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The putative imidazoline receptor agonist, harmane, promotes intracellular calcium mobilisation in pancreatic β-cells

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Abstract

β-Carbolines (including harmane and pinoline) stimulate insulin secretion by a mechanism that may involve interaction with imidazoline I_3-receptors but which also appears to be mediated by actions that are additional to imidazoline receptor agonism. Using the MIN6 β-cell line, we now show that both the imidazoline I_3-receptor agonist, efaroxan, and the β-carboline, harmane, directly elevate cytosolic Ca^{2+} and increase insulin secretion but that these responses display different characteristics. In the case of efaroxan, the increase in cytosolic Ca^{2+} was readily reversible, whereas, with harmane, the effect persisted beyond removal of the agonist and resulted in the development of a repetitive train of Ca^{2+}-oscillations whose frequency, but not amplitude, was concentration-dependent. Initiation of the Ca^{2+}-oscillations by harmane was independent of extracellular calcium but was sensitive to both dantrolene and high levels (20 mM) of caffeine, suggesting the involvement of ryanodine receptor-gated Ca^{2+}-release. The expression of ryanodine receptor-1 and ryanodine receptor-2 mRNA in MIN6 cells was confirmed using reverse transcription-polymerase chain reaction (RT-PCR) and, since low concentrations of caffeine (1 mM) or thimerosal (10 μM) stimulated increases in $[Ca^{2+}]_i$, we conclude that ryanodine receptors are functional in these cells. Furthermore, the increase in insulin secretion induced by harmane was attenuated by dantrolene, consistent with the involvement of ryanodine receptors in mediating this response. By contrast, the smaller insulin secretory response to efaroxan was unaffected by dantrolene. Harmane-evoked changes in cytosolic Ca^{2+} were maintained by nifedipine-sensitive Ca^{2+}-influx, suggesting the involvement of L-type voltage-gated Ca^{2+}-channels. Taken together, these data imply that harmane may interact with ryanodine receptors to generate sustained Ca^{2+}-oscillations in pancreatic β-cells and that this effect contributes to the insulin secretory response.

Keywords: Imidazoline receptor; Ryanodine; KU14R; β-Carboline; Insulin secretion; Islets of Langerhans

1. Introduction

It is now widely accepted that certain synthetic ligands bearing an imidazoline moiety are able to stimulate insulin secretion from pancreatic β-cells and that this effect results from the activation of a subtype of imidazoline receptor (Eglen et al., 1998; Morgan and Chan, 2001; Efendic et al., 2002). The mechanism by which this promotes insulin secretion remains enigmatic but recent evidence suggests that imidazoline receptors regulate a distal step in the pathway of insulin exocytosis rather than controlling intracellular signal generation (Morgan and Chan, 2001; Efendic et al., 2002; Chan et al., 2001; Hoy et al., 2003; Efanov et al., 2001).

The imidazoline receptor involved in the regulation of insulin secretion belongs to a wider class of similar receptors that are expressed in multiple tissues and comprise at least three distinct subtypes, I_1, I_2 and I_3 (Eglen et al., 1998; Morgan and Chan, 2001). These are defined according to their pharmacological characteristics

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and many ligands interact with one or more subtypes. The imidazoline I_3-receptor subtype is responsible for the ability of imidazoline compounds to stimulate insulin secretion and is activated by a number of synthetic ligands including efaroxan (Eglen et al., 1998; Morgan and Chan, 2001) phentolamine (Schulz and Hasselblatt, 1988) antazoline (Berdeu et al., 1997) and 2-(*N*-phenylindoyl)imidazole hydrochloride (RX871024; Efendic et al., 2002). The imidazoline I_3-receptor can also be activated by a putative endogenous ligand (termed "clonidine displacing substance"; Chan et al., 1997; Prell et al., in press) suggesting that activation of this receptor may be of physiological relevance for control of insulin secretion from the β-cell.

The molecular characterisation of clonidine displacing substance is ongoing (Prell et al., in press) and formal identification of the various active components remains an important priority. In this context, recent data indicate that β-carbolines may be present in crude preparations of clonidine displacing substance (Husbands et al., 2001; Hudson et al., 2001; Robinson et al., 2003) and that one member of this class, harmane, displaces radioligands from imidazoline I_1-receptors and may be agonistic at these receptors in the rostrolateral ventral medulla (Musgrave and Badoer, 2000; Piletz et al., 2000). We have recently shown that harmane stimulates insulin secretion from isolated human islets of Langerhans and have found that this response shares features in common with that mediated by the imidazoline-ligand, efaroxan, suggesting that imidazoline I_3-receptors might be involved (Cooper et al., 2003; Morgan et al., 2003).

However, when comparing the effects of harmane and efaroxan in β-cells, it became clear that the two agents do not exert exactly equivalent effects. In particular, harmane was observed to potentiate insulin secretion beyond the maximal response elicited by glucose, whereas efaroxan did not do so (Cooper et al., 2003). These observations led to the proposal that the ability of harmane to stimulate insulin secretion might involve both the activation of imidazoline I_3 receptors and an additional, undefined, mechanism (Cooper et al., 2003; Morgan et al., 2003). We have noted that harmane displays a strong structural similarity with the eudistomins, a group of molecules isolated from marine invertebrates that have been shown to regulate the mobilisation of Ca^{2+} from ryanodine sensitive stores in mammalian cells (Seino et al., 1991; Lahouratate et al., 1997; Seino-Umeda et al., 1998), including pancreatic β-cells (Bruton et al., 2003). Certain eudistomins possess a β-carboline skeleton and these molecules appear to bind to ryanodine receptors leading to the enhancement of Ca^{2+} release (Seino et al., 1991; Lahouratate et al., 1997; Seino-Umeda et al., 1998; Bruton et al., 2003). Therefore, in the present work, we have explored the possibility that one of the actions of harmane in β-cells might be to promote intracellular Ca^{2+} release by a mechanism involving ryanodine receptors. The results reveal that harmane evokes the generation of sustained Ca^{2+} oscillations in MIN6 β-cells and suggest that this may involve ryanodine receptors.

2. Materials and methods

2.1. Maintenance and preparation of MIN6 cells

All experiments were performed with the mouse insulinoma cell line, MIN6. These cells (passage 38–44) were maintained at 37 °C (95% O_2/5% CO_2) in Dulbecco's Modified Eagle's Medium (Sigma, Poole, Dorset, UK) supplemented with 15% foetal calf serum, 2 mM glutamine and 100 U/ml penicillin/0.1 mg/ml streptomycin. Medium was changed every 3–4 days and the monolayers were trypsinised (0.1% trypsin, 0.02% EDTA) for experiments when approximately 80% confluent. MIN6 cells were retrieved by trypsinisation and seeded onto 3-aminopropyltriethoxysilane (APES)-coated (Sigma) glass coverslips at a density of 30,000 cells per coverslip which was sufficient to allow the adherent cells to form two-dimensional cell clusters.

2.2. Single-cell microfluorimetry

Cells were loaded for 20 min at 37 °C with 2.5 μM of the Ca^{2+}-fluorophore Fura-2/AM (Sigma). The coverslips were washed and placed in a steel chamber, the volume of which was approximately 500 μl. A single 22-mm coverslip formed the base of the chamber, which was mounted into a heating platform on the stage of an Axiovert 200 Research Inverted microscope (Carl Zeiss, Welwyn Garden City, UK). All experiments were carried out at 37 °C using a Na^+-rich balanced salt solution as the standard extracellular medium. A low pressure, rapid superfusion system (flow rate 1–2 ml/min) was used to change the solutions in the bath. Cells were illuminated alternatively at 340 and 380 nm using a Metafluor Imaging Workbench (Universal Imaging, Marlow Bucks, UK). Emitted light was filtered using a 510-nm-long pass barrier filter and detected using a CoolSnap HQ CCD camera (Roper Scientific). Changes in the emission intensity of fura-2 expressed as a ratio of dual excitation were used as an indicator of changes in $[Ca^{2+}]_i$, using established procedures. Data were collected every 3 s for multiple regions of interest in any one field of view. All records have been corrected for background fluorescence (determined from cell-free coverslip).

2.3. Insulin secretion

Monolayers of MIN6 cells were cultured in 24-well plates then washed and incubated in physiological saline solution (Gey and Gey, 1936) containing glucose (2 or 6 mM according to the experiment), bovine serum albumin (1 mg/ml) and test reagents. Cells were incubated for 1 h at 37 °C

and the medium sampled for measurement of insulin by radioimmunoassay.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of ryanodine receptor expression

Total RNA was isolated from cultures of MIN6 cells using Trizol reagent and stored at −80 °C until use. RNA was reverse transcribed and amplified using single tube RT-PCR reagents (Abgene) according to the manufacturer's instructions. Reverse transcription was carried out at 42 °C for 1 h followed by PCR amplification (30 cycles) at 95 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min. Specific primer sets were employed which selectively amplify each of the three cloned mouse Ryanodine receptor isoforms:

Ryanodine receptor-1-forward: gaaggttctggacaaacacggg; reverse: tcgctcttgttgtagaatttgcgg
Ryanodine receptor-2-forward: ctgaagagcctgaagaagca; reverse: gccggcatttgggttgtgag
Ryanodine receptor-3-forward: ttctttgctgctcatctgtt; reverse: aatgacgaagaagaagaacg

PCR products were separated by electrophoresis on 1% agarose gels and visualised by post-staining with ethidium bromide.

3. Results

3.1. The effects of β-carbolines and efaroxan on $[Ca^{2+}]_i$ in insulin-secreting cells

When MIN6 cells were incubated with 2 mM glucose, harmane evoked an increase in cytosolic Ca^{2+} over the concentration range 1–100 μM (Fig. 1A and B). This effect was seen in more than 85% of the cells and was observed consistently in multiple experiments. It was slow in onset and similar oscillations were also seen when MIN6 cells were exposed to harmane in the presence of 20 mM glucose (not presented). The amplitude of the Ca^{2+} oscillations appeared to be independent of the harmane concentration although the frequency increased as the drug concentration was raised (6.9±0.6 oscillations/300 s post application at 1 μM vs. 10.8±0.5 oscillations/300 s at 100 μM; $P<0.0001$; unpaired Student's t-test). The harmane-evoked changes in Ca^{2+} were irreversible over the time scale of the experiments (≥10 min) and at higher concentrations (>10 μM), increases in $[Ca^{2+}]_i$ oscillated above a mean elevated plateau, even following removal of the agonist from the extracellular medium.

A second β-carboline, pinoline (100 μM), was less effective at evoking an oscillatory change in cytosolic Ca^{2+}, and elicited lower amplitude responses compared to a similar concentration of harmane (12/33 cells (36%) in three separate experiments, Fig. 1C).

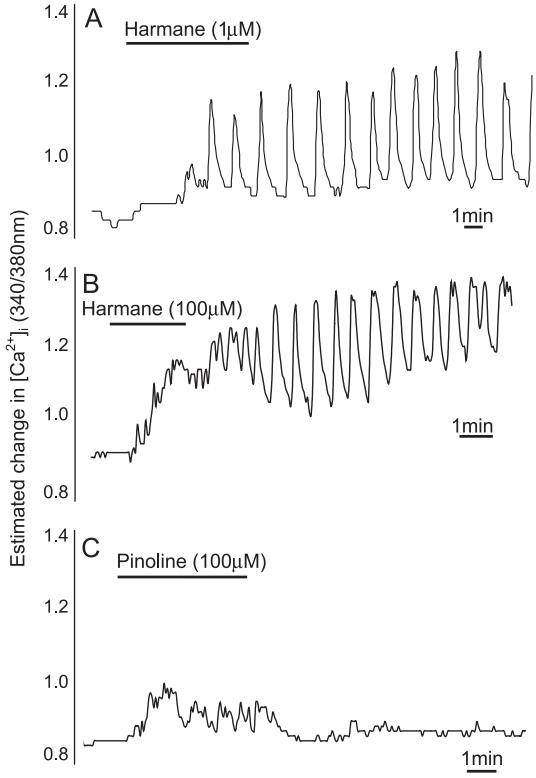

Fig. 1. The effect of β-carbolines on $[Ca^{2+}]_i$ in insulin-secreting cells. When MIN6 cells were incubated with 2 mM glucose, the addition of harmane evoked a repetitive train of Ca^{2+}-oscillations that arose either from basal levels of cytosolic Ca^{2+} (Panel A) or from an elevated plateau of $[Ca^{2+}]_i$ (Panel B). In each case, the response was irreversible and continued beyond the removal of the agonist. In Panel C, pinoline (100 μM) evoked a small, reversible increase in $[Ca^{2+}]_i$.

The imidazoline I_3-receptor agonist, efaroxan (which is not a β-carboline), consistently increased Ca^{2+} levels in MIN6 cells (Fig. 2A,B) although it did not always provoke Ca^{2+} oscillations (Fig. 2A). When Ca^{2+} oscillation were present, their amplitude was smaller than those induced by 100 μM harmane. Moreover, in marked contrast to the effects of harmane, the response to efaroxan was rapidly and completely reversible upon removal of the drug (Fig. 2B).

The initiation of harmane (10 μM)-induced Ca^{2+}-oscillations did not require extracellular Ca^{2+} (Fig. 3A), but maintenance of the response was dependent on Ca^{2+}-influx. Thus, oscillatory activity returned below unstimulated levels in Ca^{2+}-free media (+1 mM EGTA; 35/35 cells in four separate experiments, Fig. 3B). Ca^{2+}-transients returned following re-addition of external Ca^{2+}, without the need for a further application of the β-carboline. The effect of removing extracellular Ca^{2+} was mimicked by the addition of the voltage-gated Ca^{2+}-channel blocker nifedipine (Fig. 3C; 39/39 (100%) cells in four separate experiments), suggesting that Ca^{2+}-influx and the maintenance of the response was mediated via L-type Ca^{2+} channels.

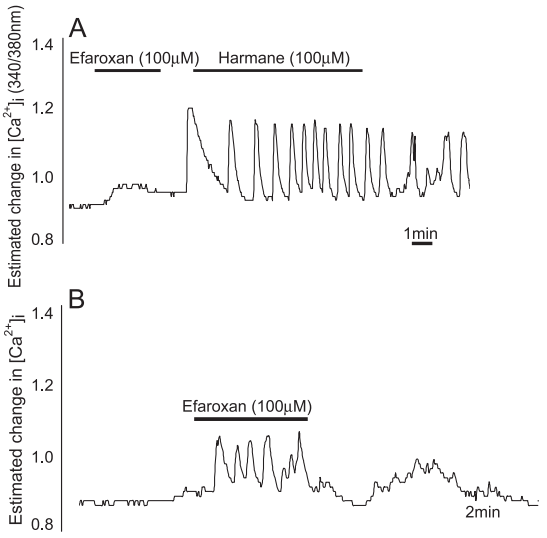

Fig. 2. Effects of efaroxan and harmane on $[Ca^{2+}]_i$ in insulin-secreting cells. Addition of efaroxan (100 μM) to MIN6 cells resulted in a rise in $[Ca^{2+}]_i$ (Panels A and B) although this was not always oscillatory (Panel A) and any oscillations were invariably of lower amplitude than those elicited by 100 μM harmane. The response to efaroxan was rapidly and completely reversible (Panels A, B) whereas Ca^{2+} oscillations induced by harmane persisted after removal of the drug.

3.2. Effects of the imidazoline I_3-receptor antagonist, KU14R, on harmane-evoked changes in $[Ca^{2+}]_i$

The imidazoline I_3-receptor antagonist 2-(2-ethyl-2,3-dihydrobenzo[*b*]furan-2-yl)-1*H*-imidazole (KU14R; Chan et al., 1998) was used to investigate the possibility that the response to harmane involved interaction with an imidazoline I_3-receptor. As shown in Fig. 4A, KU14R (100 μM) reduced the amplitude of harmane-evoked Ca^{2+}-oscillations in MIN6 cells, although it failed to prevent oscillatory activity or to return the elevated Ca^{2+} plateau to basal levels. In the presence of KU14R, there was a tendency for the frequency of the harmane-induced Ca^{2+}-oscillations to increase even though their amplitude was reduced (Fig. 4A). The amplitude of the Ca^{2+}-transients was restored upon removal of KU14R and without the need for a further harmane application (21/37 cells (57%) in three separate experiments). In the small number of cells (<15%) that failed to exhibit a robust harmane-evoked change in $[Ca^{2+}]_i$, KU14R itself evoked an increase in $[Ca^{2+}]_i$, a phenomenon reflecting the known inhibition of ATP-sensitive potassium channels (K_{ATP}^+) by KU14R (Chan et al., 1998).

3.3. Effects of caffeine, thimerosal and dantrolene on Ca^{2+}-release in insulin-secreting cells

Low concentrations of caffeine were used to access ryanodine-sensitive Ca^{2+}-stores in MIN6 cells. Caffeine (1 mM) evoked Ca^{2+}-oscillations of a comparative amplitude and periodicity to the harmane-evoked transients in MIN6 cells (17/21 cells, Fig. 5A) consistent with the possibility that these agents may access equivalent stores of Ca^{2+}. High concentrations of caffeine (20 mM) partially and reversibly inhibited harmane-evoked Ca^{2+}-oscillations (19/27 cells (70%) in three separate experiments; Fig. 5B).

The thiol reagent thimerosal can evoke heparin-sensitive increases in $[Ca^{2+}]_i$, suggesting that it can access both ryanodine receptor and inositol trisphosphate (IP_3)-sensitive Ca^{2+}-stores under appropriate conditions. In pancreatic β-cells, the thimerosal-insensitive type 3 isoform of the $InsP_3$-receptor predominates (Blondel et al., 1993), suggesting that any thimerosal-evoked change in $[Ca^{2+}]_i$ would be due to ryanodine receptor-gated store release. In the current study, thimerosal (10 μM) elicited transitory and reversible, low amplitude oscillations with a long latency period (180±30 s) in 18/21 cells studied (Fig. 6A).

Addition of the ryanodine receptor-antagonist, dantrolene (10 μM) to MIN6 cells caused a rapid but reversible inhibition of harmane-induced Ca^{2+} oscillations (Fig. 6B)

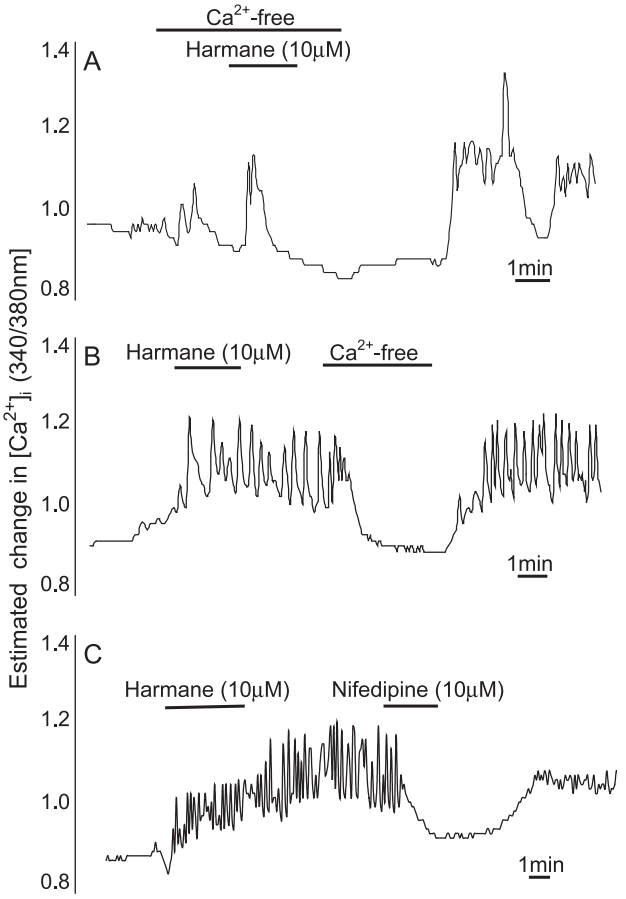

Fig. 3. The effect of alterations in extracellular Ca^{2+} availability on harmane-evoked changes in $[Ca^{2+}]_i$. In the absence of $[Ca^{2+}]_e$, harmane (10 μM) evoked a transient increase in $[Ca^{2+}]_i$ (Panel A). The response to harmane declined within 1–2 min but could be restored by replacement of $[Ca^{2+}]_e$. In Panel B, harmane-evoked changes in $[Ca^{2+}]$i were abolished following removal of external Ca^{2+} but were reinstated by the re-addition of Ca^{2+}, without the need for further application of the agonist. In Panel C, a similar inhibitory response was observed when the L-type voltage-gated Ca^{2+}-channel blocker, nifedipine, was added to cells exposed to harmane in the presence of extracellular Ca^{2+}.

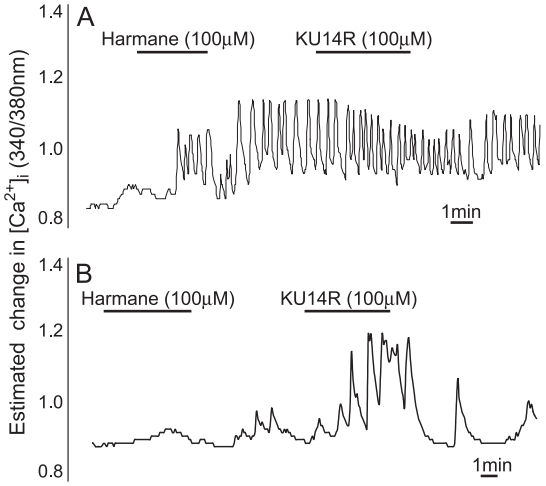

Fig. 4. The effect of KU14R on harmane-evoked changes in $[Ca^{2+}]_i$ in MIN6 cells. The I_3-imidazoline antagonist KU14R, reduced the amplitude of harmane-evoked Ca^{2+}-oscillations without affecting the elevated plateau of the response (Panel A). In those cells that failed to exhibit an increase in $[Ca^{2+}]_i$ in response to harmane, KU14R stimulated basal Ca^{2+} (Panel B).

suggesting that harmane may access Ca^{2+} stores by a mechanism involving the activation of ryanodine receptors.

3.4. Expression of ryanodine receptors in MIN6 cells

RT-PCR analysis with isoform-specific primer sets confirmed the recent observation (Mitchell et al., 2003) that MIN6 cells express two of the three known ryanodine receptor subtypes. Appropriate products were generated with either ryanodine receptor-1 or ryanodine receptor-2-specific primers whereas no amplification was achieved with primers designed to amplify ryanodine receptor-3 (Fig. 7) even when the annealing temperature was decreased to reduce stringency.

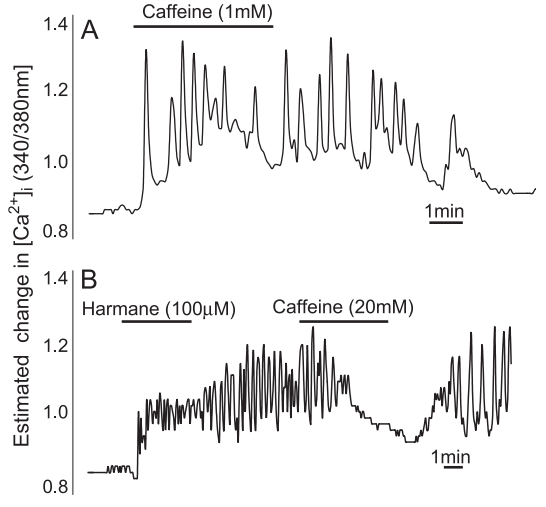

Fig. 5. The effect of caffeine on $[Ca^{2+}]_i$ in MIN6 cells. Low concentrations of caffeine (1 mM; Panel A) stimulated repetitive trains of Ca^{2+}-oscillations above a mean elevated level of cytosolic Ca^{2+}. The Ca^{2+}-transients persisted for >3 min beyond the removal of the methylxanthine. In Panel B, harmane-evoked increases in $[Ca^{2+}]_i$ were reversibly inhibited by high concentrations of caffeine (20 mM).

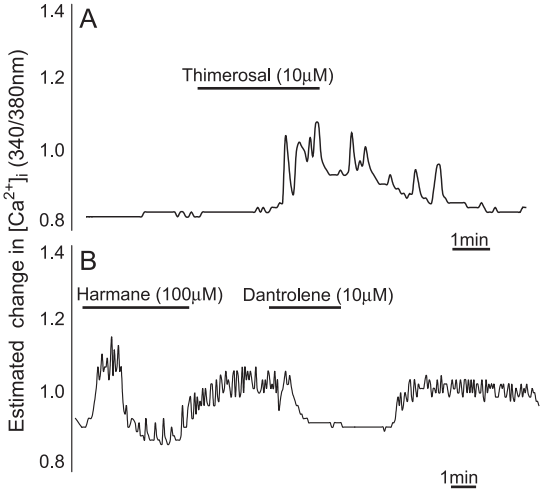

Fig. 6. The effect of thimerosal and dantrolene on $[Ca^{2+}]_i$ in MIN6 cells. (Panel A) Addition of the thiol agent, thimerosal, increased $[Ca^{2+}]_i$ in MIN6 cells after an initial latency period. The response was reversible and the cells displayed a pattern of slow oscillations above a mean elevated plateau. (Panel B) Dantrolene (10 μM) reversibly inhibited harmane-evoked changes in $[Ca^{2+}]_i$ in MIN6 cells.

3.5. Effects of dantrolene on harmane and efaroxan-induced insulin secretion from MIN6 cells

In view of the finding that harmane-induced Ca^{2+}-release was sensitive to the ryanodine receptor antagonist, dantrolene, the effects of dantrolene were tested on insulin secretion from MIN6 cells (Fig. 8). Harmane and efaroxan each caused a marked increase in insulin secretion but, as observed in previous studies with human islets (Cooper et al., 2003), the extent of this increase was greater with harmane than with efaroxan (Fig. 8). Harmane increased insulin secretion to a similar extent in MIN6 cells that were incubated with 2 or 6 mM glucose (2 mM glucose alone: 1.61±0.12 ng/well; 2 mM glucose+100 μM harmane: 3.07±0.21; 6 mM glucose alone: 1.75±0.21; 6 mM glucose+100 μM harmane: 3.45±0.16; n=12). Dantrolene (10 μM) did not alter the increase in insulin secretion caused by efaroxan whereas it significantly attenuated the secretory response to harmane (Fig. 8). Indeed, under these conditions, insulin secretion was reduced to the same level as that observed with efaroxan alone.

4. Discussion

In the current study, we have examined the proposition that the β-carboline, harmane, may elevate cytosolic calcium ($[Ca^{2+}]_i$) in a model insulin-secreting cell line, MIN6. This hypothesis was based on the observation that harmane is structurally similar to a class of agents (eudistomins) known to regulate intracellular Ca^{2+} fluxes

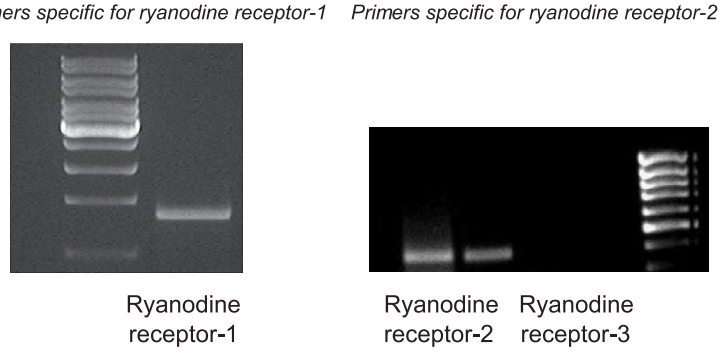

Fig. 7. Expression of ryanodine receptor isoforms in MIN6 cells. Total RNA was extracted from MIN6 cells, reverse transcribed and then amplified with primers specific for ryanodine receptor-1, ryanodine receptor-2 and ryanodine receptor-3, as shown. PCR products were visualised under UV illumination after electrophoresis on agarose gels and staining with ethidium bromide. Molecular weight markers were run in parallel with experimental samples. Amplification of ryanodine receptor-1 (left) and ryanodine receptor-2 (centre) was observed consistently but no product could be amplified with primers designed to the ryanodine receptor-3 sequence (right).

in mammalian cells (Seino et al., 1991; Lahouratate et al., 1997; Seino-Umeda et al., 1998) and that both harmane and certain eudistomins stimulate insulin secretion (Bruton et al., 2003; Cooper et al., 2003; Morgan et al., 2003). The results revealed that harmane (1–100 μM) evoked an increase in $[Ca^{2+}]_i$ in MIN6 cells; a response that was manifest as the development of trains of Ca^{2+} oscillations having similar amplitude but variable frequency, according to the harmane concentration. The dose-dependence was similar to that seen for harmane-induced insulin secretion (Cooper et al., 2003) consistent with the possibility that the generation of Ca^{2+} oscillations may contribute to the secretory response. At a low concentration (1 μM), the harmane-evoked train of Ca^{2+}-transients originated just above the resting Ca^{2+}-level. Higher concentrations increased the frequency of oscillations, producing Ca^{2+}-transients above a mean elevated plateau level. In all cases, the response was not reversible over the time course of the experiment, with Ca^{2+}-oscillations persisting beyond the removal of the agonist, for periods in excess of 10 min. A second β-carboline, pinoline, was less effective at evoking changes in $[Ca^{2+}]_i$ which is consistent with our previous studies suggesting that pinoline is less potent than harmane as a stimulus for insulin secretion (Cooper et al., 2003).

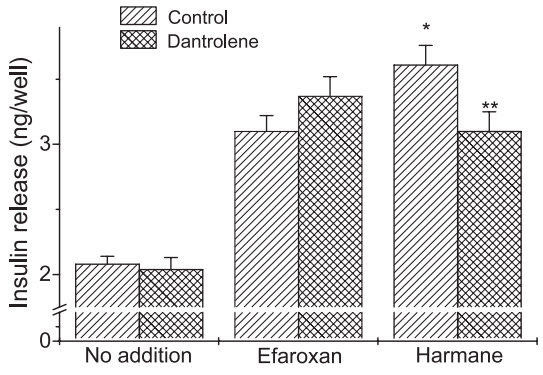

Fig. 8. Effects of dantrolene on the insulin secretory response to harmane or efaroxan. MIN6 cells responded to either harmane (100 μM) or efaroxan (100 μM) with an increase in insulin secretion. The magnitude of this response was significantly greater with harmane than efaroxan (*$p<0.05$). Dantrolene attenuated the secretory response to harmane (**$p<0.05$) whereas it failed to alter insulin secretion mediated by efaroxan. Mean values (±S.E.M.) are shown for six replicate incubations. The experiment was repeated twice with similar results.

Importantly, it was observed that harmane transiently increased cytosolic Ca^{2+} when cells were incubated in the absence of extracellular calcium, suggesting that harmane may mobilise Ca^{2+} from intracellular stores in MIN6 cells. However, it was also clear that development of the full response required Ca^{2+}-influx since the sustained oscillations mediated by harmane were abolished by removal of extracellular Ca^{2+} or by addition of the L-type voltage-gated Ca^{2+}-channel (VGCC) blocker, nifidipine. This suggests that the influx component was primarily mediated via L-type VGCCs.

Insulin secreting cells are known to express several different intracellular Ca^{2+}-stores that can be mobilised either via changes in $InsP_3$ (Morgan et al., 1985; Prentki et al., 1984) or by the plant alkaloid ryanodine (Mitchell et al., 2003; Islam, 2002; Holz et al., 1999; Graves and Hinkle, 2003). These stores are functionally independent and are released upon binding of relevant ligands to either IP_3 receptors or to ryanodine receptors. Low concentrations of methylxanthines (e.g. caffeine) are known to access stored Ca^{2+} via ryanodine receptors (Graves and Hinkle, 2003) and, consistent with this, 1 mM caffeine generated an oscillatory train of Ca^{2+}-oscillations in MIN6 cells. Indeed, these oscillations displayed a similar amplitude and periodicity to those evoked by harmane consistent with the proposal that the β-carboline may also access stored Ca^{2+} via ryanodine receptors. In the case of caffeine, the response was reversible, although oscillations were recorded for at least 3 min following removal of the drug, confirming similar findings from primary mouse islets (Bruton et al., 2003). These data are in accord with the hypothesis that ryanodine-gated store release (Mitchell et al., 2003; Graves and Hinkle, 2003; Koizumi et al., 1999) may mediate the oscillatory response to harmane.

There has been controversy about the expression and functional roles of ryanodine receptors in β-cells and some authors have argued that they may be relatively unimportant since cADP-ribose (a putative ryanodine receptor ligand) is relatively ineffective as a Ca^{2+}-mobilising agent in clonal β-cells (Rutter et al., 1994; Islam and Berggren, 1997). However, recent studies have begun to clarify this issue and at least four independent groups have now observed the expression of ryanodine receptors in primary islets or β-cell lines (Mitchell et al., 2003; Holz et al., 1999; Islam, 2002; Takasawa et al., 1998). In confirmation of this, we observed that both ryanodine receptor-1 and ryanodine receptor-2 are expressed in MIN6 cells, whereas no detectable signal was produced when primers designed to amplify ryanodine receptor-3 were employed in the PCR reactions. These results are exactly in accord with those of Mitchell et al. (2003).

The conclusion that ryanodine receptors are expressed and are functionally important in MIN6 cells was supported by additional studies with the thiol reagent thimerosal. This agent is known to evoke heparin-sensitive increases in $[Ca^{2+}]_i$ in certain cell types (Thorn et al., 1992) suggesting that it can access both ryanodine receptor and IP_3-sensitive Ca^{2+} stores under appropriate conditions. However, the type 3 isoform of the $InsP_3$-receptor predominates in β-cells (Blondel et al., 1993) and this receptor is relatively insensitive to thimerosal (Islam, 2002; Missiaen et al., 1998). Consequently, thimerosal-evoked stimulation of Ca^{2+}-release in pancreatic β-cells can be taken as further evidence of a functional role for ryanodine receptors.

Still more convincing support for the involvement of ryanodine receptor in β-cell Ca^{2+} homeostasis was obtained from experiments with the skeletal muscle relaxant, dantrolene (Xu et al., 1998). This agent effectively blocks Ca^{2+} mobilisation in secretory and neuronal cells (Rossier et al., 1987; Frandsen and Schousboe, 1991) and was suggested to specifically target ryanodine-gated stores in early studies (Palade et al., 1989). This was subsequently confirmed by the direct demonstration that dantrolene inhibits the binding of ryanodine, but not $InsP_3$, to intracellular membrane preparations (Shoshan-Barmatz et al., 1990; Zhao et al., 2001). Indeed, it is now clear that dantrolene binds to ryanodine receptor-1 and ryanodine receptor-3 but not to ryanodine receptor-2 (Zhao et al., 2001). Thus, it can be used to differentiate between Ca^{2+} mobilisation mediated by the various ryanodine receptor isoforms as well as between ryanodine receptor and IP_3-sensitive pools. In the current study, dantrolene inhibited the sustained generation of Ca^{2+} oscillations evoked by harmane, providing direct support for the involvement of ryanodine-gated store release in this response. Moreover, since dantrolene blocks ryanodine receptor-1 and ryanodine receptor-3 but not ryanodine receptor-2 (Zhao et al., 2001), these results further imply that harmane activates ryanodine receptor-1 to mediate its effects in MIN6 cells since neither we, nor others (Mitchell et al., 2003), could find evidence for ryanodine receptor-3 expression. In parallel studies, it was observed that dantrolene also attenuated harmane-induced insulin secretion (Fig. 8) which provides direct evidence that the ryanodine receptor-mediated Ca^{2+} oscillations contribute to the secretory response.

We have recently reported that harmane potentiates glucose-induced insulin secretion from human islets, and can antagonize diazoxide-mediated inhibition of K_{ATP}^+-channel activity (Cooper et al., 2003). These data suggested that harmane might be acting at the level of the imidazoline I_3-receptor in β-cells since imidazoline I_3-receptor agonists are known to exhibit these characteristics (Eglen et al., 1998; Morgan and Chan, 2001). Moreover, harmane has recently been reported to reproduce certain imidazoline receptor-mediated responses in brain (Husbands et al., 2001; Musgrave and Badoer, 2000; Piletz et al., 2000), consistent with the possibility that it may be a ligand for these molecules. In support of this, we also found previously that the insulin secretory response to harmane was inhibited by the imidazoline I_3-receptor antagonist, KU14R (Cooper et al., 2003). Therefore, in the present work we considered it important to investigate the effects of KU14R on harmane-induced Ca^{2+} mobilisation. The results revealed that KU14R (100 μM) reduced the amplitude of harmane-evoked Ca^{2+}-oscillations but that it did not negate this response. Thus, it can be concluded that the generation of Ca^{2+} oscillations by harmane in MIN6 cells resulted from an action that was independent of the imidazoline I_3-receptor and that the ability of KU14R to attenuate harmane-induced insulin secretion must be mediated by an action that lies distal to Ca^{2+} mobilisation. As such, these results are consistent with the recent suggestion that the imidazoline I_3-receptor regulates a late step in the exocytotic pathway that lies downstream of intracellular signal generation (Morgan and Chan, 2001; Chan et al., 2001; Hoy et al., 2003; Efanov et al., 2001).

In order to verify these conclusions, we also studied the Ca^{2+} and insulin secretory responses to the well-characterised imidazoline I_3-receptor agonist, efaroxan, and compared these with harmane. Like harmane, efaroxan increased cytosolic Ca^{2+} in MIN6 cells. However, efaroxan was less effective at generating Ca^{2+} oscillations than harmane (Fig. 2A) and, where these were present, they were smaller in amplitude than those generated by harmane (Fig. 2). Moreover, the Ca^{2+} response subsided rapidly on withdrawal of efaroxan, whereas cells exposed to harmane continued to oscillate for many minutes after removal of the drug. Important differences were also observed when the secretory responses were compared. Most significantly, harmane caused a larger increase in insulin secretion than efaroxan (as seen previously in human islets (Cooper et al., 2003)) and dantrolene attenuated the response to harmane but failed to alter insulin-secretion in response to efaroxan (Fig. 8). In fact, dantrolene caused a reduction in the response to harmane to a level equivalent to that seen with efaroxan.

Taken together, the present data support our earlier conclusion (Cooper et al., 2003; Morgan et al., 2003) that harmane activates at least two distinct mechanisms to promote insulin release. One of these may involve binding to imidazoline I_3-receptors, while a second arises from the interaction of harmane with ryanodine receptor-1, leading to the generation of sustained Ca^{2+} oscillations. The concerted interaction of these two responses then accounts for the ability of harmane to increase insulin secretion beyond the level evoked by efaroxan.

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