1 μM or 5 μM). After 30 min, the islets were exposed to 6 mM glucose and either TPA (500 nM) or efaroxan (100 μM) and incubated for 1 h at 37°C. After this time, the medium was sampled and its insulin content determined by RIA. Date are expressed as mean rates ± S.E.M. for 24 observations. * $P < 0.01$ relative to TPA in the absence of Ro31-8220.

centration of inhibitors and a sub-stimulatory concentration of glucose (4 mM). One hundred microliters of buffer containing test substances and the appropriate concentration of glucose was then added and the islets incubated for a further 1 h, prior to removal of samples of the medium for insulin assay.

2.4. Down-regulation of islet protein kinase C

For certain experiments, islets were incubated in the presence of the phorbol ester, TPA, in order to down-regulate protein kinase C. To achieve this, they were cultured for 18–24 h in RPMI-1640 containing either 200 nM TPA or vehicle (DMSO). After washing, the islets were incubated in batches of three for 1 h in 200 μl of incubation medium containing appropriate concentrations of glucose and test substances. At the end of the incubation period, an aliquot of the medium was removed and assayed for insulin.

2.5. Measurement of islet cAMP

Groups of 10 isolated cultured rat islets were incubated for 15 or 60 min at 37°C in 500 μl of incubation medium containing 6 mM glucose and test substances. At the end of the incubation time, the tubes were centrifuged briefly ($500 \times g$; 15 s) and the islet pellets were extracted with 6% v/v trichloroacetic acid and lyophilised. The extracts were assayed for cAMP using a commercial ELISA kit, according to the manufacturer's instructions.

2.6. Statistical analysis

Data are presented as mean values ± S.E.M. Analysis of variance was used to assess levels of statistical significance between different groups. Differences were considered significant when $P < 0.05$ or less.

3. Results

3.1. Effects of Ca^{2+} depletion or Ca^{2+} channel blockade on efaroxan-induced insulin secretion from isolated rat islets of Langerhans

As expected from earlier studies (Chan and Morgan, 1990; Chan et al., 1991) the imidazoline efaroxan (100 μM) significantly potentiated insulin secretion from islets incubated with 6 mM glucose (Fig. 1). However, when the incubation was performed in Ca^{2+} free medium, efaroxan failed to increase insulin secretion, and the insulin secretory response to 20 mM glucose was also abolished (Fig. 1). Addition of the L-type Ca^{2+} channel antagonist nifedipine, to islets incubated in medium containing 1 mM Ca^{2+} also abrogated the insulin secretory response to both efaroxan and 20 mM glucose.

3.2. Effects of selective inhibitors of protein kinases on efaroxan-induced insulin secretion

In order to test the hypothesis that activation of protein kinases A and C may participate in the insulin secretory response to imidazolines, selective inhibitors of these kinases were employed. In initial experiments the purported inhibitor of protein kinase A, KT5720, was employed. This agent is reported to be a selective and highly potent ($K_i \sim 50$ nM) inhibitor of protein kinase A (Gadbois et al., 1992), but, in control experiments, the secretory response to the broad spectrum phosphodiesterase inhibitor IBMX

Table 1
Effects of efaroxan and RX871024 on glucose-induced insulin secretion from cultured rat islets

	Insulin secretion (ng/islet per h)		
	No addition	100 μM efaroxan	100 μM RX871024
6 mM glucose	2.6 ± 0.36	4.4 ± 0.33[a]	4.0 ± 0.40[a]
20 mM glucose	4.2 ± 0.41[a]	4.3 ± 0.49	5.9 ± 0.39[b]

Groups of islets were cultured for 18 h in RPMI-1640 then washed and incubated for 60 min at 37°C with either 6 mM or 20 mM glucose and in the presence or absence of efaroxan or RX871024, as shown. Samples of medium were removed and assayed for insulin secretion by radioimmunoassay. Results are mean values for insulin secretion ± S.E.M. from 24 observations.
[a] $P < 0.001$ relative to 6 mM glucose control.
[b] $P < 0.001$ relative to 20 mM glucose control.

Table 2
Effects of protein kinase inhibitors on the insulin secretory response to RX871024

[Glucose] (mM)	[Kinase inhibitor]	RX871024 (100 μM)	Insulin secretion (ng/islet per h)
6	−	−	0.67 ± 014
20	−	−	1.18 ± 0.11[a]
20	−	+	1.79 ± 0.12[a]
20	1 μM Go6976	+	1.83 ± 0.21[a]
20	5 μM Ro31-8220	+	1.71 ± 0.14[a]
20	200 μM Rp-cAMPs	+	0.79 ± 0.11[b]

Isolated rat islets were cultured for 18–24 h then washed and treated for 30 min at 37°C with various different kinase inhibitors as indicated. The islets were then exposed to 20 mM glucose and RX871024 (100 μM) for 1 h. At the end of the incubation time, an aliquot of the medium was taken for measurement of insulin release by RIA. Values are means ± S.E.M. for 16 observations.
[a] $P < 0.01$ relative to 20 mM glucose alone.
[b] $P < 0.01$ relative to RX871024 in the absence of Rp-cAMPs.

was unaffected by KT5720 (data not shown) suggesting that, despite its high potency in some systems, KT5720 does not function as an effective protein kinase A inhibitor in intact rat islets. Thus, a second specific protein kinase A inhibitor (Rp-cAMPs; Dostmann and Taylor, 1991) was investigated.

By contrast with the response to KT5720, IBMX-induced insulin secretion was significantly diminished upon inclusion of Rp-cAMPs (200 μM) in the islet incubation medium (Fig. 2a). However, under identical conditions, insulin secretion in response to efaroxan was not affected (Fig. 2a), nor was the ability of efaroxan to antagonise diazoxide-induced inhibition of insulin secretion (Fig. 2b).

To investigate a possible role for protein kinase C in the effects of efaroxan, experiments were performed using two different potent and selective protein kinase C inhibitors; Go6976 (IC_{50} = 7.9 nM) and Ro31-8220 (IC_{50} = 10 nM). TPA (500 nM) was used as a control and stimulated insulin secretion from islets incubated in the presence of 6 mM glucose. This response was significantly reduced in the presence of Go6976 (Fig. 3a) or Ro31-8220 (Fig. 4a). By contrast, neither inhibitor modified the secretory response to efaroxan in either the absence (Fig. 3a, Fig. 4a) or presence (Fig. 3b, Fig. 4b) of diazoxide.

In parallel with these studies, we also investigated the effects of kinase inhibitors on the secretory response to a second imidazoline, RX871024. The effects of this agent on insulin secretion have been described only recently (Zaitsev et al., 1996) and the proposal advanced that this compound may exert its influence at more than one level within the stimulus–secretion coupling pathway. In accordance with previous work, RX871024 significantly increased the rate of insulin release from cultured rat islets incubated with either 6 mM or 20 mM glucose whereas efaroxan was effective at 6 mM glucose but did not potentiate the response to 20 mM glucose (Table 1). The ability of RX871024 to enhance glucose-induced insulin secretion was not modified by the protein kinase C inhibitors Go6976 and Ro31-8220 (Table 2) under conditions when they attenuated the secretory response to the phorbol ester, TPA. By contrast, (and at variance with the response to efaroxan; Fig. 2) the protein kinase A inhibitor Rp-cAMPs significantly attenuated RX871024-induced insulin secretion (Table 2).

Table 3
Effect of down-regulation of protein kinase C on insulin secretory responses to efaroxan and RX871024

[Glucose] (mM)	Additions to the medium	Control islets	Down-regulated islets
6	None	0.62 ± 0.15	0.73 ± 0.11
6	500 nM TPA	1.74 ± 0.20[a]	0.77 ± 0.11
6	100 μM Efaroxan	2.0 ± 0.20[a]	1.86 ± 0.16[b]
20	None	2.16 ± 0.24[a]	2.0 ± 0.16[b]
20	100 μM RX871024	2.82 ± 0.14[c]	3.24 ± 0.21[c]
20	200 μM Diazoxide	0.85 ± 0.11	0.93 ± 0.15
20	200 μM Diazoxide + 100 μM efaroxan	2.10 ± 0.29[d]	1.79 ± 0.18[d]

Groups of isolated rat islets were cultured for 18 h in control medium ('Control islets') or in medium supplemented with TPA (200 nM—'Down-regulated islets'). Islets were washed and incubated for 75 min at 37°C under the indicated conditions. At the end of the incubation, samples of the medium were removed and their insulin content determined. Data are presented as mean values ± S.E.M. for 24 observations.
[a] $P < 0.001$ relative to 6 mM glucose alone in control islets.
[b] $P < 0.001$ relative to 6 mM glucose alone in down-regulated islets.
[c] $P < 0.01$ relative to 20 mM glucose in absence of RX871024.
[d] $P < 0.001$ relative to diazoxide in the absence of efaroxan.

3.3. Effects of protein kinase C down-regulation on insulin secretory responses to efaroxan and RX871024

To evaluate further a possible role for protein kinase C in the stimulation of insulin secretion by efaroxan and RX871024, the effects of both drugs were tested in islets which had been cultured in the presence of 200 nM TPA. This procedure has been used widely as a means to down-regulate protein kinase C activity in islets (Gembal et al., 1993; Persaud et al., 1989, 1991). Islets cultured with vehicle alone (DMSO) served as controls. In islets cultured in the presence of DMSO, TPA strongly amplified the secretory response to 6 mM glucose (Table 3). The secretory responses to 20 mM glucose, diazoxide and efaroxan were also normal in these control islets (Table 3). After overnight culture with TPA (200 nM) 20 mM glucose was still an effective secretagogue and this response was strongly inhibited by diazoxide. However, in the down-regulated islets, the acute amplification of insulin secretion by TPA was abolished (Table 3). By contrast, down-regulation of protein kinase C did not prevent the secretory responses to either efaroxan or RX871024 in either the presence or absence of diazoxide (Table 3).

3.4. Effect of RX871024 on islet cAMP accumulation

Since the stimulatory effect of RX871024 on insulin secretion was attenuated by the protein kinase A inhibitor Rp-cAMPs (Table 2) we considered it important to investigate the effect of this drug on islet cAMP production. The cAMP content of cultured rat islets incubated for 15 min in medium containing 6 mM glucose was increased slightly by IBMX but was not altered by RX871024, whereas both agents significantly increased insulin release over this time period (Table 4). By contrast, over a 60 min incubation period, RX871024 provoked a marked increase in both insulin secretion and the islet content of cAMP (Table 4). Under these conditions, the rise in cAMP induced by RX871024, was not significantly different from that seen in islets treated with IBMX (Table 4).

4. Discussion

A substantial body of evidence now exists suggesting that imidazoline insulin secretagogues cause membrane depolarisation in pancreatic β-cells (Chan et al., 1991; Dunne, 1991; Ishida-Takahashi et al., 1996; Jonas et al., 1992; Plant and Henquin, 1990; Proks and Ashcroft, 1997; Zaitsev et al., 1996) leading to an increase in Ca^{2+} influx. This was recently confirmed using fluorescent Ca^{2+} indicator dyes in experiments which also suggested that the Ca^{2+} influx pathway involves voltage-sensitive Ca^{2+} channels (Shepherd et al., 1996; Rustenbeck et al., 1995). We now demonstrate that, in the case of efaroxan, removal of extracellular Ca^{2+} or blockade of L-type (voltage-sensitive) Ca^{2+} channels by nifedipine leads to abolition of the insulin secretory response. This suggests that the stimulus–secretion coupling pathway activated by efaroxan is crucially dependent on changes in Ca^{2+} influx. However, it can also be inferred that a rise in cytosolic Ca^{2+} concentration may not be the sole determinant of the final secretory response to efaroxan since the imidazoline does not act as an initiator of secretion in the absence of glucose (Chan et al., 1991; Chan, 1993). Thus, it seems probable that a further signal, generated in response to metabolic changes in the β-cell, is also required for stimulation of insulin secretion.

Recently, Zaitsev et al. (1996) have reported that another imidazoline, RX871024, activates at least two intracellular effector systems which are separate from, and distal to, an increase in Ca^{2+} influx in rat islets. This raises the interesting possibility that imidazolines may have intracellular targets which are separate from the K_{ATP} channel. The nature of these sites must await the outcome of further studies but Zaitsev et al. (1996) presented evidence that their activation is accompanied by increases in the activities of protein kinase A and members of the protein kinase C family. We now confirm that a highly selective, cell permeable antagonist of protein kinase A (Rp-cAMPs) is able to attenuate the stimulation of insulin secretion by RX871024 (Table 2). This agent also reduced

Table 4
Effect of RX871024 on islet cAMP formation and insulin secretion

[Glucose] (mM)	Incubation time (min)	Test reagents	Insulin secretion (ng/islet)	cAMP (fmol/10 islets)
6	15	–	0.31 ± 0.06	146 ± 34
6	15	50 μM IBMX	0.63 ± 0.07[a]	282 ± 40[a]
6	15	100 μM RX871024	0.56 ± 0.06[a]	154 ± 37
6	60	–	2.10 ± 0.28	211 ± 29
6	60	50 μM IBMX	ND	593 ± 98[a]
6	60	100 μM RX871024	3.95 ± 0.33[a]	471 ± 85[a]

Isolated rat islets were cultured for 18–24 h. They were washed and incubated for 15 or 60 min at 37°C in the presence of 6 mM glucose and test reagents as shown, prior to measurement of either insulin secretion by radioimmunoassay or cAMP levels by ELISA. Data represent mean values ± S.E.M. from three islet preparations.

[a] $P < 0.01$ relative to 6 mM glucose alone.

ND, not determined.

the secretory response to the phosphodiesterase inhibitor IBMX, confirming that its actions are likely to be mediated by blockade of protein kinase A. By contrast, secretory responses to efaroxan were not affected by Rp-cAMPs (Fig. 2). This was true for both the direct potentiation of glucose-induced insulin secretion (which could reflect activation of multiple intracellular effectors) and reversal of the inhibitory action of diazoxide (which may be principally related to blockade of K_{ATP} channels) by efaroxan (Fig. 2). Thus, there appears to be an important difference in the secretory responses induced by efaroxan and RX871024 in rat islets and that only the latter compound promotes activation of protein kinase A.

In mammalian cells, activation of protein kinase A is invariably associated with a rise in cAMP. Thus, one explanation for the difference in sensitivity between efaroxan and RX871024 to Rp-cAMPs, would be that RX871024 causes an increase in islet cAMP levels. In support of this, we observed that, over a 60-min incubation period, RX871024 provoked a substantial increase in islet cAMP levels (Table 4) which was equivalent in magnitude to that elicited by 50 μM IBMX. This effect was not seen within a 15-min period of incubation suggesting that it is a relatively slowly developing effect. It is noteworthy that RX871024 caused a rise in insulin secretion within 15 min, despite failing to increase cAMP within this period (Table 4). This suggests that the ability of RX871024 to promote insulin secretion does not depend entirely on a rise in cAMP (and probably results primarily from blockade of K_{ATP} channels; Zaitsev et al., 1996) but that a rise in cAMP does play a role in the potentiation of glucose-induced insulin secretion by RX871024 during longer term incubations. Since previous studies have established that efaroxan does not raise islet cAMP during 60 min of incubation (Chan et al., 1991) this is consistent with the present finding that its effects on insulin secretion were insensitive to Rp-cAMPs. This difference may also explain why RX871024 can further enhance insulin secretion in response to a maximal concentration of glucose (a characteristic shared by other agents which raise islet cAMP; Sharp, 1979; Hughes and Ashcroft, 1992) whereas efaroxan does not (Table 1).

We are not able to identify the precise mechanism by which RX871024 causes a rise in islet cAMP but, since certain other imidazoline derivatives can act as cAMP-phosphodiesterase inhibitors (Brackeen et al., 1995; Michel et al., 1995) it seems possible that this mechanism could account for the response. It is unlikely that the rise in cAMP was mediated by Ca^{2+}-dependent activation of adenylate cyclase since the response was not seen with efaroxan.

By contrast with the effect of blockade of protein kinase A, manoeuvres designed to inhibit activation of islet protein kinase C isoforms failed to influence the secretory response to either efaroxan or RX871024. Thus, whereas the selective inhibitors Go6976 and Ro31-8220 attenuated the secretory response to the phorbol ester TPA (a direct activator of protein kinases C; Figs. 3 and 4; Table 3) they did not alter responses mediated by efaroxan or RX871024. Moreover, culture of islets in the presence of TPA to down-regulate protein kinase C activity, abolished the secretory response to TPA during a subsequent incubation (Table 3) but failed to prevent either the potentiation of glucose-induced insulin secretion or reversal of the inhibitory effects of diazoxide, by efaroxan or RX871024.

These data provide strong evidence that protein kinase C activation does not contribute to the enhancement of insulin secretion seen when islets are treated with imidazolines. This conclusion is at variance with that of Zaitsev et al. (1996) who reported that the protein kinase C inhibitor, Calphostin C, inhibited insulin secretion induced by RX871024. One possible explanation for this difference may lie in the finding that although Calphostin C is a potent inhibitor of protein kinase C ($IC_{50} \sim 50$ nM) it can also inhibit other protein kinases (including Ca^{2+} and cAMP-dependent enzymes) at higher concentrations (Kobayashi et al., 1989). However, we cannot formally exclude the possibility that Calphostin C may act as an inhibitor of an isoform of protein kinase C which is insensitive to Go6976 and Ro31-8220 and not subject to down-regulation by TPA but stimulated by RX871024.

Overall, therefore, the present results confirm that the ability of imidazolines to promote insulin secretion is critically dependent on membrane depolarisation and an increase in Ca^{2+} influx through L-type Ca^{2+} channels. The results do not exclude the possibility that imidazolines may exert effects on intracellular targets that are distal to the K_{ATP} channel but these sites remain to be identified.

Acknowledgements

We thank the Wellcome Trust and BBSRC for financial support of this work.

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