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The effects of ZD6169 on the ATP-dependent K^+ current ($I_{K_{ATP}}$) in isolated cat ventricular myocytes

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Abstract

The effect of the K_{ATP} channel opener ZD6169 [(*S*)-*N*-(4-benzoyl-phenyl)-3,3,3-trifluoro-2-hydroxy-2-methyl-propionamide] currently under development for the treatment of urinary incontinence was explored in acutely isolated adult feline ventricular myocytes. ZD6169 activated a current over a wide range of concentrations (0.1–100 μM) that is completely blocked by 10 μM glyburide thereby identifyinga it as $I_{K_{ATP}}$. The maximum activation of K_{ATP} current was observed at 10 μM; higher concentrations decreased current activation. In contrast, the standard K_{ATP} channel opener cromakalim showed a more usual concentration–response relationship, with increasing current for increased concentrations and no signs of saturation or reversal. The bell-shaped dose–response relationship for ZD6169 activation of $I_{K_{ATP}}$ has also been seen in bladder myocytes, albeit at a lower concentration, and it has been proposed to contribute to the reported lack of in vivo cardiovascular side effects. We compared the effects of ZD6169 to cromakalim and showed that both compounds dramatically shorten cardiac myocyte action potential duration and that ZD6169 does so in spite of the bell-shaped concentration–response relationship for activation of K_{ATP} current.

Keywords: K_{ATP}; $I_{K_{ATP}}$; Cardiac myocyte; Cromakalim; K^+ channel

1. Introduction

Urge urinary incontinence is characterized by abnormal spontaneous detrusor contractions that are often unrelated to the urine volume stored in the bladder. These contractions can produce a chronic sensation of urgency, and result in involuntary urine loss. Urge incontinence accounts for 35–65% of all incontinence cases, depending upon the age of the patient (Resnick, 1995). It has been suggested that a K^+channel opener of detrusor smooth muscle cells would be a useful treatment for urge incontinence (Foster et al.,1989). Activation of K_{ATP} channels present on the smooth muscle cells of the detrusor has been shown to hyperpolarize membrane potential, thus decreasing the probability of opening voltage-dependent Ca^{2+} channels (Zografos et al., 1992; Bonev and Nelson, 1993). This would in turn reduce Ca^{2+} entry and muscle contraction and relax the hyperactive bladder (Quast, 1993; Li et al., 1995).

ZD6169 [(*S*)-*N*-(4-benzoyl-phenyl)-3,3,3-trifluoro-2-hydroxy-2-methyl-propionamide] has been developed along these lines as a selective opener of K_{ATP} channels in bladder. A bell-shaped concentration–response relationship for ZD6169 has been reported for guinea pig detrusor K_{ATP} channels (Hu and Kim, 1997). The authors proposed that the unusual concentration–response relationship might account for the modest in vivo hemodynamic effects reported with ZD6169 (Howe et al., 1995). However, the effects of ZD6169 on cardiac K_{ATP} channels are still unknown. In this study, we used the patch-clamp technique on isolated feline ventricular myocytes to examine the effects of ZD6169 on ionic currents and action potential. We also compared ZD6169 to cromakalim, a nonselective K_{ATP} channel opener.

2. Methods

2.1. Isolation of cat ventricular myocytes

Ventricular myocytes were isolated from 2.5- to 3-kg cats by collagenase dissociation according to a modification of the Langendorff coronary perfusion method (Spinelli et al., 1993). The cells were cultured for up to 2 days in

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DMEM/F-12 (1:1 mix) culture media (Bio Whittaker, Walkersville, MD, USA) supplemented with streptomycin sulfate (200 μg/ml) plus penicillin-G sodium salt (200 units/ml). The cells solution were maintained at pH = 7.2 in an incubator at room temperature and they were studied within 2 days. Culturing under these conditions produced no changes in ionic currents or action potential parameters. The average cell capacitance of the myocytes was 106 ± 1 pF ($n = 26$) and did not vary upon storage. The protocol used for myocyte isolation was approved by the Wyeth-Ayerst Institutional Animal Care and Use Committee and was performed in accordance with the guidelines of the Animal Welfare Act and the American Association for Accreditation of Laboratory Animal Care.

2.2. Patch-clamp recording

Dialysis pipette techniques were used to record whole-cell current. Pipettes were made from square-bore borosilicate capillary tubing (1.0 or 1.5 mm OD) with resistance of 2–3 MΩ when filled with recording solution. The pipette solution contained (mM): 120 K-aspartate, 20 KCl, 2 $MgCl_2$, 5 EGTA, 5 ATP-K_2, 10 HEPES, 5 phosphocreatine-Na_2. The pH was adjusted to 7.2 by addition of KOH. Free $[Mg^{2+}]_i$ was calculated to be 46 μM. Outward currents and action potentials were recorded during superfusion of the myocyte with HEPES Tyrode's solution (138 NaCl, 4 KCl, 2 $CaCl_2$, 0.5 $MgCl_2$, 5 $NaHCO_3$, 1.6 NaH_2PO_4, 5.5 Glucose, 10 HEPES, pH = 7.35) at 37°C. Perfusion of myocytes with ZD6169 or cromakalim activated $I_{K_{ATP}}$ within 1 min and reached steady state in 5 min. Cells were clamped at −50 mV and ionic currents were activated by a voltage-ramp protocol (from −100 mV to +50 mV at a speed of 30 mV/s). Ionic currents were amplified with an Axopatch-200B and filtered at 1 kHz. Action potentials were elicited at a frequency of 1 Hz by injection of a brief depolarizing current pulse. Data acquisition and analysis were performed using pCLAMP6 software. Statistics were obtained using Microsoft EXCEL and graphs plotted with Microcal ORIGIN Software. All data are reported as mean ± S.E.M.

2.3. Compounds

ZD6169 was synthesized in-house. Glyburide and cromakalim were obtained from Sigma Chemical (St. Louis, MO). Drugs were dissolved in dimethylsulfoxide (DMSO) to prepare a 10 mM stock. Final concentrations were prepared by dilution with the Tyrode's solution and the final concentration of DMSO was less than 1%.

3. Results

Fig. 1 shows representative results from a cardiac myocyte where ionic current elicited by voltage-ramps and

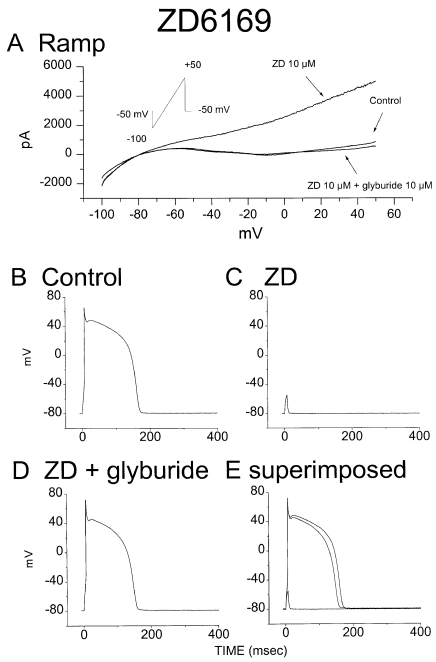

Fig. 1. ZD6169 activates $I_{K_{ATP}}$ current in isolate cardiac myocytes. At 10 μM ZD6169 activates a large outward current which reverses near −80 mV and is blocked by 10 μM glyburide (A). Action potentials could not be triggered in the presence of 10 μM ZD6169 (C) but were restored when glyburide (10 μM) was also added (D). Panel E summarizes the results by showing superimposed action potential traces. (A–E) Representative results from the same cell.

action potentials were recorded from the same cell in the presence and absence of ZD6169. A linear outward current was activated after 5 min of superfusion with ZD6169 (10 μM). The outward current reversed near −80 mV and was sensitive to glyburide, which has been shown to be a selective blocker of K_{ATP} current ($I_{K_{ATP}}$) in the heart (Sanguinetti et al., 1988). The effects of ZD6169 on the cardiac action potential were also explored by periodically switching to current clamp throughout the experiment. Fig. 1B shows a control action potential prior to drug treatment with the characteristic plateau region seen in cat (Spinelli et al., 1993). Perfusion with ZD6169 (10 μM) shunted the cell to the K^+ equilibrium potential such that no action potential could be elicited. Addition of glyburide (10 μM) blocked the underlying K_{ATP} current seen in Fig. 1A and restored the cells ability to generate an action potential. Fig. 1E summarizes the results by showing superimposed action potential traces. The changes in action potential therefore paralleled the changes in outward current in this and another cell studied with the same protocol.

Fig. 2 shows representative results from another cell where ionic current ramp traces and action potentials were recorded in the presence of increasing concentrations of ZD6169. The cell was switched between current clamp and voltage clamp at the various concentrations tested. Under voltage clamp, in control conditions, a voltage ramp from −100 to +50 mV lasting 5 s elicited a small outward

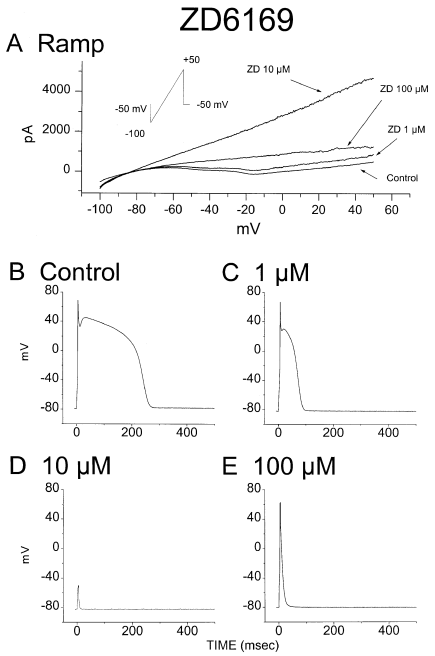

Fig. 2. ZD6169 activates $I_{K_{ATP}}$ in a dose dependent manner. (A) shows ionic current in response to a voltage ramp in the presence of ZD6169 (1, 10, and 100 μM). The outward current increases with increasing doses up to 10 μM and then decreases. The voltage ramp protocol is shown in the inset. ZD6169 (1 μM) (C) greatly reduced the duration of the action potential and made the myocyte unexcitable at 10 μM (D) ($APD_{95} = 0$ ms). At 100 μM, the myocyte could again generate action potentials (E), but was severely truncated in duration ($APD_{95} = 21$ ms). (A–E) Representative data from the same cell.

current at positive potentials. ZD6169 (1 μM) produced an increase in the outward current. Increasing the concentration to 10 μM eliciting a large linear outward current, whose amplitude at +50 mV, was larger than 4 nA. Surprisingly, further increasing the concentration of ZD6169 to 100 μM caused a reduction in this outward current to levels near those seen with 1 μM. The effect on the action potentials reflected the changes in K_{ATP} current in that ZD6169 (1 μM) greatly reduced action potential duration, while a 10-μM concentration rendered the myocyte unexcitable. In the presence of 100 μM, the cell was again excitable, although the duration for 95% decay of the action potential peak amplitude (APD_{95}) was severely shortened (21 ms) and lacked entirely a plateau region.

Fig. 3A shows the bell-shaped concentration–response relationship of ZD6169 on the K_{ATP} current. The drug-induced current ($\Delta I = I_{drug} - I_{control}$) was determined from the current values measured at +50 mV (peak outward current) with the voltage-ramp protocol. The threshold concentration for the increase in outward current was 0.1 μM ZD6169, producing 102 ± 8 pA of current at +50 mV; $n = 10$. The current increased to 389 ± 25 pA ($n = 9$) with 1 μM, and reached a maximum at 10 μM of 3210 ± 172 pA ($n = 12$). When the drug concentration was further increased to 30 μM, the amplitude of the outward current was reduced (1927 ± 125 pA; $n = 8$) in comparison to that observed at 10 μM. The reduction was even more pronounced at 100 μM, the highest concentration studied (440 ± 21 pA; $n = 12$). A Gaussian curve was fitted to the histogram to illustrate the bell-shaped characteristics of the relationship.

The effects of ZD6169 on APD_{95} are shown in Fig. 3B. At 0.1 μM, ZD6169 had a small but statistically significant effect on APD_{95} ($13.9 \pm 2.4\%$ reduction; $n = 8$), while at 1 μM it greatly reduced the APD_{95} ($65.5 \pm 5.2\%$ reduction; $n = 7$). When the drug concentration was increased to 10 and 30 μM, the APD_{95} was drastically reduced by $99.6 \pm 0.4\%$; ($n = 10$) and $96.2 \pm 2.4\%$; ($n = 4$) and it was frequently (90% for 10 μM and 50.0% for 30 μM) not possible to trigger an action potential. At 100 μM, action potentials were again triggered and the reduction in APD_{95} began to reverse, although the action potential was still shortened by $86.2 \pm 2.0\%$; ($n = 11$). A Gaussian relationship was fitted to this data. These results show that ZD6169 is an effective K_{ATP} channel opener in the myocardium and that it does shorten action potential duration at all concentrations higher than 0.1 μM (Fig 3B).

The effects of the standard K_{ATP} channel opener cromakalim was also examined for comparison. Fig. 4 shows the effects of cromakalim (10 and 100 μM) on both outward current and action potentials recorded from a typical myocyte. Cromakalim (1 μM) elicited a K_{ATP} current (270 pA) comparable in size to that seen with ZD6169 (1 μM) and produced a similar shortening of action potential duration (Fig. 4A,C). At 10 μM, the increase was more pronounced (3690 pA), and even greater at 100 μM (6750 pA) with no signs of saturation. When the concentration of cromakalim was increased to 10 μM (Fig. 4D), action potential duration was drastically reduced ($APD_{95} = 12$ ms). At 100 μM (Fig. 4E), it became impossible to trigger the action potential due to the very large

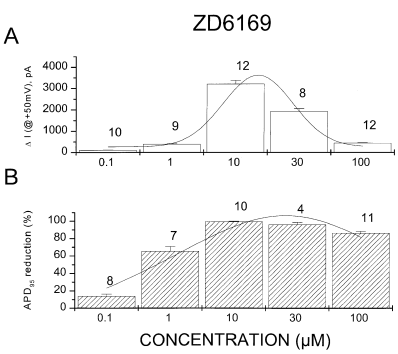

Fig. 3. ZD6169 has a bell shaped dose–response relationship for $I_{K_{ATP}}$. The drug-induced current ($\Delta I = I_{drug} - I_{control}$) was determined from the current values measured at +50 mV with the voltage-ramp protocol and plotted against drug concentrations (A). A Gaussian curve is fitted to the data to demonstrate the attenuation of currents at higher concentrations. The effects of ZD6169 on APD_{95} are shown in (B). Action potential duration (APD_{95}) was severely shortened by all but the lowest concentration of ZD6169. Cell number shown above each column.

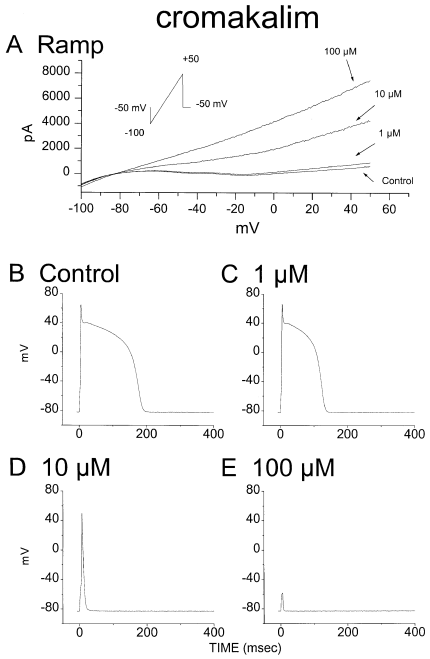

Fig. 4. Cromakalim activation of K_{ATP} channel. (A) shows a number of superimposed ionic ramp traces before and after various concentrations of cromakalim. Voltage ramp protocol shown in inset. In contrast to ZD6169, cromakalim activated increased amount of current with increasing concentrations. The cardiac action potential duration was also greatly reduced by cromakalim (1–100 μM). Action potential generation failed at 100 μM. Data from representative cell recorded in both voltage and current-clamp mode.

increase in outward $I_{K_{ATP}}$ current at this high concentration (Fig. 4A).

Fig. 5 shows that the concentration–response relationship for the cromakalim current is not bell-shaped but shows a continuous increase with increasing doses. No evidence for saturation was seen even in the presence of 100 μM cromakalim. A comparison of cromakalim- vs. ZD6169-induced K_{ATP} current at lower doses follows: 0.1 μM {74 vs. 102 pA}; 1 μM {286 vs. 389 pA}; 10 μM {4118 vs. 3210 pA} respectively. Although the two compounds appear to elicit comparable levels of K_{ATP} current over this range (0.1 to 10 μM), they differ at higher concentrations. At 100 μM cromakalim increased $I_{K_{ATP}}$ to 6378 ± 158 pA; $n = 17$ while the effect of ZD6169 was attenuated at 30 and 100 μM. The effects of cromakalim on APD_{95} were very similar to those seen with ZD6169, producing modest shortening at 0.1 μM cromakalim (11% vs. 14% reduction for ZD6169); moderate shortening at 1 μM (41% vs. 66%) and a severe reduction in action potential duration accompanied by a failure of excitation at 10 μM (96% vs. 99%) and 100 μM (100% vs. 86%), respectively (Fig. 5B).

In an attempt to elucidate more about the mechanism of ZD6169 and cromakalim's effects on the cardiac K_{ATP} channel, we explored the interaction of ZD6169 with cromakalim on the same cell. Fig. 6 shows action potential data for a cardiac myocyte before and after 100 μM cromakalim followed by addition of 100 μM of both

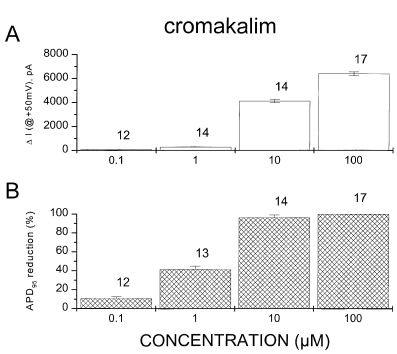

Fig. 5. The dose–response relationship of cromakalim is not bell shaped. The drug-induced current ($\Delta I = I_{drug} - I_{control}$) was determined from the current values measured at +50 mV with the voltage-ramp protocol and plotted against drug concentrations (A). Increasing concentrations of cromakalim increased $I_{K_{ATP}}$ with no sign of saturation. APD_{95} was greatly reduced at concentrations greater than 1 μM (B).

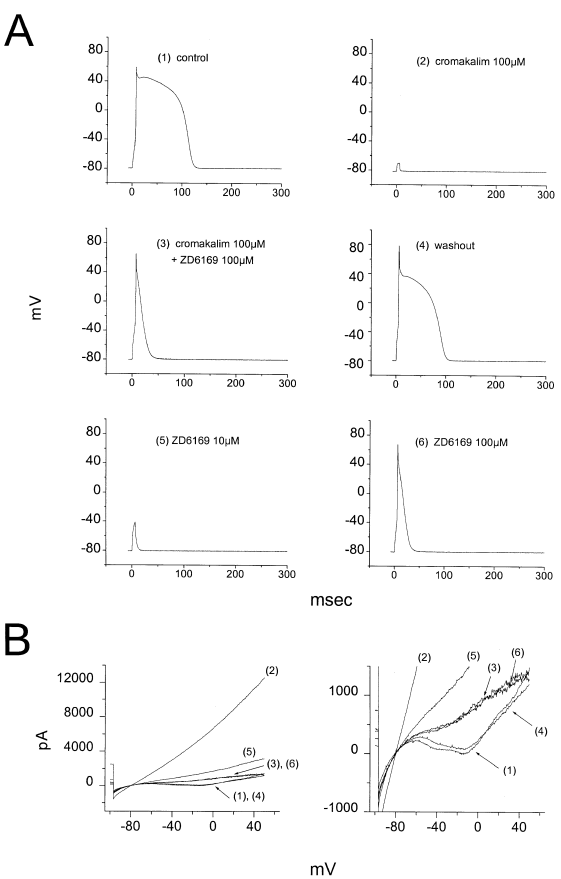

Fig. 6. ZD6169 and cromakalim compete for a common interaction site in activation of the cardiac K_{ATP} current. (A) Action potential recordings showing a control action potential (1), the effects of 100 μM cromakalim (2), 100 μM cromakalim + 100 μM ZD6169 (3), washout (4), 10 μM ZD6169 (5), and 100 μM ZD6169 (6) all from the same cardiac myocyte. (B) Current data in response to a 5-s voltage ramp from −100 mV to +50 mV for the same drug conditions listed above (1–6) taken from the same cell as in (A).

cromakalim and ZD6169. The ZD6169 was able to partially restore the action potential. Both compounds were successfully washed out and ZD6169 reapplied at 10 and 100 μM still exhibited the bell-shaped response for action potential duration. Fig. 6B shows voltage clamp data from the same cell for the drug exposures outlined above. The resulting currents directly show the suppression of the K_{ATP} current, elicited by 100 μM cromakalim, by coapplication of 100 μM ZD6169 [Fig. 6B, (2) vs. (3)]. Similar results were seen for coapplication of ZD6169 and cromakalim in two other myoctyes.

4. Discussion

The data show that ZD6169, a K_{ATP} channel opener in bladder smooth muscle cells (Li et al., 1995; Trivedi et al., 1995; Heppner et al., 1996), can activate a current in isolated cat ventricular myocytes whose voltage dependency and reversal potential is very similar to that of $I_{K_{ATP}}$ (De Lorenzi et al., 1995). In addition, this current is inhibited by the K_{ATP} channel blocker glyburide showing that ZD6169 in ventricular myocytes activates K_{ATP} channels.

At positive voltages, corresponding to the plateau of the cardiac action potential, $I_{K_{ATP}}$ induced by the openers is outward and quite large, and would be expected to affect the action potential. The effects of ZD6169 on $I_{K_{ATP}}$ show a bell-shaped concentration–response relationship with a peak at 10 μM. Its effect on APD_{95} exhibits a somewhat different response relationship. ZD6169, at 10 and 30 μM, causes a maintained and near-maximal reduction of action potential duration (99% and 96%, respectively) with 100 μM having less effect, although the shortening of APD_{95} (88% at 100 μM) is still near the maximum observed.

Cromakalim, on the other hand, showed a standard concentration–response relationship with increasing K_{ATP} current for increases in dose. The relationship showed no signs of reversal or saturation even with the highest concentration used (100 μM). The I_{KATP} current and the shortening of the action potential duration produced by the lower concentrations of cromakalim (0.1–10 μM) was very similar in magnitude to that of ZD6169 although at 100 μM the two compounds were clearly different.

A bell-shaped concentration–response relationship for ZD6169 has already been reported for guinea pig detrusor K_{ATP} channels (Hu and Kim, 1997). The authors proposed that the unusual concentration–response relationship might account for the modest in vivo hemodynamic effects reported with ZD6169 (Howe et al., 1995), despite a profile of effects in detrusor smooth muscle similar to that of cromakalim. Here, we reported that the ZD6169 reaches its maximal effect on activating cardiac $I_{K_{ATP}}$ and shortening the action potential duration at a concentration of 10 μM. This value is close to the value reported in guinea pig smooth bladder muscle cells, where 5 μM is the most effective concentration in activating smooth muscle K_{ATP} channels. However, there is a significant difference in the reduction of K_{ATP} current seen at higher concentrations of ZD6169 between the two studies. In guinea pig bladder smooth muscle, ZD6169 at 50 μM ,was able to completely close $I_{K_{ATP}}$ resulting in a negative change in current (−29.6%). However, in cat ventricular myocytes, ZD6169 at 100 μM could still activate cardiac K_{ATP} channels to 13% of the maximum seen at 10 μM and still exerted an almost maximal effect with respect to the shortening of the action potential duration. Part of this difference may reside from the different animal species used in the two studies, but it is still difficult to explain how ZD6169 has such minimal cardiovascular effects (Howe et al., 1995). The data presented here suggest that additional mechanisms must be involved in explaining the modest in vivo hemodynamic effects.

ZD6169 represents a new generation of K_{ATP} channel openers. The mechanisms underlying the bell-shaped concentration response of ZD6169 on cardiac K_{ATP} channels are not yet clear and are currently under investigation. One hypothesis is that there is one interaction site for both ZD6169 and cromakalim on the K_{ATP} channel. Cromakalim acts as a pure agonist at this site by displacing ATP from it's inhibitory position. ZD6169 acts in a more complex manner, acting as an agonist at low concentrations by also displacing ATP but as an partial antagonist at higher concentrations by blocking the channel possibly by mimicking the effects of ATP.

Cardiac K_{ATP} channels appear to consist of the inward rectifier Kir6.2 coupled to the sulfonylurea receptor subtype SUR2A (Chutkow et al., 1996; Yokoshiki et al., 1998), while vascular smooth muscle appears to contain Kir6.1 and SUR2B (Yamada et al., 1997). The composition of the bladder K_{ATP} channel is still being explored and once this is elucidated may help to explain some of the pharmacological differences we see for ZD6169 on cardiac cells (Inagaki et al., 1996). The selectivity of ZD6169 for bladder K_{ATP} channels over cardiac K_{ATP} channels presumably reflects changes in bioavailability and pharmacokinetics.

These results suggest that ZD6169 can activate or inhibit $I_{K_{ATP}}$ in isolated cat ventricular myocytes depending on the concentrations used. The mechanism of this effect is at present unknown. In contrast, in the same assay, cromakalim activates increasing amounts of K_{ATP} current from 0.1 μM to 100 μM. The bell-shaped concentration–response relationship of ZD6169 on $I_{K_{ATP}}$ can not explain the minimal effects of this drug on the heart and cardiovascular system as considerable shortening of the action potential duration (88%) occurred even at the highest concentration of ZD6169 used (100 μM).

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