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ARTICLE

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Essential role of the imidazoline moiety in the insulinotropic effect but not the K_{ATP} channel-blocking effect of imidazolines; a comparison of the effects of efaroxan and its imidazole analogue, KU14R

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Abstract *Aims/hypothesis:* Imidazolines are a class of investigational antidiabetic drugs. It is still unclear whether the imidazoline ring is decisive for insulinotropic characteristics. *Materials and methods:* We studied the imidazoline efaroxan and its imidazole analogue, KU14R, which is currently classified as an imidazoline antagonist. The effects of both on stimulus secretion-coupling in normal mouse islets and beta cells were compared by measuring K_{ATP} channel activity, plasma membrane potential, cytosolic calcium concentration ($[Ca^{2+}]_c$) and dynamic insulin secretion. *Results:* In the presence of 10 mmol/l but not of 5 mmol/l glucose, efaroxan (100 μmol/l) strongly enhanced insulin secretion by freshly isolated perifused islets, whereas KU14R (30, 100 or 300 μmol/l) was ineffective at both glucose concentrations. Surprisingly, the insulinotropic effect of efaroxan was not antagonised by KU14R. K_{ATP} channels were blocked by efaroxan (IC_{50} 8.8 μmol/l, Hill slope −1.1) and by KU14R (IC_{50} 31.9 μmol/l, Hill slope −1.5). Neither the K_{ATP} channel-blocking effect nor the depolarising effect of efaroxan was antagonised by KU14R. Rather, both compounds strongly depolarised the beta cell membrane potential and induced action potential spiking. However, KU14R was clearly less efficient than efaroxan in raising $[Ca^{2+}]_c$ in single beta cells and whole islets at 5 mmol/l glucose. The increase in $[Ca^{2+}]_c$ induced by 10 mmol/l glucose was affected neither by efaroxan nor by KU14R. Again, KU14R did not antagonise the effects of efaroxan. *Conclusions/interpretation:* The presence of an imidazole instead of an imidazoline ring leads to virtually complete loss of the insulinotropic effect in spite of a preserved ability to block K_{ATP} channels. The imidazole compound is less efficient in raising $[Ca^{2+}]_c$; in particular, it lacks the ability of the imidazoline to potentiate the enhancing effect of energy metabolism on Ca^{2+}-induced insulin secretion.

Keywords Cytosolic calcium concentration · Imidazolines · Insulin secretion · K_{ATP} channel · Pancreatic islets · Plasma membrane potential

Abbreviations $[Ca^{2+}]_c$: cytosolic free calcium concentration · HEK: human embryonic kidney · K_{ATP} channel: ATP-sensitive K^+ channel

Introduction

Imidazolines are a group of investigational oral antidiabetic drugs. Their insulinotropic effect differs from that of sulfonylureas in two respects: (1) insulin secretion is increased only in the presence of stimulatory glucose concentrations; and (2) insulin secretion is increased beyond the extent achieved by sulfonylureas [1]. However, these characteristics do not apply equally to each insulinotropic imidazoline compound [2, 3].

Originally, antagonism at alpha-adrenoceptors was supposed to be the mechanism of action of the prototypical imidazoline, phentolamine [4, 5]. When it became clear that the insulinotropic property of this compound was not due to alpha-antagonism but was related to the presence of an imidazoline moiety [6, 7], it was hypothesised that, like other tissues, beta cells express specific imidazoline binding sites [8–10]. A number of observations supported the view that these binding sites are receptors activating an intracellular signalling cascade, in particular that long-term exposure to imidazolines induces a homologous desensitisation and that enantiomers of efaroxan differ widely in their insulinotropic efficacy [11, 12].

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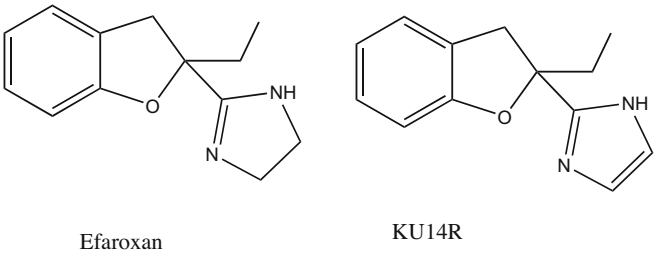
It remained unclear how the functionally defined imidazoline receptor was related to the blocking of K_{ATP} channels exerted by these compounds [13]. The demonstration that phentolamine interacted directly with Kir6.2 (also known as KCNJ11, potassium inwardly rectifying channel, subfamily J, member 11), the pore-forming subunit of the K_{ATP} channel [14], led to the view that the binding site on Kir6.2 was the beta cell imidazoline receptor [1, 15]. However, there are multiple imidazoline binding sites in insulin-secreting cells [9]. Whereas it is likely that Kir6.2 is one of them [16], it is accepted that additional effects are essential for the specific secretory characteristics of the imidazolines [17], which are hard to explain solely by the closure of K_{ATP} channels.

Thus, a specific antagonist of the insulinotropic imidazolines is a valuable tool to elucidate the signalling mechanisms underlying therapeutically favourable characteristics. KU14R, the imidazole analogue of efaroxan (Fig. 1), was reported to abolish the enhancement of insulin secretion produced by a number of imidazolines, such as efaroxan, RX 821002 and phentolamine. This antagonism was ascribed to KU14R-induced opening of K_{ATP} channels which had been closed by imidazolines [18]. It was also found that KU14R had an inhibitory effect on K_{ATP} channels in inside-out patches from beta cells, but it was concluded that this effect was too weak to stimulate insulin secretion [18]. Consequently, this compound was classified as a weak partial agonist that is capable of antagonising the effects of the pure imidazoline agonists at the hypothetical imidazoline receptor (I3 site) of pancreatic beta cells [10, 19].

In a recent investigation, KU14R was used to delineate the mechanisms of several insulinotropic drugs. Unexpectedly, KU14R did not antagonise the effect of efaroxan, but rather enhanced it [20]. This lack of antagonism could well be due to the peculiarities of the BRIN BD11 cell line employed in this study [19]. However, KU14R also decreased blood glucose levels in mice, suggesting that it could also have an insulinotropic effect on normal islets [21]. We now sought to clarify how efaroxan and its imidazole analogue differ in their ability to affect the beta cell signalling pathways leading to enhanced secretion. Implicitly, this meant testing whether the group name ‘imidazolines’ is a misnomer or refers to a pharmacologically relevant moiety of these insulin secretagogues.



Efaroxan KU14R

Fig. 1 Structural formulae of efaroxan and KU14R. KU14R differs from efaroxan in the presence of an imidazole instead of an imidazoline moiety [18]. The second double bond in the imidazole ring leads to delocalised π-electrons. However, this leaves the spatial arrangement of the molecule practically unchanged

Materials and methods

Chemicals

Efaroxan and KU14R were purchased from Tocris-Cookson (Bristol, UK). Collagenase P was supplied by Roche (Mannheim, Germany) and fura-2/AM by Molecular Probes (Leiden, the Netherlands). Cell culture medium RPMI 1640 was purchased from Gibco BRL (Gaithersburg, MD, USA) and fetal calf serum from Biochrom (Berlin, Germany). All other reagents of analytical grade were from E. Merck (Darmstadt, Germany).

Tissue culture

Islets were isolated from the pancreas of NMRI mice by a collagenase digestion technique and hand-picked under a stereomicroscope. Single cells were obtained by incubation of the islets for 10 min in a Ca^{2+}-free medium and subsequent vortex-mixing for 1 min. Single islet cells and human embryonic kidney (HEK) cells stably transfected with *Kir6.2* ΔC26 were cultured in cell culture medium RPMI 1640 (5 mmol/l glucose) with 10% fetal calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Electrophysiological recordings

K_{ATP} channel activity was measured by a standard patch-clamp technique using the cell-attached and whole-cell configurations [22]. Pipettes were pulled from borosilicate glass (outside diameter 2 mm, inside diameter 1.4 mm; Hilgenberg, Malsfeld, Germany) by a two-stage vertical puller (List Electronic, Darmstadt, Germany) and had resistances between 3 and 6 MΩ when filled with solution. Currents were recorded with an EPC 7 patch-clamp amplifier (List Electronic), and low pass-filtered with a four-pole Bessel filter at 2 kHz and stored on a video tape. The pipette holding potential was 0 mV in cell-attached and −70 mV in whole-cell recordings. In whole-cell recordings currents were evoked by hyperpolarising and depolarising steps of 10 mV [23]. The membrane potential of beta cells was determined using the whole-cell or perforated patch mode under current-clamp conditions. The patch perforation was achieved with 100 μg/ml nystatin in the pipette solution [24]. The compositions of the bath and pipette media were as given by Zünkler et al. [25]. All experiments were performed at room temperature (21–23°C). Measurement of Kir6.2 ΔC26 channels was performed as described recently [16]. Data were analysed off-line using pClamp 6.03 software (Axon Instruments, Foster City, CA, USA).

Microfluorimetric measurements of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)

Islet cells and intact islets from NMRI mice were cultured on glass coverslips in Petri dishes and were used from day 2 to day 4 after isolation. Fura-2/AM was loaded at a concentration of 2 μmol/l for 30 min (cells) or 45 min (islets) at 37°C. The coverslip with the attached cells or islets was inserted in a purpose-made perifusion chamber on the stage of an epifluorescence microscope fitted with a Zeiss Fluar (40×) objective. The fluorescence (excitation at 340 or 380 nm, emission at >470 nm) was recorded by a slow-scan CCD camera (Till Photonics, Gräfelfing, Germany). All perifusions were performed at 34°C using a HEPES-buffered Krebs–Ringer bicarbonate medium. Image pairs were taken at intervals as indicated in the figures; illumination time for each image was 800 ms.

Measurement of insulin secretion

Batches of 50 NMRI mouse islets were perifused at 37°C with a HEPES-buffered Krebs–Ringer medium containing the respective secretagogue. The insulin content in the fractionated effluate was determined by ELISA (Mercodia, Uppsala, Sweden).

Data handling and statistics

Statistical calculations were performed using Prism and Instat software (GraphPad, San Diego CA, USA). If not specified otherwise, differences were considered significant if $p<0.05$.

Results

To test for antagonism between efaroxan and KU14R at the level of insulin secretion, the compounds were used at equimolar concentrations. When 100 μmol/l efaroxan was added to a perifusion medium containing 10 mmol/l glucose, insulin secretion from freshly isolated islets increased steadily, achieving a plateau within 20 min. The secretion rate at steady state amounted to 480% of the prestimulatory value. The additional presence of 100 μmol/l KU14R for 20 min did not affect the rate of secretion (Fig. 2a).

In the presence of 5 mmol/l glucose, neither 100 μmol/l efaroxan nor 100 μmol/l KU14R increased the rate of insulin secretion from freshly isolated islets (Fig. 2b). Raising the glucose concentration from 5 to 10 mmol/l in the continued presence of efaroxan led to a strong, biphasic increase in secretion, whereas raising glucose in the presence of 30, 100 or 300 μmol/l KU14R resulted in a transient, monophasic increase in secretion. Within 10 min the secretory rate receded to a plateau which was not significantly higher than the secretion in the presence of 5 mmol/l glucose alone (Fig. 2b). Comparing the integrals

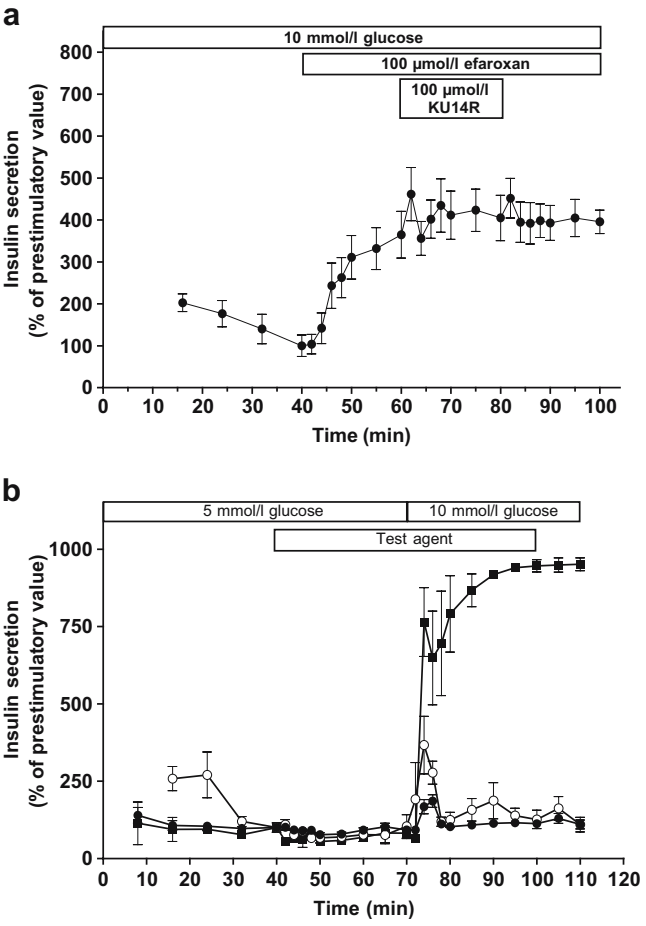


Fig. 2 Insulinotropic characteristics of efaroxan and KU14R. **a** Effects of efaroxan and of efaroxan and KU14R combined on insulin secretion. Mouse islets were perifused with Krebs–Ringer medium containing 10 mmol/l glucose throughout the experiment. From 40 to 100 min, 100 μmol/l efaroxan was present, and from 60 to 80 min 100 μmol/l efaroxan and 100 μmol/l KU14R were present. Values are means±SEM of four experiments. **b** Glucose-dependent stimulatory effect of efaroxan and KU14R on insulin secretion. Freshly isolated islets were perifused with Krebs–Ringer medium containing 5 mmol/l glucose for 40 min. From 40 to 100 min the medium contained either 100 μmol/l efaroxan (closed squares) or 30 μmol/l KU14R (open circles) or 300 μmol/l KU14R (closed circles). From 70 to 110 min the glucose concentration was increased to 10 mmol/l. Secretion in the presence of 100 μmol/l KU14R was virtually identical to that in the presence of 30 μmol/l KU14R. Values are means±SEM of three (efaroxan) or four (KU14R) experiments

of secretion in the presence of the secretagogues, raising glucose from 5 to 10 mmol/l increased insulin secretion to 1308% in the presence of efaroxan, but only to 226% in the presence of KU14R. Even when KU14R was used at 300 μmol/l, a concentration sufficient to completely block K_{ATP} channels in intact beta cells (see below), the secretory response remained as modest as with the lower concentrations (Fig. 2b). In view of the virtual inability of KU14R to potentiate glucose-induced insulin secretion, the effects of both compounds on typical parameters of stimulus-secre-

tion coupling were compared to localise the step at which the imidazoline moiety is essential and cannot be replaced by the closely similar imidazole ring.

Concentration-dependency of the K_{ATP} channel blocking effect of efaroxan and KU14R was set up using the whole-cell mode of the patch-clamp technique. The effect of both drugs was reversible during washout (Fig. 3a) and both efaroxan and KU14R, achieved a virtually complete block of K_{ATP} channel activity. The IC_{50} concentration for efaroxan was 8.8 μmol/l with a Hill slope of −1.1; the corresponding values for KU14R were 31.9 μmol/l and −1.5. At 300 μmol/l, efaroxan reduced channel activity to 16.6±1.7% (n=3) and KU14R to 14.9±2.2% (n=4, Fig. 3b). For comparison, 500 μmol/l tolbutamide reduced K_{ATP} channel activity to 18.0±2.2% (n=3) under the same conditions.

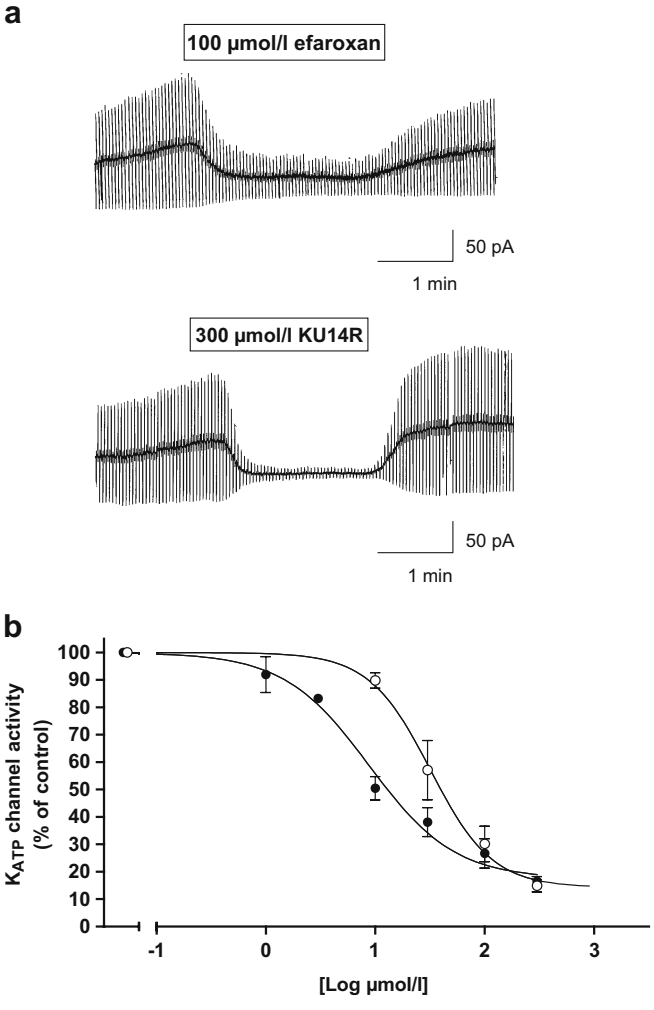


Fig. 3 K_{ATP} channel-blocking effect of efaroxan and KU14R as measured in the whole-cell configuration of the patch-clamp technique. **a** Original recordings of the effects of efaroxan (100 μmol/l) and KU14R (300 μmol/l). Currents were evoked by hyperpolarising and depolarising steps of 10 mV from a holding potential of −70 mV. **b** Concentration dependence of the K_{ATP} channel-blocking effect. Values are expressed as percentages of control and are means±SEM of four to six experiments. The maximal effect was the same for efaroxan and KU14R and corresponded to the inhibitory effect of 500 μmol/l tolbutamide

In intact mouse beta cells, 100 μmol/l KU14R had a marked inhibitory effect on K_{ATP} channel activity (reduction to 49.6±10.5% of control), which was significantly weaker (p<0.05, t-test) than the effect of an equimolar concentration of efaroxan (19.1±5.3%). At 300 μmol/l, however, KU14R achieved a practically complete block of channel activity (2.1±1.2%, n=4) in intact beta cells, whereas efaroxan at 300 μmol/l was somewhat less effective (8.8±2.4%, n=9). The combined presence of efaroxan and KU14R (100 μmol/l each) resulted in a reduction in channel activity (11.7±4.6%, n=6) which was not significantly different from that elicited by 100 μmol/l efaroxan alone. Both compounds inhibited the activity of Kir6.2 ΔC26 channels expressed in HEK cells. Using the cell-attached mode, 100 μmol/l KU14R reduced channel activity to 45.2±10.3% of control, which was significantly weaker (p<0.05, t-test) than the effect of 100 μmol/l efaroxan (reduction to 21.4±4.5%, n=5 each).

Measured under current-clamp condition in the whole-cell mode, the resting beta cell membrane potential was −73.4±1.2 mV (n=16). Efaroxan 100 μmol/l reduced the membrane potential to −28.6±3.4 mV (n=7) and 100 μmol/l KU14R reduced it to −33.4±4.0 mV (n=9) (Fig. 4a and b). KU14R 100 μmol/l, when added to beta cells which had been depolarised by 100 μmol/l efaroxan, did not repolarise the membrane potential. Rather, there was a slight further depolarisation to −24.9±3.8 mV (n=7) (Fig. 4a and b). KU14R also had no repolarising effect on beta cells depolarised by 10 or 100 μmol/l of the imidazolines phentolamine and alinidine (data not shown).

When the membrane potential was measured in quasi-intact cells using the perforated patch mode, 100 μmol/l efaroxan depolarised the beta cell membrane from −70.5±2.3 mV to a plateau value of −52.3±3.3 mV, whereas 100 μmol/l KU14R depolarised it from −65.5±2.5 to −59.5±4.1 mV only (n=6 each). Thus, in intact but not in open beta cells 100 μmol/l KU14R was significantly (p<0.05, t-test) less effective than 100 μmol/l efaroxan on depolarising the plasma membrane (Fig. 4c). At 300 μmol/l, however, KU14R and efaroxan were equally effective in depolarising the membrane of intact beta cells (−44.9±2.9 and −46.6±2.7 mV, respectively, n=4 each) (Fig. 4c). Whereas the depolarisation by efaroxan displayed an oscillatory pattern in about half of the perforated patch experiments, such a response was only seen in two out of eight experiments with KU14R.

In perifused fura 2-loaded islets, 100 μmol/l efaroxan induced slow, large-amplitude oscillations of the cytosolic calcium concentration ($[Ca^{2+}]_c$). These oscillations persisted when the glucose concentration was raised from 5 to 10 mmol/l (Fig. 5a). KU14R 100 μmol/l was virtually ineffective in raising $[Ca^{2+}]_c$ in the presence of 5 mmol/l glucose, but increasing the glucose concentration in the continued presence of KU14R produced the typical large-amplitude oscillations of $[Ca^{2+}]_c$ (Fig. 5b). With 300 μmol/l KU14R an increase in $[Ca^{2+}]_c$ in the presence of 5 mmol/l glucose was obvious, but this effect was still less marked than that produced by 100 μmol/l efaroxan (Fig. 6a). This was also true when KU14R was applied before efaroxan

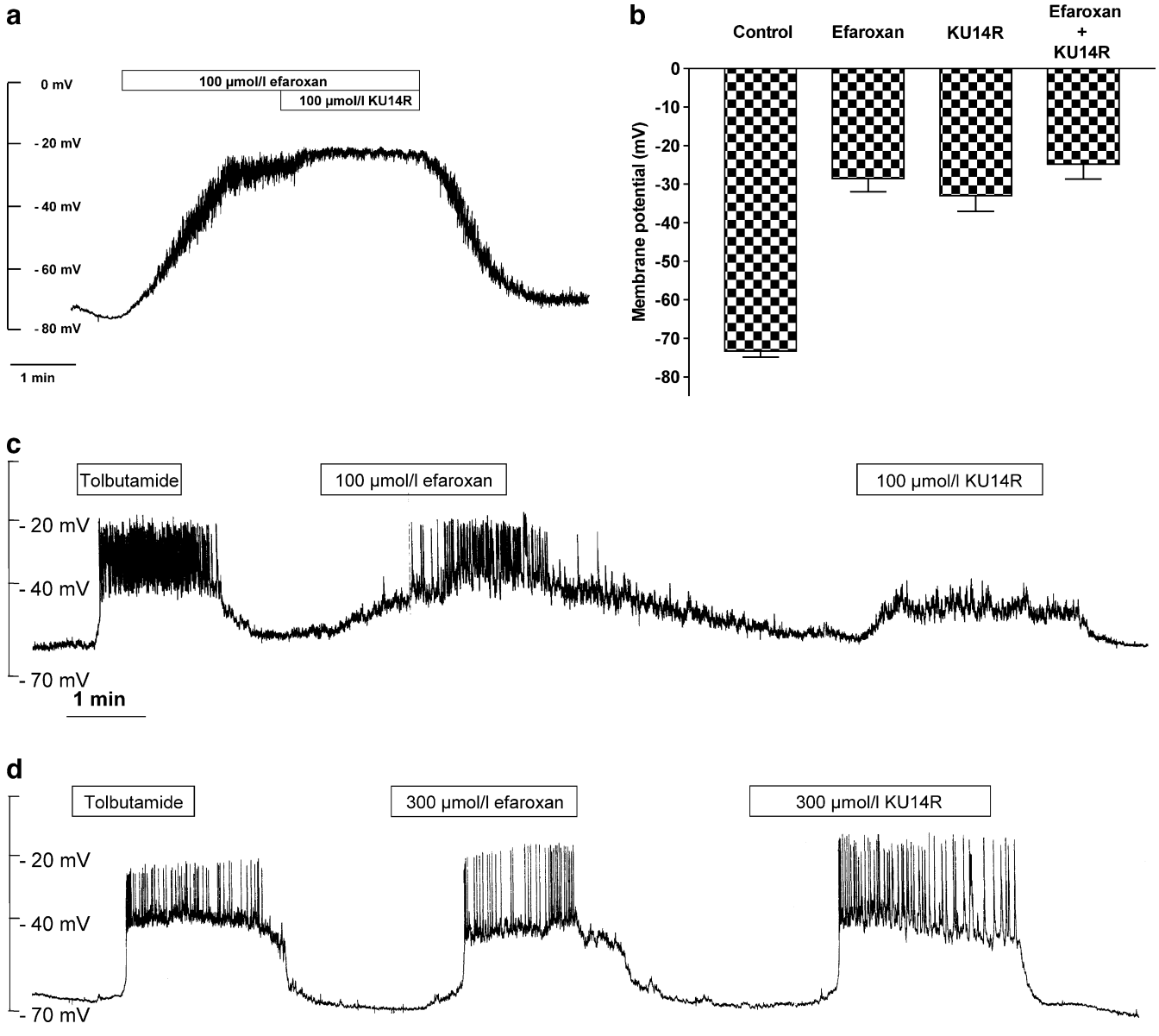


Fig. 4 Depolarisation of the membrane potential of mouse pancreatic beta cells by efaroxan and KU14R. **a** Original recording of the membrane potential of mouse pancreatic beta cells in the conventional whole-cell configuration. Within 4 min, 100 µmol/l efaroxan strongly depolarised the beta cell membrane. The additional presence of 100 µmol/l KU14R led to a small further depolarisation. Washout led to virtually complete repolarisation. **b** KU14R 100 µmol/l does not repolarise the membrane potential depolarised by 100 µmol/l efaroxan. Data were obtained from measurements as shown in **a** and are means±SEM of seven experiments. **c**, **d** Original recordings of the membrane potential of mouse pancreatic beta cells in the perforated patch configuration. At 100 µmol/l (**c**), the depolarisation produced by KU14R was clearly weaker than that produced by efaroxan. At 300 µmol/l, KU14R elicited a depolarisation similar to that by 300 µmol/l efaroxan (**d**). Representative traces of five experiments

(not shown). Essentially the same observations were made when $[Ca^{2+}]_c$ was measured in single perifused beta cells. Efaroxan increased $[Ca^{2+}]_c$ in the absence of glucose as well as in the presence of 5 and 10 mmol/l glucose. The increase in $[Ca^{2+}]_c$ produced by KU14R, even at 300 µmol/l, was much less marked than that produced by efaroxan under comparable conditions (data not shown).

With both drugs the $[Ca^{2+}]_c$ increase was oscillatory, in contrast to the $[Ca^{2+}]_c$ increase produced by K^+ depolarisation (Fig. 6a). The reason why KU14R elicited a comparatively small increase in $[Ca^{2+}]_c$ in spite of marked depolarisation was investigated by measuring the effect of KU14R on the increase in $[Ca^{2+}]_c$ elicited by K^+ depolarisation. While efaroxan left the typical $[Ca^{2+}]_c$ pattern unchanged, KU14R induced a moderate, reversible decrease in the steady-state $[Ca^{2+}]_c$ level (Fig. 6b). When $[Ca^{2+}]_c$ levels in islets had been elevated by 100 µmol/l efaroxan, KU14R had no decreasing effect (Fig. 6c). This observation could be confirmed by measuring $[Ca^{2+}]_c$ in single perifused beta cells: there was no reduction but also

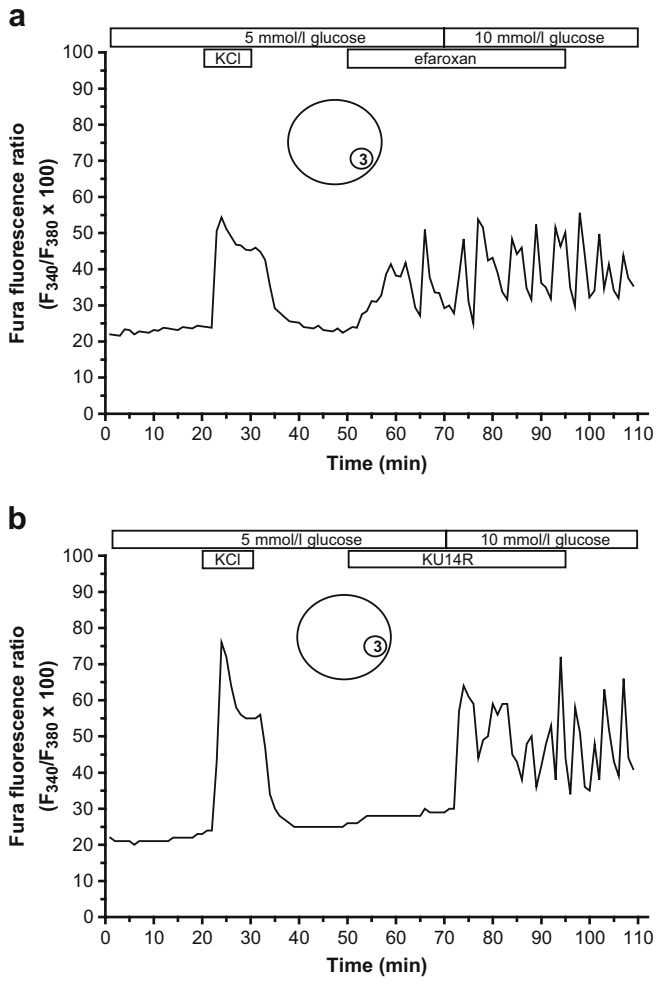


Fig. 5 Comparison of the effects of equimolar concentrations of efaroxan and KU14R on $[Ca^{2+}]_c$ of perifused pancreatic islets. Fura 2-loaded islets were perifused with Krebs–Ringer medium containing 5 mmol/l glucose. After depolarisation with 40 mmol/l K^+ for 10 min, the islets were perifused with 100 μmol/l efaroxan (**a**) or 100 μmol/l KU14R (**b**). In the continued presence of the drugs, the glucose concentration was raised from 5 to 10 mmol/l. This perifusion protocol corresponds to that employed in the secretion measurements. A recording from a single islet subregion is shown, demonstrating the oscillatory character of the $[Ca^{2+}]_c$ increase. Traces are representative of four experiments each

no further increase in $[Ca^{2+}]_c$ when 100 μmol/l KU14R was added to a perifusion with 100 μmol/l efaroxan in the presence of 5 or 10 mmol/l glucose (data not shown).

Discussion

Originally, KU14R was reported to abolish the insulinotropic effect of efaroxan and other imidazolines at

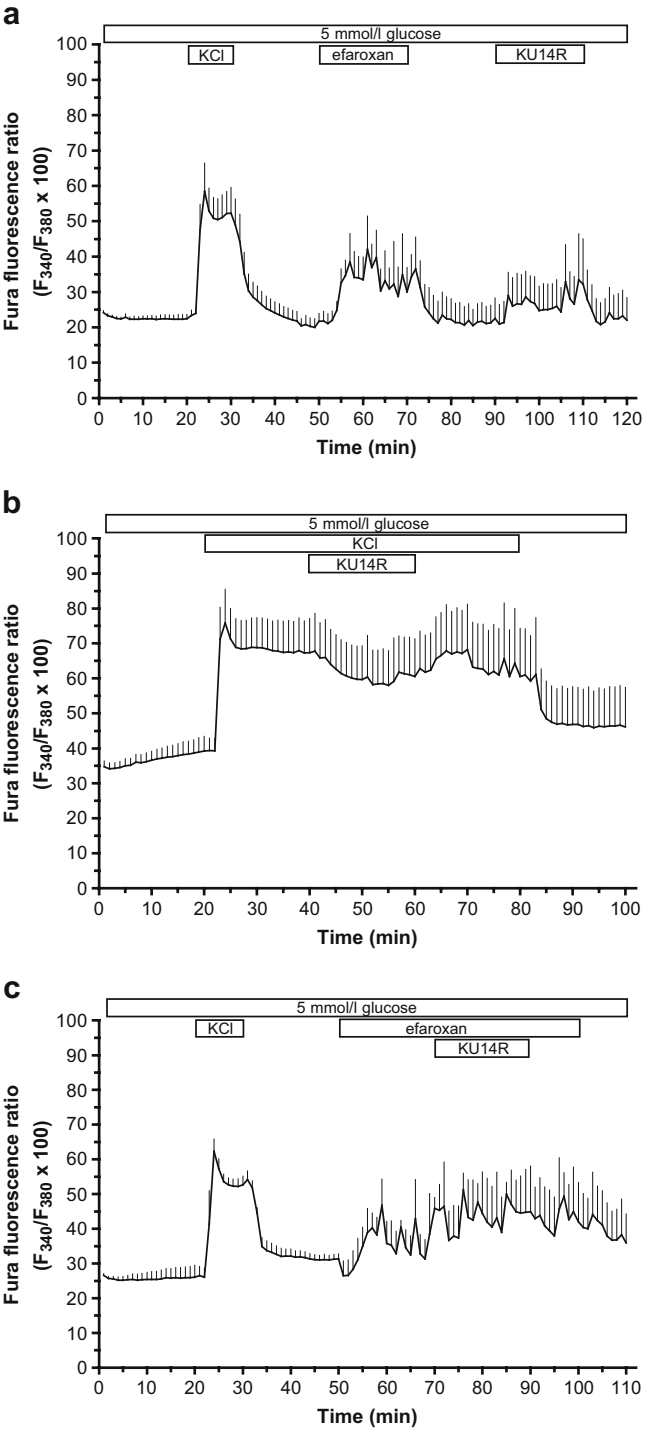


Fig. 6 Effects of efaroxan and a strongly depolarising KU14R concentration on $[Ca^{2+}]_c$ of perifused pancreatic islets. Fura 2-loaded islets were perifused with Krebs–Ringer medium containing 5 mmol/l glucose throughout the experiments. **a** After depolarisation with 40 mmol/l K^+ for 10 min, the islets were perifused with 100 μmol/l efaroxan and subsequently, after a 20-min interval, with 300 μmol/l KU14R. Data are means±SEM of five experiments. **b** From 20 to 80 min, the K^+ concentration was increased to 40 mmol/l. From 40 to 60 min, 300 μmol/l KU14R was additionally present. Data are means±SEM of six experiments. **c** Islets were perifused with Krebs–Ringer medium containing 5 mmol/l glucose throughout. From 50 to 100 min the perifusion medium contained 100 μmol/l efaroxan. From 70 to 90 min 300 μmol/l KU14R was additionally present. Data are means±SEM of four experiments

equimolar concentrations on statically incubated rat islets [18]. In the present investigation no antagonism between KU14R and efaroxan could be found. The virtually unchanged rate of secretion of perifused mouse islets when KU14R was added to efaroxan (100 μmol/l each) corresponded to the unchanged K_{ATP} channel activity, membrane potential and $[Ca^{2+}]_c$ under the same conditions. On the other hand, we did not find an additive effect of KU14R and efaroxan on insulin secretion, as was recently found in in vitro experiments [20] and indirectly shown in vivo [21]. The typical modest secretory response of mouse islets to an increase in the glucose concentration from 5 to 10 mmol/l was practically unchanged in the presence of KU14R, whereas the presence of efaroxan transformed this response into a strong, biphasic increase in secretion. Thus, the question of why the secretagogue properties differ considerably even though the two compounds differ only by one minor structural feature—the presence of one additional double bond (Fig. 1)—required a new answer.

Previous work suggested that the essential difference between KU14R and efaroxan lies in their ability to inhibit K_{ATP} channel activity [18]. By setting up a concentration dependency in the whole-cell configuration, we found that KU14R, like efaroxan, is able to completely block K_{ATP} channels in normal mouse beta cells. In this context it must be mentioned that about 15% of the current amplitude elicited by our stimulation protocol in these experiments cannot be blocked by high sulfonylurea concentrations and is probably not due to K_{ATP} channel activity [26]. Our IC_{50} value for the efaroxan block (9 μmol/l) is in good agreement with an early estimate [27], though in some later investigations the channel-blocking effect of efaroxan appeared to be considerably more potent [18]. The Hill coefficient was very close to unity, suggesting that efaroxan interacts with one site on the K_{ATP} channel. This site is probably located on the Kir6.2 subunit, as can be concluded from the ability of efaroxan to inhibit the activity of heterologously expressed Kir6.2 ΔC26 channels.

The somewhat lower potency and steeper concentration-dependency of the K_{ATP} channel block by KU14R may be explained by a lower affinity of KU14R for the binding site on Kir6.2 in conjunction with greater accumulation in the membrane, resulting from the higher lipophilicity of the imidazole ring. Since KU14R, like efaroxan, blocked Kir6.2 ΔC26 channels with a similar potency to that for native K_{ATP} channels, it can be assumed that the mechanism of K_{ATP} channel block by the imidazole compound is essentially the same as that described previously for a number of imidazolines [14, 16, 28]. In dialysed cells (whole-cell mode) as well as in intact cells (cell-attached mode), KU14R was somewhat less effective than efaroxan at 100 μmol/l but at least equally effective at 300 μmol/l. Apparently, the K_{ATP} channel-blocking property of both compounds is not modulated by an imidazoline-sensitive signalling system, which would be operative in intact but not in dialysed beta cells.

The membrane potential changes induced by both secretagogues closely reflected the changes in K_{ATP} channel activity. In the conventional whole-cell mode, 100 μmol/l KU14R was only slightly less efficient than 100 μmol/l efaroxan in depolarising the plasma membrane, corresponding to a slightly lower inhibitory effect on K_{ATP} channels in this configuration. The more marked difference at 100 μmol/l in the perforated patch mode corresponded to the respective effects on K_{ATP} channels in intact cells, as did the virtual equal efficiency when both compounds were tested at 300 μmol/l. Apparently, KU14R does not induce an ion channel activity that counteracts the depolarising effect of K_{ATP} channel closure. This is in agreement with the observation that KU14R, like efaroxan, is an antagonist at alpha 2-adrenoceptors [18, 21].

Thus, the difference between the insulinotropic effects of KU14R and efaroxan is located at a step distal to depolarisation of the plasma membrane. In fact, KU14R was clearly less effective than efaroxan in raising $[Ca^{2+}]_c$, even at a concentration at which both compounds completely blocked K_{ATP} channel activity. Since this observation was made in whole islets as well as in single beta cells, it appears unlikely that efaroxan and KU14R differ primarily in their effects on non-beta cells, which would then indirectly lead to different insulinotropic characteristics. On the other hand, the straightforward explanation that KU14R has a blocking effect on voltage-dependent Ca^{2+} channels in beta cells, thereby diminishing the depolarisation-induced Ca^{2+} influx, is not easily compatible with the observation that the efaroxan-induced $[Ca^{2+}]_c$ increase and insulin secretion were not reduced by KU14R. Furthermore, the spiking activity during a depolarisation phase, which is regarded as an expression of Ca^{2+} channel activity, was not diminished when the depolarisation was caused by KU14R at a maximally effective concentration. However, when $[Ca^{2+}]_c$ levels were increased by a strongly depolarising K^+ concentration, there was a moderate reduction in $[Ca^{2+}]_c$ by KU14R, but not efaroxan. So, it is possible that efaroxan and KU14R differ in their ability to influence intracellular Ca^{2+} transport. In this context it should be mentioned that the imidazoline RX871024 was found to induce Ca^{2+} release from thapsigargin-sensitive stores [29].

Our results suggest that there must be an additional step in stimulus-secretion coupling at which the effects of efaroxan and KU14R differ. The large increase in secretion when raising glucose from 5 to 10 mmol/l in the presence of efaroxan cannot be attributed to a further increase in $[Ca^{2+}]_c$. Rather, it can be hypothesised that efaroxan enhances the metabolic signal from the amplifying pathway, which requires elevated $[Ca^{2+}]_c$ as a permissive condition [30]. Since the increase in $[Ca^{2+}]_c$ produced by raising glucose from 5 to 10 mmol/l was not impaired by KU14R, the lack of secretion enhancement under this condition points to an additional decisive difference between the imidazoline and the imidazole compound: in the terms of the above hypothesis, KU14R would not enhance the metabolic signal. Another observation pertaining to the imidazoline effects on exocytosis is that recently we described a dissociation between an efaroxan-induced increase in $[Ca^{2+}]_c$ and a lack of stimulated secretion in freshly isolated mouse islets when the glucose concentration was 5 mmol/l [3]. This is confirmed by the present

study. Earlier investigations reported a moderate increase in secretion by efaroxan at 5 mmol/l glucose. However, rat islets were used in these investigations, which are more sensitive to glucose than mouse islets [31, 32].

The demonstration that the insulinotropic property of α-adrenoceptor ligands was not correlated with the α-antagonistic property but with the presence of an imidazoline moiety in the molecule [6, 33] was the basis for the group name of insulinotropic imidazolines. This has led to the implicit assumption that these insulin secretagogues have a homogeneous pharmacological profile, which is not the case [2, 3]. Also, it remained unclear whether the imidazoline moiety was essential for the insulinotropic characteristics. In fact, there are structurally closely related guanidinium compounds which bind to non-adrenergic imidazoline sites in insulin-secreting cells and close K_{ATP} channels [9]. One guanidinium compound with insulinotropic property (BTS 67 582) was explored as an oral antidiabetic agent, assuming that it acts as an imidazoline analogue [20, 34, 35]. On the other hand, several imidazoline compounds have been synthesised recently that do not inhibit K_{ATP} channel activity, but nevertheless stimulate insulin secretion [36, 37]. Whether the insulinotropic effect of these second-generation imidazolines [38] is simply due to the K_{ATP} channel-independent mechanism of the conventional imidazolines [17, 38] or represents something new is unclear thus far.

The present investigation shows that the imidazoline moiety is in fact decisive for the insulinotropic characteristics, but not for K_{ATP} channel block. While the block of the K_{ATP} channel block via Kir6.2 permits considerable structural variability, there are two additional steps in stimulus-secretion coupling of the beta cell, at which the minor structural difference between an imidazoline and an imidazole compound is recognised with major functional consequences. One step is the depolarisation-induced increase in cytosolic calcium and the other is the metabolism-dependent enhancement of secretion. At the former step the imidazole compound is inhibitory, whereas the imidazoline compound is probably without effect; consequently this step may involve an imidazole—but not an imidazoline—specific site. At the latter step the imidazoline potentiates the enhancing effect of energy metabolism on Ca^{2+}-induced secretion, whereas the imidazole is apparently without effect. It appears that an imidazoline-specific binding site exists, which is responsible for the therapeutically interesting features of this class of compounds. A better understanding of the amplifying pathway of insulin secretion [30] should help to identify the molecular target.

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Duality of Interest The authors declare that they have no conflicts of interest.

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