British Journal of Pharmacology (1996) 119, 911-916

Elevation of cytosolic calcium by imidazolines in mouse islets of Langerhans: implications for stimulus-response coupling of insulin release

Ruth M. Shepherd, Molly N. Hashmi, Charlotte Kane, Paul E. Squires & [1]Mark J. Dunne

Department of Biomedical Science, University of Sheffield, Alfred Denny Building, Western Bank, Sheffield, S10 2TN

1 Microfluorimetry techniques with fura-2 were used to characterize the effects of efaroxan (200 μM), phentolamine (200-500 μM) and idazoxan (200-500 μM) on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse isolated islets of Langerhans.

2 The imidazoline receptor agonists efaroxan and phentolamine consistently elevated cytosolic Ca^{2+} by mechanisms that were dependent upon Ca^{2+} influx across the plasma membrane; there was no rise in $[Ca^{2+}]_i$ when Ca^{2+} was removed from outside of the islets and diazoxide (100-250 μM) attenuated the responses.

3 Modulation of cytosolic $[Ca^{2+}]_i$ by efaroxan and phentolamine was augmented by glucose (5-10 mM) which both potentiated the magnitude of the response and reduced the onset time of imidazoline-induced rises in $[Ca^{2+}]_i$.

4 Efaroxan- and phentolamine-evoked increases in $[Ca^{2+}]_i$ were unaffected by overnight pretreatment of islets with the imidazolines. Idazoxan failed to increase $[Ca^{2+}]_i$ under any experimental condition tested.

5 The putative endogenous ligand of imidazoline receptors, agmatine (1 μM-1 mM), blocked K_{ATP} channels in isolated patches of β-cell membrane, but effects upon $[Ca^{2+}]_i$ could not be further investigated since agmatine disrupts fura-2 fluorescence.

6 In conclusion, the present study shows that imidazolines will evoke rises in $[Ca^{2+}]_i$ in intact islets, and this provides an explanation to account for the previously described effects of imidazolines on K_{ATP} channels, the cell membrane potential and insulin secretion in pancreatic β-cells.

Keywords: Mouse islets; Ca^{2+}; imidazoline; fura-2; efaroxan; idazoxan; phentolamine; tolbutamide; agmatine; K_{ATP} channels; I-receptor

Introduction

The pharmacological manipulation of ATP-sensitive potassium (K_{ATP}) channels in insulin-secreting cells has important implications for our understanding of stimulus-response coupling events. These channels establish the resting cell membrane potential in β-cells, and close following glucose metabolism to initiate a depolarization of the cell, voltage-gated Ca^{2+} influx and insulin secretion (see Dunne & Petersen, 1991). K_{ATP} channels are inhibited by sulphonylureas, such as tolbutamide and glibenclamide, which mimic the effects of glucose stimulation and initiate insulin secretion (see Ashcroft & Ashcroft, 1992). Conversely, agonists of K_{ATP} channels such as diazoxide, inhibit insulin release by hyperpolarizing the membrane potential and preventing the influx of Ca^{2+} by voltage-dependent events (Dunne & Petersen, 1991).

Certain imidazoline derivatives such as the I_1 agonist efaroxan and the I_1/I_2 agonist phentolamine are also insulin secretagogues (Ahrén & Lundquist, 1985; Schulz & Hasselblatt, 1989) and inhibitors of ATP-sensitive potassium channels (Plant & Henquin, 1990; Chan *et al.*, 1991; Dunne, 1991; Jonas *et al.*, 1992). The underlying mechanisms responsible for the link(s) between electrical activity and insulin release are incompletely understood. Indeed, the I_2 agonist idazoxan inhibits K_{ATP} channels (Chan *et al.*, 1991), but does not promote secretion of insulin (Chan & Morgan, 1990; Chan *et al.*, 1991). In this study we examined the effects of phentolamine, efaroxan, tolbutamide and idazoxan, on cytosolic Ca^{2+} homeostasis in intact glucose-responsive islets of Langerhans of the mouse.

[1] Author for correspondence.

Methods

Experimental procedures

Microfluorimetry with fura-2 was used to monitor changes in cytosolic Ca^{2+} levels (Grynkiewicz *et al.*, 1985). Mouse islets were prepared by use of established methods (Gilon & Henquin, 1992). Briefly, pancreata from five MF1 mice were washed and finely minced with scissors in a Krebs solution containing (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24, 4-(2-hydroxyethoyl)-1-piperazineethane-sulphonic acid (HEPES) 5 and glucose 10. The medium was gassed for 20 min with 95% O_2/5% CO_2 and the pH set to 7.35 with NaOH. Bovine serum albumin was then added at 1 mg 1 ml^{-1}. Islets were liberated from the pancreas first by collagenase digestion (7 mg, Serva collagenase, Basle, Switzerland) for 7-12 min at 37°C and, then by microdissection to prevent damage of the islet cell capsule. Whole islets were cultured for between 18 and 72 h in RPMI 1640 medium (Gibco, UK) supplemented with 10% foetal calf serum, and 100 u/0.1 mg per ml penicillin/streptomycin, respectively, on poly-D-lysine (50 μg ml^{-1}) treated coverslips. The islets were loaded with 0.5 μM fura-2-AM (Sigma) in 1 ml of RPMI 1640 medium for 8 min at 37°C. The composition of basic perifusion medium was (mM): NaCl 137, KCl 5.36, $MgSO_4$ 0.81, Na_2PO_4 0.34, KH_2PO_4 0.44, $NaHCO_3$ 4.17, HEPES 10 and $CaCl_2$ 1.26. This medium was gassed with air for 20 min, glucose was added as 2.02 mM and pH set to 7.4 with NaOH. For studies involving the removal of external Ca^{2+}, $CaCl_2$ was omitted and 1 mM ethylenebis-(oxonitrilo) tetraacetate (EGTA) added (Squires *et al.*, 1994). All experiments were carried out at 37°C. For electrophysiology and microfluorimetry studies of isolated β-cells, tissue was prepared as previously described (Findlay *et al.*, 1985). Single K_{ATP}

channel currents were recorded by use of patch-clamp techniques (Hamill *et al.*, 1981) at 0 mV voltage-clamp with the cell-free inside-out patch configuration with a Na^+-rich solution in the pipette; composition (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.13, glucose 2.5, HEPES 10 and $CaCl_2$ 2.5 (pH 7.4) and a K^+-rich solution in the bath; composition (mM): KCl 140, NaCl 10, $MgCl_2$ 1.13, glucose 2.5, HEPES 10 and EGTA 1 (pH 7.2) (Lebrun *et al.*, 1996). For experiments involving down-regulation of imidazoline receptors, procedures were followed from Chan *et al.* (1993). All compounds were added to the medium from dimethylsulphoxide stocks; tolbutamide 100 μM, thapsigargin 100 mM or from distilled water stocks of efaroxan 200 mM, idazoxan 500 mM (Sigma). Phentolamine myslate (Rogitine, Ciba, Switzerland) was added as a saline stock solution.

Quantification of data

Microfluorimetry; (i) an *in vitro* calibration procedure (Grynkiewicz *et al.*, 1985) was used to estimate changes in $[Ca^{2+}]_i$, (ii) basal values of $[Ca^{2+}]_i$ were estimated in the presence of 2 mM glucose, (iii) peak changes in cytosolic Ca^{2+} ($\Delta[Ca^{2+}]_i\uparrow$) are presented as the difference between basal $[Ca^{2+}]_i$ and the mean rise in $[Ca^{2+}]_i$, (iii) 't_{basal}' – time in seconds from the point of removal of an agonist to the return to basal Ca^{2+}, and (iv) '$t_{1/2}$ onset' – time in seconds from application of an agonist to the attainment of half the mean rise in $[Ca^{2+}]_i$. Electrophysiology; changes in K_{ATP} channel open-state probability were estimated by published procedures (Dunne, 1991; Lebrun *et al.*, 1996).

Results

Imidazoline-evoked changes in cytosolic Ca^{2+}

Islets of Langerhans were isolated on 14 occasions from batches of 5 mice. The mean basal cytosolic calcium level was established to be 57±3 nM (n=92) (mean±s.e.mean). Figures 1, 2 and Table 1 summarise the responsiveness of isolated islets to a number of agonists. Glucose (10 mM, n=12 and 15 mM, n=11) typically induced a rise in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) that was associated with fast oscillations, on top of slow waves of cytosolic Ca^{2+}, superimposed upon a plateau of elevated $[Ca^{2+}]_i$ (Figure 1). Quantitatively, in the presence of 2 mM glucose the responses of the islets to efaroxan (200 μM, n=7) and tolbutamide (100 μM, n=6) were similar; both elevated $[Ca^{2+}]_i$ in a readily reversible manner and each initiated the appearance of fast Ca^{2+} oscillations on a number of occasions (Figure 2 and Table 1). Phentolamine (500 μM, n=5), on the other hand, produced a rise in $[Ca^{2+}]_i$ that was comparable in magnitude with the other agonists, but the time-course of the response was markedly different with a long delay in both the onset and reversibility times (Figure 2, Table 1). At lower concentrations efaroxan was also effective (Dunne *et al.*, 1995), but there were no actions of phentolamine on $[Ca^{2+}]_i$ at concentrations <500 μM (n=6). Idazoxan (200–500 μM) failed to evoke any significant increase in $[Ca^{2+}]_i$ (Figure 2, n=7).

Mechanism of action; Ca^{2+} influx vs. Ca^{2+} mobilization

Several complementary protocols were undertaken to investigate the mechanisms that underlie Ca^{2+} increases; (i) islets

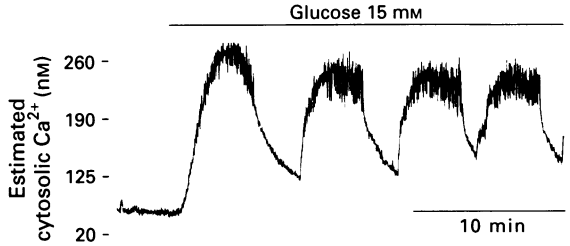

Figure 1 Effect of glucose on cytosolic Ca^{2+} levels in mouse islets. The glucose concentration of the perifusion medium was raised from 2 to 15 mM as indicated. The trace is representative of 11 similar experiments.

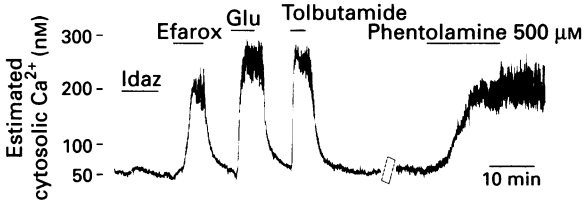

Figure 2 Ca^+ profiles of an islet stimulated by imidazolines and insulin secretagogues. The islets were exposed to idazoxan (Idaz) 200 μM, efaroxan (Efarox) 200 μM, phentolamine, glucose (Glu) 15 mM and tolbutamide 100 μM as indicated. The trace is representative of 6 similar complete experiments.

Table 1 Agonist induced changes in $[Ca^{2+}]_i$

Agonist	$\Delta[Ca^{2+}]_i\uparrow$ *(nM)*	*t½ onset (s)*	t_{basal} *(s)*	$[Ca^{2+}]_i$ *oscillations*
Glucose 15 mM	139±14	199±40	467±25	9/9
Glucose 2 mM + efaroxan	88±10	130±17	316±39	4/15
Glucose 2 mM + phentolamine	118±10	1078±90	*	11/12
Glucose 2 mM + idazoxan	#			0/7
Glucose 2 mM + tolbutamide	143±14	21±4	461±48	4/7
Diazoxide + efaroxan	35±5	299±55	417±97	2/5
In 6 other islets no Δ $[Ca^{2+}]_i$				
Diazoxide + phentolamine	73±7	1542±153	**	5/6
In 6 other islets no Δ $[Ca^{2+}]_i$				
Glucose 5 mM + efaroxan	132±21	33±6	–	6/6
Glucose 5 mM + phentolamine	99±17	501±79	**	5/5
Glucose 10 mM + phentolamine	129±22	201±82	**	6/6

Agonist concentrations: phentolamine 500 μM, efaroxan 200 μM, idazoxan 200 μM, tolbutamide 100 μM and diazoxide 100–250 μM. All values are expressed as mean±s.e.mean. *No recovery over a 10–20 min period; **No recovery over a 15–20 min period; #No response; –not quantifiable.

were pre-exposed to a Ca^{2+}-free medium and the agonist added, (ii) external Ca^{2+} was acutely removed during an agonist-induced rise in cytosolic Ca^{2+}, (iii) interactions between agonists and the K_{ATP} channel activator diazoxide were studied, and (iv) effects were examined in combination with the intracellular Ca^{2+} ATPase inhibitor thapsigargin.

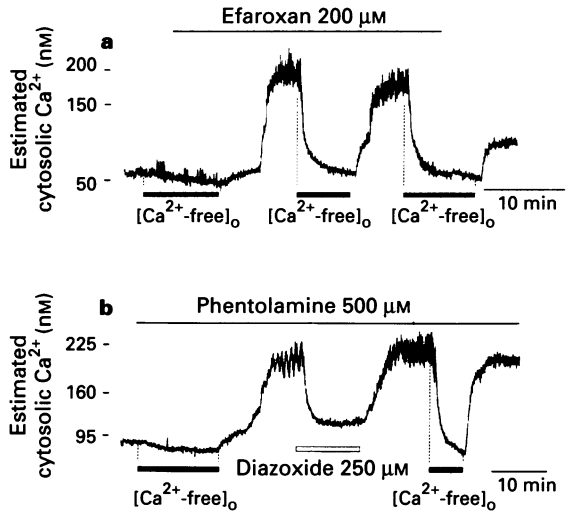

Figure 3 Efaroxan and phentolamine require extracellular Ca^{2+} to raise $[Ca^{2+}]_i$. Traces in (a) and (b) are from separate islets and show the $[Ca^{2+}]_i$ profiles for efaroxan and phentolamine, respectively. A Ca^{2+}-free medium or diazoxide (250 μM) were applied as indicated. Traces are representative of 5 and 6 islets, respectively.

Our findings show that in the absence of external Ca^{2+} neither efaroxan ($n=4$, Figure 3a) nor phentolamine ($n=6$, Figure 3b) were able to initiate a rise in $[Ca^{2+}]_i$ and that acute removal of external Ca^{2+} in the presence of either agonist completely attenuated the responses (efaroxan ($n=5$), phentolamine ($n=6$), Figure 3). The K_{ATP} channel opener diazoxide was used to investigate the involvement of voltage-dependent Ca^{2+} influx since it produces a hyperpolarization of the cell membrane potential and inhibits glucose-induced Ca^{2+} responses (Dunne *et al.*, 1990). In the presence of 250 μM diazoxide there was little effect of either efaroxan or phentolamine on $[Ca^{2+}]_i$ ($n=7/10$), and diazoxide attenuated agonist-induced rises in cytosolic Ca^{2+} ($n=9$, Figure 3b). However, when the diazoxide concentration was lowered to 100 μM, both phentolamine and efaroxan increased $[Ca^{2+}]_i$ (Figure 4), despite the fact that the responses were generally smaller and the time of onset considerably slower (Table 1). Finally, the involvement of Ca^{2+} release from cytoplasmic Ca^{2+} stores was studied by examining the effects of efarozan in the presence of thapsigargin, which failed to prevent agonist-evoked increases in $[Ca^{2+}]_i$ ($n=5$, not shown).

Glucose dependency

When the responses of islets to efaroxan and phentolamine were studied in the presence of 5–10 mM glucose, two predominant effects were noted; first the magnitude of the imidazoline-induced rise in $[Ca^{2+}]_i$ was enhanced, and secondly there was a marked decrease in the onset time of the cytosolic Ca^{2+} rise (efaroxan $n=6$, phentolamine, $n=11$, Figure 5, Table 1). These effects are consistent with glucose causing an augmentation of the imidazoline-induced rise in $[Ca^{2+}]_i$.

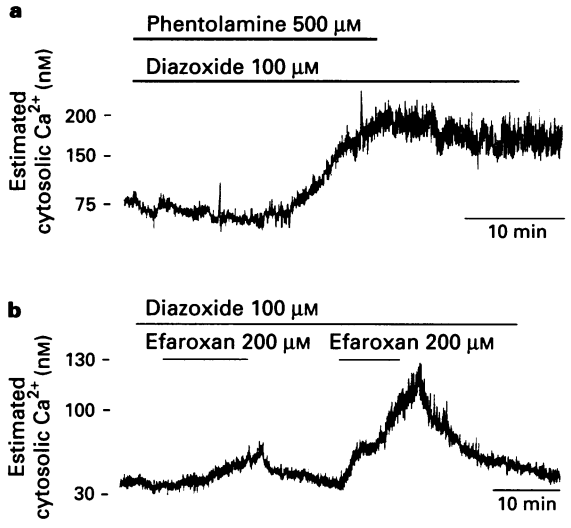

Figure 4 Effects of the hyperpolarizing K_{ATP} channel agonist diazoxide on the imidazoline-induced rises in $[Ca^{2+}]_i$. Diazoxide 100 μM did not prevent the elevation of $[Ca^{2+}]_i$ by phentolamine (a) or efaroxan (b) on 6/6 and 5/11 occasions, respectively.

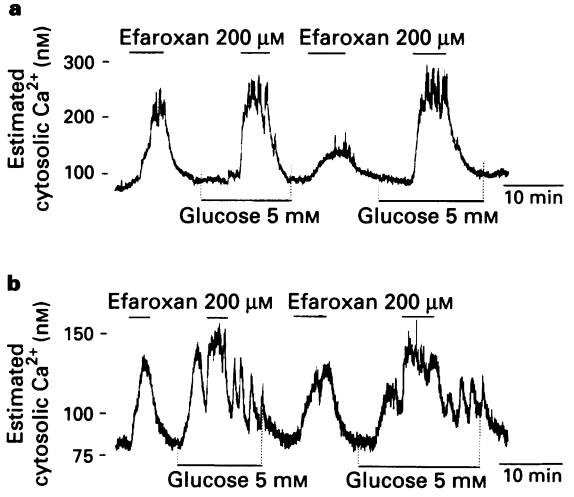

Figure 5 Potentiation of the efaroxan-induced rises in $[Ca^{2+}]_i$ by glucose. When efaroxan was applied in the presence of 5 mM glucose there was a marked augmentation of the response, and this also occurred when glucose did not raise $[Ca^{2+}]_i$, $n=2/6$ islets (a).

Table 2 Ca^{2+} responses from a range of agonists following overnight pretreatment of islets with either efaroxan or phentolamine

	Efaroxan (50–500 μM) Pretreatment				*Phentolamine (200–500 μM) Pretreatment*			
	$\Delta[Ca^{2+}]_i\uparrow$ (nM)	t½ *onset* (s)	t_{basal} (s)	n	$\Delta[Ca^{2+}]_i\uparrow$ (nM)	t½ *onset* (s)	t_{basal} (s)	n
Efaroxan	52±14	225±53	266±54	6	97±23	32±8	385±42	5
Phentolamine	99±14	542±45	*	6	91±14	457±55	726±84	8
Glucose	125±22	134±34	451±32	6	86±14	159±82	507±42	6
Tolbutamide	133±23	24±5	378±32	6	91±18	27±5	443±39	6
Idazoxan	#			6	#			6

Agonist concentrations: glucose 15 mM, phentolamine 500 μM, efaroxan 200 μM, idazoxan 200 μM and tolbutamide 100 μM. All values are expressed as mean±s.e.mean. *No recovery over 10–20 min period; #No response.

Receptor down-regulation

The actions of the imidazoline agonists efaroxan and phentolamine can be down-regulated by long-term exposure of insulin-secreting cells to the agonists (Chan *et al.*, 1993). Table 2 and Figure 6 show a summary of the findings related to imidazoline receptor down-regulation on $[Ca^{2+}]_i$ signalling. Pre-exposure of islets to either phentolamine or efaroxan for 18 h at 50 μM, 200 μM and 500 μM did not significantly impair Ca^{2+} signalling events in response to phentolamine, efaroxan, tolbutamide or glucose.

Effects of agmatine on ion channels and Ca^{2+} signalling in β-cells

Agmatine has been suggested to be the endogenous agonist for imidazoline receptors (Li *et al.*, 1994). As agmatine will promote insulin release from β-cells (Sener *et al.*, 1989) we investigated the mechanisms underlying this effect. By use of patch-clamp techniques we found that agmatine directly inhibits K_{ATP} channels in a concentration-dependent manner, and that the effects are sustained and reversible ($n=7$, Figure 7). However, the actions of agmatine on $[Ca^{2+}]_i$ homeostasis could not be fully assessed since in β-cells agmatine interferes with the fluorescence of fura-2 ($n=6$) (Figure 8).

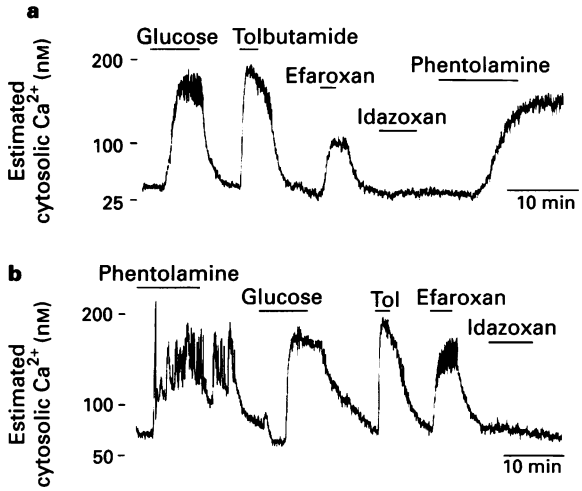

Figure 6 Pretreatment of islets with imidazolines: (a) 50 μM efaroxan and (b) 500 μM phentolamine. Ca^{2+} profiles are shown for glucose (15 mM), tolbutamide (Tol, 100 μM), efaroxan (200 μM), idazoxan (200 μM) and phentolamine (500 μM) in islets pretreated for 18 h with either 50 μM efaroxan (a) or 500 μM phentolamine (b). Both traces are representative of 6 islets.

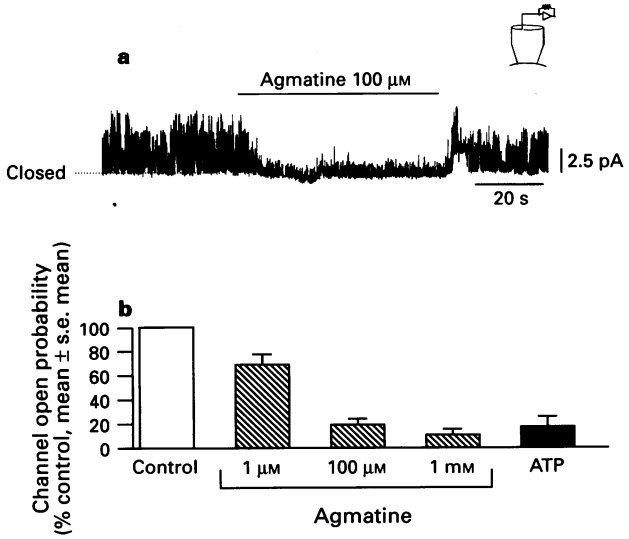

Figure 7 Block of K_{ATP} channels by agmatine. Experiments were carried out with the cell-free inside-out variation of the patch-clamp technique; see cartoon in (a). (b) Agmatine caused a concentration-dependent inhibition of K_{ATP} channels. Upward deflections represent outward current events. Trace in (a) is typical of 6 other patches; average data in (b) obtained from 6 patches. ATP = 500 μM concentration.

Discussion

The purpose of this study was to investigate the effects of imidazolines on cytosolic Ca^{2+} signalling events in pancreatic islets of Langerhans. Many of these compounds are insulin secretagogues that facilitate their action through modulation of K^+ channels. However, the subcellular signalling events involving these compounds are incompletely resolved. We have previously shown that there is no accumulation of intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels resulting from exposure of β-cells to imidazolines (Chan *et al.*, 1991), but the involvement of $[Ca^{2+}]_i$ homeostasis has not been thoroughly investigated (Rustenbeck *et al.*, 1995). Overall our data show that phentolamine and efaroxan elevate cytosolic Ca^{2+} levels in islets, and that this could explain how the compounds promote insulin secretion following K^+ channel modulation.

In β-cells, glucose-induced insulin secretion is a Ca^{2+} dependent process involving the influx of Ca^{2+} across the plasma membrane (Wollheim & Sharp, 1981). Calcium-selective ion channels in β-cells are predominantly voltage-operated, and open upon a depolarization of the cell membrane potential. This depolarization is facilitated by closure of K_{ATP} channels which occurs following glucose metabolism and a concomitant rise in the adenosine 5'-triphosphate:adenosine 5'-diphosphate (ATP:ADP) ratio (Ashcroft *et al.*, 1984; Dunne & Petersen, 1991). K_{ATP} channels are also inhibited by the antidiabetic sulphonylureas which depolarize the cell membrane, elevate cytosolic Ca^{2+} and promote the release of insulin (Ashcroft & Ashcroft, 1992). By contrast, rather less is known about the stimulus-response coupling events involving imidazolines in β-cells. Efaroxan, phentolamine and idazoxan are blockers of K_{ATP} channels, and whilst efaroxan and phentolamine will depolarize the β-cell membrane potential (Plant & Henquin, 1990; Dunne, 1991; Dunne & Petersen, 1991) and promote insulin secretion (Schulz & Hasselblatt, 1989), idazoxan will

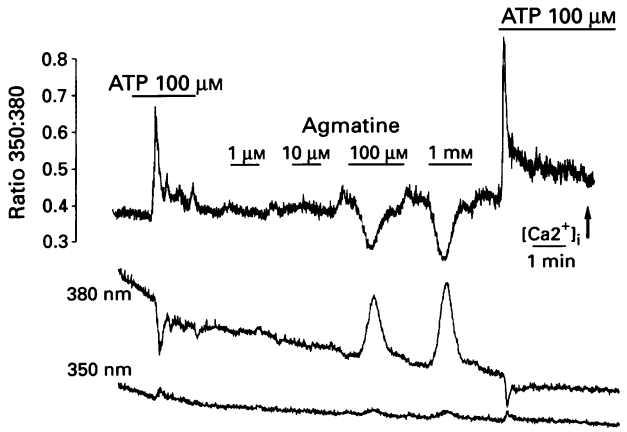

Figure 8 Anomalous effects of agmatine on fura-2 fluorescence in β-cells. Microfluorimetry data show that whilst ATP, through stimulation of β-cell purinoceptor elevates $[Ca^{2+}]_i$ (Squires *et al.*, 1994), agmatine had a deleterious effect on fura-2. Unlike ATP which causes a decrease in 380 nm fluorescence and a concomitant rise at 350 nm, agmatine increases the fluorescence at both wavelengths.

not (Chan & Morgan, 1990). In this study we show that both phentolamine and efaroxan are able to effect a marked elevation of $[Ca^{2+}]_i$ in intact islets of Langerhans, and that the subcellular events are critically dependent upon Ca^{2+} influx and not mobilization of stored Ca^{2+}; (i) in the absence of external Ca^{2+} there is no increase in intracellular Ca^{2+}, (ii) acute removal of external Ca^{2+} attenuated agonist-evoked increases in $[Ca^{2+}]_i$, (iii) the hyperpolarizing K^+ channel agonist diazoxide attenuated both efaroxan- and phentolamine-evoked increases in $[Ca^{2+}]_i$ and caused a marked delay in the onset times of the responses, and (iv) imidazoline-mediated rises in $[Ca^{2+}]_i$ were unaffected by the intracellular Ca^{2+}-ATPase inhibitor thapsigargin. The actions of idazoxan on $[Ca^{2+}]_i$ are also relevant since in contrast to efaroxan and phentolamine, idazoxan failed to elevate $[Ca^{2+}]_i$ (Figure 2) despite blocking K_{ATP} channels at much lower concentrations (Chan *et al.*, 1991). Since K_{ATP} channel inhibition is associated with a depolarization of the cell membrane potential and the opening of voltage-gated Ca^{2+} channels, we investigated whether this inconsistency could be due to a deleterious effect of idazoxan on the influx of Ca^{2+}. Voltage-dependent calcium influx was therefore initiated by exposing islets to high external KCl, and under these conditions we found that idazoxan attenuated the KCl-induced rises in $[Ca^{2+}]_i$ ($n=3$, mean block by $18 \pm 1\%$ of the control value) suggesting that the compound could inhibit the entry of Ca^{2+}.

Interactions between glucose and efaroxan or phentolamine, suggest that the imidazolines are able not only to initiate Ca^{2+} signalling events in β-cells (Figure 2), but that they will also potentiate the actions of glucose (Figure 5). Our data show that not only did glucose facilitate faster onset times for the actions of both phentolamine and efaroxan (Table 1), but that there was also an increase in the magnitude of the Ca^{2+} response. These effects were also seen in islets that themselves did not respond to 5 mM glucose through a rise in $[Ca^{2+}]_i$ (Figure 5a). The effects of glucose availability upon phentolamine-induced rises in $[Ca^{2+}]_i$ were significant. In the presence of 2 mM glucose the mean time for phentolamine to initiate a rise in $[Ca^{2+}]_i$ was approximately 20 min. This is not related to the solubility of the compound in aqueous solution, since the onset time in the presence of 5 mM glucose was 8 min, and 3 min at 10 mM glucose, Table 1. One possible explanation for these findings is that the actions of the imidazoline are augmented in a metabolically-dependent manner through modulation of K_{ATP} channels (Dunne *et al.*, 1995).

Efaroxan, idazoxan and phentolamine are all imidazoline derivatives with differing affinities for the two broad classes of imidazoline receptors: efaroxan is an I_1 ligand, idazoxan an I_2 agonist and phentolamine will interact with both I_1 and I_2 receptors (Michel & Insel, 1989; Michel & Ernsberger, 1992). In β-cells the putative imidazoline receptor has many features in common with the I_1 and I_2 receptor subtypes, but has properties that distinguish it from these receptors (Chan *et al.*, 1994; Olmos *et al.*, 1994; for recent review see Morgan *et al.*, 1995). Li and colleagues (1994) recently showed that agmatine is a component of the 'endogenous ligand' for imidazoline receptors. Agmatine is also an insulin secretagogue (Sener *et al.*, 1989), and in this study we have been able to show that agmatine, like efaroxan and phentolamine will block K_{ATP} channels (Figure 7). However, any further examination of the mechanisms of action of agmatine are hindered by the fact that agmatine disrupts fura-2 fluorescence associated with Ca^{2+} signalling in pancreatic β-cells (Figure 8). Surprisingly, this appears specific to β-cells, since agmatine will readily induce a rise in $[Ca^{2+}]_i$ in NTERA2-derived neurones (Squires *et al.*, 1996), effects that are dependent upon Ca^{2+} influx (Squires & Dunne, unpublished observations).

The insulinotropic actions of imidazoline receptor agonists on insulin secretion (Morgan *et al.*, 1995), β-cell electrophysiology (Dunne *et al.*, 1995) and Ca^{2+} signalling (Figure 2), exhibit little or no desensitization over relatively short periods of time. By contrast, however, imidazoline-evoked insulin secretion can be inhibited by long-term exposure of islets to the compounds. These responses are also selective (Chan *et al.*, 1993). Thus, pre-incubation of islets to efaroxan or phentolamine prevented subsequent responses of cells to either agent, but not to glucose or diazoxide, whereas islets that were pre-incubated with idazoxan, responded normally to efaroxan and phentolamine. In this study we find under the same experimental conditions, that there was no inhibition of imidazoline-evoked increases in $[Ca^{2+}]_i$ (Figure 6, Table 2). This may indicate that imidazolines, like sulphonylureas (Elliasson *et al.*, 1996) will also evoke insulin secretion through more distal exocytotic events (see also Westerlund *et al.*, 1996). Such a mechanism would also help to explain the relative inefficiency of imidazolines to promote rises in $[Ca^{2+}]_i$ when compared to the action of the compounds on insulin secretion and ion channel activity. For example, phentolamine will act as an insulin secretagogue in perifused intact mouse islets at concentrations of approximately 30 μM (Schulz & Hassleblatt, 1988) and block K_{ATP} channels with an IC_{50} of <20 μM (Dunne *et al.*, 1990), yet an effective increase in $[Ca^{2+}]_i$ can only be initiated by 500 μM. Whilst it is possible that this discrepancy could be due to differences in technical procedures (primary cultured islets vs. acutely isolated tissue) and the limitation of microfluorimetry techniques to detect sub-plasma membrane levels of $[Ca^{2+}]_i$, a direct or modulating effect on exocytosis of insulin-containing granules independent of $[Ca^{2+}]_i$ cannot be ruled out. In summary, our data show that imidazoline agonists are modulators of $[Ca^{2+}]_i$ in pancreatic islets and we conclude that elevation of $[Ca^{2+}]_i$ is predominately mediated through Ca^{2+} influx.

We thank the British Diabetic Association and the Wellcome Trust for their financial support, Kate Hinchliffe for technical assistance and Christine Booth for typing this manuscript. M.N.H. and C.K.

References

AHRÉN, B. & LUNDQUIST, I. (1985). Effects of α-adrenoceptor blockade by phentolamine on basal and stimulated insulin secretion in the mouse. *Acta Physiol. Scand.*, **125**, 211–217.

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1992). The sulphonylurea receptor. *Biochem. Biophys. Acta*, **1175**, 45–59.

ASHCROFT, F.M., HARRISON, D.E. & ASHCROFT, S.J.H. (1984). Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature*, **132**, 446–448.

CHAN, S.L.F., BROWN, C.A. & MORGAN, N.G. (1993). Stimulation of insulin secretion by the imidazoline α_2-adrenoceptor antagonist efaroxan is mediated by a novel, stereoselective, binding site. *Eur. J. Pharmacol.*, **230**, 375–378.

CHAN, S.L.F., BROWN, C.A., SCARPELLO, K.E. & MORGAN, N.G. (1994). The imidazoline site involved in control of insulin secretion: characteristics that distinguish it from I_1- and I_2-sites. *Br. J. Pharmacol.*, **112**, 1065–1070.

CHAN, S.L.F., DUNNE, M.J., STILLINGS, M.R. & MORGAN, N.G. (1991). Characterisation of the interactions of efaroxan, an α_2-adrenergic antagonist, with insulin-secreting cells. *Eur. J. Pharmacol.*, **204**, 41–48.

CHAN, S.L.F. & MORGAN, N.G. (1990). Stimulation of insulin secretion by efaroxan may involve interaction with potassium channels. *Eur. J. Pharmacol.*, **176**, 97–101.

DUNNE, M.J. (1991). Block of ATP-regulated potassium channels by phentolamine and other α-adrenoceptor antagonists. *Br. J. Pharmacol.*, **103**, 1847–1850.

DUNNE, M.J., HARDING, E.A., JAGGAR, J.H., SQUIRES, P.E., LIANG, R., KANE, C., LONDON, N.J.M. & JAMES, R.L.F. (1995). Potassium channels, imidazolines and insulin-secreting cells. *Ann. New York Acad. Sci. U.S.A.*, **763**, 243–262.

DUNNE, M.J. & PETERSEN, O.H. (1991). Potassium selective ion channels in insulin-secreting cells; physiology, pharmacology and their role in stimulus secretion coupling. *Biochem. Biophys. Acta* (Reviews in Biomembranes), **1071,** 67–82.

DUNNE, M.J., YULE, D.I., GALLACHER, D.V. & PETERSEN, O.H. (1990). A comparative study of the effects of cromakalim (BRL34915) and diazoxide on membrane potential, $[Ca^{2+}]_i$, and ATP-sensitive potassium currents in insulin-secreting cells. *J. Memb. Biol.*, **114,** 54–61.

ELLIASSON, L., RENSTRÖM, E ÄMMÄLÄ, C., BERGGREN, P.O., BERTORELLO, A.M., BOKVIST, K., CHIHALIN, A., DEENEY, J.T., FLATT, P.R., GABEL, J., GROMADA, J., LARSSON, O., LINDSTRÖM, P., RHODES, C.J. & RORSMAN, P. (1996). PKC dependent stimulation of exocytosis by sulphonylureas in pancreatic β-cells. *Science*, **271,** 813–815.

FINDLAY, I., DUNNE, M.J. & PETERSEN, O.H. (1985). High conductance K^+ channel in pancreatic islet cells can be activated and inactivated by internal calcium. *J. Membr. Biol.*, **83,** 169–175.

GILON, P. & HEQUIN, J.C. (1992). Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic β-cell. *J. Biol. Chem.*, **267,** 20713–20720.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved properties. *J. Biol. Chem.*, **260,** 3440–3450.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high resolution current recordings from cells and cell-free membrane patches. *Pflügers Arch.*, **391,** 85–100.

JONAS, J.C., PLANT, T.D. & HENQUIN, J.C. (1992). Imidazoline antagonists of α_2-adrenoceptors increase insulin release *in vitro* by inhibiting ATP-sensitive K^+ channels in pancreatic β-cells. *Br. J. Pharmacol.*, **107,** 8–14.

LEBRUN, P., ANTOINE, M.-H., OUEDRAOGO, R., DUNNE, M.J., KANE, C., HERMANN, M., HERCHUELZ, A., MASEREEL, B., DELARGE, J., DE TULLIO, P. & PIROTTE, B. (1996). Activation of ATP-dependent K^+ channels and inhibition of insulin release; effect of BPDZ-62. *J. Pharmacol. Exp. Ther.*, **277,** 156–162.

LI, G., REGUNATHAN, S., BARROW, C.J., ESHRAGHI, J., COOPER, R. & REIS, D.J. (1994). Agmatine: an endogenous clonidine displacing substance in the brain. *Science*, **263,** 966–969.

MICHEL, M.C. & ERNSBERGER, P. (1992). Keeping an eye on the i site: imidazoline-preferring receptors. *Trends Pharmacol. Sci.*, **13,** 360–370.

MICHEL, M.C. & INSEL, P.A. (1989). Are there multiple imidazoline binding sites? *Trends Pharmacol. Sci.*, **10,** 342–344.

MORGAN, N.G., CHAN, S.L.F., BROWN, C.A. & TSOLI, E. (1995). Characterization of the imidazoline binding site involved in regulation of insulin secretion. *Ann. New York. Acad. Sci. U.S.A.*, **763,** 361–366.

OLMOS, G., KULKARNI, R.N., HAGUE, M. & MACDERMOT, J. (1994). Imidazolines stimulate release of insulin from RIN-5AH cells independently from imidazoline I_1 and I_2 receptors. *Eur. J. Pharmacol.*, **262,** 41–48.

PLANT, T. & HEQUIN, J.C. (1990). Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **101,** 115–120.

RUSTENBECK, I., KOWALEWSKI, R., HERRMANN, C., DICKEL, C., RATZKA, P. & HASSELBLATT, A. (1995). Effects of imidazoline compounds on cytoplasmic Ca^{2+} concentration and ATP-sensitive K^+ channels in pancreatic β-cells. *Exp. Clin. Endocrinol.*, **103,** 42–45.

SCHULZ, A. & HASSELBLATT, A. (1988). Phentolamine, a deceptive tool to investigate sympathetic nervous control of insulin release. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **337,** 637–643.

SCHULZ, A. & HASSELBLATT, A. (1989). An insulin-releasing property of imidazoline derivatives is not limited to compounds that block α-receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **340,** 712–714.

SENER, A., LEBRUN, P., BLACHIER, F. & MALAISSE, W.J. (1989). Stimulus-secretion coupling of arginine-induced insulin release. *Biochem Pharmacol.*, **38,** 327–330.

SQUIRES, P.E., JAMES, R.L.F., LONDON, N.J.L. & DUNNE, M.J. (1994). ATP-induced intracellular Ca^{2+} signals in isolated human insulin-secreting cells. *Pflügers Archives*, **427,** 181–183.

SQUIRES, P.E., WAKEMAN, J.A., CHAPMAN, H.M., KUMPF, S., FIDOCK, M., ANDREWS, P.W. & DUNNE, M.J. (1996). Regulation of intracellular Ca^{2+} in response to muscarinic and glutamate receptor agonists during the differentiation of NTERA2 human embryonal carcinoma calls into neurones. *Eur. J. Neurosci.*, **8,** 783–793.

WESTERLUND, J., HELLMAN, B. & BERGSTEN, P. (1996). Pulsatile insulin release from mouse islets occurs in the absence of stimulated entry of Ca^{2+}. *J. Clin. Invest.*, **97,** 1860–1863.

WOLLHEIM, C.B. & SHARP, G.W.G. (1981). The regulation of insulin release by calcium. *Physiol. Rev.*, **61,** 914–973.

(Received June 12, 1996
Revised July 30, 1996
Accepted July 31, 1996)