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Direct block of hERG potassium channels by caffeine

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hERG: human ether-a-go-go related gene, IBMX: 3-isobutyl-1-methylxanthine

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Abstract

The hERG potassium channel is expressed in a variety of cell types, including neurones, tumour cells and cardiac myocytes. In the heart it is important for repolarisation of the cardiac action potential. Attenuation of hERG current can cause long QT syndrome and cardiac arrhythmias such as Torsades de Pointes. Caffeine is frequently used as a pharmacological tool to study calcium-dependent transduction pathways in cellular preparations. It raises cytosolic calcium by opening ryanodine receptors and may also inhibit phosphodiesterases to increase cytosolic cAMP. In this study we show 5 mM caffeine rapidly and reversibly attenuates hERG currents expressed in HEK 293 cells to 61.1 ± 2.2% of control. Caffeine dependent inhibition of hERG current is not altered by raising cAMP with forskolin, buffering cytosolic calcium with BAPTA or inhibition of protein kinase C. Thus the effects of caffeine are unikely to be mediated by cAMP or intracellular calcium-dependent mechanisms. Further experiments showed caffeine directly blocks hERG in an open state dependent manner. Furthermore, caffeine inhibition is greatly reduced by the pore mutants Y562A and F656A hERG, which disrupt block of most previously tested hERG antagonists. Thus caffeine attenuates hERG currents by binding to a drug receptor located within the inner cavity of the channel. Dietary intake of caffeine is unlikely to cause long QT syndrome since plasma concentrations do not reach sufficiently high levels to significantly inhibit hERG currents. However, the effects of caffeine have implications for its use in examining calcium-dependent pathways in cellular preparations expressing hERG.

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Introduction

The human Ether a-go-go Related Gene (hERG) potassium channel is abundantly expressed in cardiac tissue, and is important in the repolarisation of the cardiac action potential, exerting repolarising effects in the latter stage of the action potential. In the heart, dysfunction of the channel has been implicated in long QT syndrome (LQTS), a disorder of action potential repolarisation that can predispose individuals to lethal arrhythmias. Either inherited mutations of hERG (e.g. Sanguinetti et al., 1996), or hERG channel block by a variety of medications (Roden 1998) can cause LQTS. The type of drugs that block hERG are functionally and structurally diverse, and include class III anti-arrhythmics such as ibutilide, antibiotics such as erythromycin, antihistamines such as terfenadine and anti-psychotics such as sertindole. (Kass et al., 2000; Taglialeta et al., 1998; Fermini and Fossa, 2003). This pharmacological 'promiscuity' of hERG is due, in part, to the channel having an unusually large inner cavity capable of binding and trapping diverse compounds (Mitcheson et al., 2000). The inner helix (S6) of hERG lacks the Pro-X-Pro motif that in most other voltage gated potassium channels is thought to introduce a sharp bend in the S6 subunit and consequently relatively small inner cavity (del Camino et al., 2000). In addition, hERG is unique in that it has two aromatic amino acid residues (Phe656 and Tyr652) per subunit that face into the cavity and allow hERG to form strong electrostatic as well as hydrophobic interactions with drugs. Mutation of Phe656 and Tyr652 to alanine has been shown to reduce sensitivity of hERG to a number of high and low affinity blockers (for examples see Mitcheson et al., 2000; Perry et al., 2004; Witchel et al., 2004). Polar residues close to the K ion selectivity filter are also important for high affinity block by some compounds (Perry et al., 2004).

The hERG channel is also expressed in neuronal and tumour tissue. Studies show hERG is expressed in cancerous tissues of various origins including human endometrial (Cherubini et al., 2000) and human prolactin secreting adenocarcinomers (Bauer et al., 2003). hERG may influence tumour cell proliferation and invasiveness (Wang et al., 2002; Smith et al., 2003), but the mechanisms involving hERG channels remain largely unknown. ERG channels are expressed in a number of regions of the central nervous system, including the hippocampus, hypothalamus and cerebellum (Papa et al., 2003) and may be an important component of M-like currents in the brain (Meves et al., 1999; Selyanko et al., 2000). ERG currents are modulated by many signal transduction pathways and regulation by G-protein coupled receptors may be important in controlling cellular excitability.

Elevations in cytosolic calcium play an important role in regulating numerous cellular processes, including excitation-contraction coupling in cardiac tissue (reviewed by Berridge et al., 2000), and programmed cell death (reviewed by Gupta and Pushkala, 1999). Caffeine is widely used either to elevate cytosolic calcium levels or in combination with calcium ATPase inhibitors to deplete intracellular stores; revealing relationships between the release of intracellular calcium and its physiological role. Caffeine is useful because it is highly membrane permeable, induces rapid calcium release from intracellular stores, and is rapidly reversible. It increases cytosolic calcium by shifting the calcium dependence of the ryanodine receptor to low concentrations, thus leading to a release of calcium from intracellular stores under resting physiological intracellular calcium levels (Rousseau et al., 1988). Caffeine also has calcium-independent actions. It is a known phosphodiesterase inhibitor, increasing cytosolic cyclic AMP (cAMP) levels and activating protein kinase A (PKA) (Butcher and Sutherland, 1962; Lindaman et al., 2002).

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A number of studies have suggested caffeine may act as a blocker of ion channels, for example inward rectifier potassium channels in the GH_3 rat pituitary cell line (Barros et al., 1996), the inward rectifier K current (I_{K1}) and transient outward K current (I_{to}) in rat ventricular myocytes (Chorvatova and Hussein, 2003), and both outwardly and inwardly rectifying K channel in taste receptors (Zhao et al., 2002).

In this study we report that caffeine inhibits the hERG potassium channel current, and investigate its mechanism of action. Our data suggests that caffeine acts directly on hERG channels expressed in both a mammalian system and *Xenopus* oocytes. We have shown caffeine acts as a rapid open channel blocker, and binds within in the inner vestibule of the channel. This data may have implications in interpretation of data when using caffeine on cell and tissue preparations expressing the hERG channel.

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Methods

Cell culture

HEK293 cells stably expressing hERG (hERG-HEK cells) were a kind gift from Professor Craig January (University of Wisconsin) and were maintained in Dulbecco's MEM with glutamax-1, sodium pyruvate, glucose and pyridoxine (Gibco), supplemented with 10% foetal bovine serum, (Gibco) 400 μg/ml geneticin (Sigma) and 50 μg/ml gentamycin (Sigma) at 37^{o}C under 5% CO_2. Cells were passaged every 3-4 days. *Xenopus* oocytes were isolated by enzymatic digestion and manual de-folliculation using previously described methods (Mitcheson et al, 2000). Stage V-VI oocytes were injected with 5-15 ng cRNA to *wild-type* (WT), F656A and Y652A hERG and recordings were made 1-4 days later. Oocytes were maintained at 18 ^{o}C in SuperBarths solution containing (in mM) NaCl 88, KCl 1, $CaCl_2$ 0.41, $Ca(NO_3)_2$ 0.33, $MgSO_4$ 1, $NaHCO_3$ 2.4, HEPES 10, pyruvate 1, gentamicin 50 μg ml^{-1}, pH 7.4 (adjusted with NaOH).

Electrophysiology

hERG expression in HEK293 cells was measured using the whole cell patch clamp technique. Recordings were carried out with an axopatch 200B amplifier (Axon Instruments). Membrane currents were low pass filtered at 1 kHz and sampled at 2.5 kHz with a digidata 1322 data acquisition system. Cells were perfused with extracellular Tyrode, containing (in mM) NaCl 140, $MgCl_2$ 1, KCl 4, Glucose 10, HEPES 5, $CaCl_2$ 2, pH 7.4 (adjusted using NaOH). Recordings were made at 35-37 ^{o}C. A solenoid valve-based switching system was used to fully exchange solutions in the recordings chamber within 60 seconds. All drug solutions were made up daily to the required concentrations in

extracellular Tyrode. Borosilicate glass pipettes (Harvard Apparatus) were pulled and fire polished to get final resistances of 2 to 5 MΩ. These were filled with an internal recording solution containing (in mM) KCl 130, MgATP 5, HEPES 10, pH 7.2 (adjusted using KOH). In some experiments 5mM BAPTA free acid was added and the pH of the internal solution was re-adjusted. Whole cell capacitance measurements ranged between 8 pF and 29 pF, and series resistances were between 2 MΩ and 8 MΩ and were compensated by 60-85%.

Membrane currents in *Xenopus* oocytes were recorded with the two electrode voltage clamp technique using a Geneclamp 500B amplifier (Axon) and 1322 digidata (Axon). Data aquistion was performed with Clampex8.1 (Axon Instruments).
Cells were perfused with a low-chloride solution containing (in mM) Na MES 96, K MES 2, $Ca(MES)_2$ 2, HEPES 5, $MgCl_2$ 1, pH 7.6 (adjusted with NaOH). Recordings were obtained at 20-21 ^{o}C. Microelectrodes were filled with 3 M KCl and the tipes broken to give resistances of 1-2 MΩ.

Measurement of cytosolic calcium levels

To monitor cytosolic calcium, hERG-HEK cells were loaded with 2 μM Fura-2-AM (Molecular Probes) for 20 minutes at room temperature. In some experiments cells were incubated with BAPTA-AM at the same time. The experiments were performed in non-voltage clamped cells to avoid dialysis of intracellular solutes via the patch pipette. Fluorescence was excited with light from a monochromator at 340 and 380 nm and emitted light at >520 nm was imaged at 2 s intervals with a CCD camera (Photon Technology International). Data acquisition was performed using PTI Imagemaster software to produce a 340/380 ratio of Fura-2 emission intensities. Experiments were conducted at 35 ± 1^{o}C.

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Voltage protocols and data analysis

Electrophysiological recordings were analysed using Clampfit (Axon Instruments) and GraphPad Prism 3.0 (GraphPad software, SanDiego, USA). To measure the effects of caffeine and other reagents on hERG currents hERG-HEK cells were held at a membrane potential of –80 mV and 5 s depolarisations to 0 mV was applied. Tail currents were evoked by a 2 or 3 s repolarisation to –50 mV. This protocol was repeated every 15 s to give sufficient time for current deactivation between test pulses. Cells were repetitively pulsed in control Tyrode until current amplitudes stabilised before reagents were applied. Experiments to determine the caffeine binding site were done in *Xenopus* oocytes. For these experiments, cells were held at a membrane potential of –90 mV and depolarised to 0 mV for 5 s before being repolarised to –70 mV for 400 ms. This voltage protocol was repeated every 6 s to ensure the channels were open (and inactivated) and the inner cavity accessible to caffeine for the majority of the time. For currents measured in both HEK cells and oocytes, peak tail current amplitudes were measured for each test pulse and normalised to currents before compound application. To analyse the voltage dependence of hERG channel activation in HEK cells, cells were held at a membrane potential of –80 mV, and depolarisations to potentials between –40 mV and +40 mV applied in 10 mV increments for 5 s. Tail currents were evoked by a 2 or 3 s repolarisation to –50 mV. In this protocol and all others in HEK cells, the start to start test pulse interval was 15 s. Peak tail current amplitudes were measured and normalised to the maximal amplitude, plotted against test pulse potential and fitted with a Boltzman curve, to obtain values for half maximal activation ($V_{0.5,act}$) and slope factors. To measure steady state inactivation of hERG, a three pulse voltage protocol was used. Membrane potential was depolarised to +40 mV for 500 ms from a holding potential of –80 mV. A 5 ms voltage pulse to

potentials between –140 mV and +20 mV was applied, and current through non-inactivated channels measured at the beginning of a third pulse to +40 mV. Peak current at the beginning of the third pulse was normalised to maximum amplitude, plotted against the voltage of the preceeding pulse and fitted with a Boltzman function to obtain values for half maximal inactivation ($V_{0.5,inact}$) and slope factors. The 'envelope of tails' voltage protocol was used to measure the time dependence of hERG block by caffeine. Cells were held at –80 mV and depolarisations to 0 mV were initially applied for 20 ms. This interval was increased in 20 ms increments to a maximum of 400 ms. After each depolarisation, current activation was monitored with repolarising pulses to –50 mV for 60 ms to elicit tail currents, which were measured and normalised to the maximum tail current in control conditions, and plotted as a function of the test pulse duration.

Statistical analysis was by unpaired Student's t-tests unless indicated differently with significance accepted at $p<0.05$.

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Results

Effects of caffeine on hERG currents in HEK cells

Extracellular application of 5 mM caffeine to HEK293 cells expressing the hERG channel attenuated hERG current in a rapid and fully reversible fashion. Cells were held at a membrane potential of –80 mV, and hERG channel currents were elicited by a 5 s depolarising pulse to 0 mV. Tail currents were elicited by a 2 second repolarisation to –50 mV. Cells were repeatedly pulsed until currents stabilised before caffeine application. Figure 1A shows currents in control conditions and during application of caffeine. Responses to caffeine were seen within the first pulse after application and reached steady state after 2 pulses (Figure 1B). Peak tail current remaining during caffeine application was 61.2 ± 2.2% of control current (n=7, $p<0.05$).

The decrease of hERG current was seen at all potentials (Figure 1C) and there was no significant shift in the voltage dependence of activation (Figure 1D). $V_{0.5,act}$ and slope factor values in control conditions were –22.3 ± 1.9 mV and 7.8 ± 0.3 respectively, compared to –23.1 ± 2.3 mV and 7.8 ± 0.4 (n=6) in the presence of caffeine. The voltage dependence of inactivation was also not significantly affected by caffeine application. $V_{0.5,inact}$ values in control conditions were –73.7 ± 0.3 mV compared to –73.8 ± 1.0 mV in the presence of caffeine (n=6, data not shown). Washout of caffeine was rapid. Currents began to recover on the first pulse after switching to control Tyrode, with currents recovering to 99.1 ± 1.6% of control current within 30 s after the switch.

The effects of caffeine were concentration-dependent (Figure 1E). Small effects of caffeine were seen at concentrations of 100 nM, with tail currents 88.1 ±

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1.5% of control (n=5). With 20mM caffeine, only 12.7 ± 1.1% (n=5) of control current remained.

The suppression of hERG currents by caffeine is not due to elevation of cytosolic calcium

To investigate if the actions of caffeine were due to an increase in cytoplasmic calcium the time course of calcium and hERG current changes were compared in parallel experiments. Cytoplasmic calcium was monitored with fura-2. The fura-2 ratio increased slowly to a mean value of 2.0 ± 0.2 after 4 minutes of caffeine application from a mean baseline value of 0.6 ± 0.2 ($n \geq 12$). Washout of the effects of caffeine also took more than 4 minutes. The elevation of calcium was abolished by extracellular application of 50 μM BAPTA-AM (Figure 2A), a membrane permeant calcium chelator. The slow changes of calcium are in contrast to calcium transients measured with the same apparatus in isolated rat cardiac myocytes, which peak in less than 5 s and decay in less than 20 s (Dr G. Rodrigo, personal communication). This suggests the density of ryanodine receptors is low in this HEK293 cell line. Clearly, the time courses of calcium elevation and hERG current suppression were different; suggesting the hERG current response to caffeine is not mediated by cytosolic calcium. To further investigate if the effects seen with caffeine are due to an increase in cytosolic calcium, voltage clamped cells were dialysed via the patch pipette with a solution containing 5 mM BAPTA. Cells were left for 10 minutes after attaining the whole cell configuration to allow complete dialysis of BAPTA into the cell. Application of caffeine in these conditions continued to cause a rapid attenuation of current to 53.9 ± 5.5% of control (n=5, Figure 2B) that was rapidly reversed upon caffeine washout, providing further evidence that caffeine does not exert its effects by

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an increase in calcium. Dialysis of cells with intracellular BAPTA depletes calcium stores by buffering released calcium and preventing replenishment. 5 mM BAPTA should be far in excess of that needed to buffer calcium since 50 μM BAPTA-AM was sufficient to abolish the calcium transient. Nevertheless, to experimentally test if the hERG response to caffeine could be due to incomplete calcium buffering by BAPTA, we measured the hERG current response to a second application. If the effect of caffeine was calcium-dependent, we would expect a greatly reduced response with a second application, since calcium stores would be significantly depleted. However this was not the case. hERG current was reduced to 62.63 ± 2.05% of control with the second application (n=5), which was not significantly different from the first application (Figure 2C). These results provide strong evidence that caffeine does not act on hERG currents via a direct action of calcium on the channel.

Caffeine induced effects on hERG currents are not PKC or cAMP -dependent

Several studies suggest that hERG currents can be attenuated by protein kinase C (PKC) and this modulation can be blocked by a PKC-selective inhibitor bisindolylmalemide-1 (bis-1) (e.g. Thomas et al., 2003). To examine if caffeine was exerting its effects via a PKC-dependent pathway, caffeine was applied to cells superfused for 3 minutes with 300 nM bis-1, a concentration that we have previously shown to be sufficient to inhibit PKC in these cells (Cockerill et al., manuscript in preparation). 300 nM bis-1 had a minor influence on hERG current amplitude, with current amplitudes 95.9 ± 1.6% of control. Caffeine was then co-applied with bis-1. Bis-1 failed to reduce the caffeine response. Current ampplitudes during caffeine application were 51.3 ± 5.5% of control (n=6, Figure 2D), which was not significantly different from the response in the absence of bis-1. Interestingly, attenuation of the

hERG current upon caffeine application was slower in the presence of bis-1 compared to control, with maximal effects of caffeine being reached within 105 seconds, compared to 30 seconds in control conditions. The effects of caffeine in the presence of bis-1 were reversible upon washout, although recovery was also slower, reaching 90.9 ± 1.9% of control after 2 minutes. These results suggest that stimulation of PKC is not responsible for the attenuation of current by caffeine.

Caffeine is also a phosphodiesterase inhibitor, increasing the cytosolic cyclic AMP (cAMP) levels, and therefore activating protein kinase A (PKA) (Butcher and Sutherland, 1962; Lindaman et al., 2002). Effects of both cAMP and PKA on the hERG channel have been previously documented (e.g. Cui et al. 2000; Cui et al., 2001), so we determined if caffeine exerts its effects through either of these mechanisms. Forskolin, an adenylyl cyclase agonist, raises cAMP and causes slow, non-reversible attenuation of hERG current under our experimental conditions. The time course of action of forskolin was slower than caffeine (Figure 2E), and forskolin application reduced current amplitudes to 75.9 ± 0.7% of control, which is less than caffeine. If cAMP levels are already high, it is unlikely that caffeine-induced phospodiesterase inhibition would further modulate current. However, 5 mM caffeine continued to cause a rapid attenuation of current (to 65.14 ± 4.75% of control, n=5, Figure 2E), that also largely reversed upon washout. Thus, caffeine still attenuates hERG current in the presence of high cytosolic levels of cAMP. Taken together, our results suggest that caffeine does not affect the hERG channel current amplitude by calcium, PKC, PKA or cAMP dependent pathways.

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Open channel block and trapping of caffeine within the inner cavity of hERG

The rapid changes in current amplitude upon caffeine application and washout suggest caffeine may directly inhibit hERG currents. This possibility was explored with voltage protocols to investigate state dependent block of hERG. Most blockers of hERG exhibit open state-dependent block, and are thought to bind within the inner cavity of the channel (Sanguinetti and Mitcheson, 2005; Perry et al., 2004; Witchel et al., 2004; Mitcheson et al., 2000). The rapid action of caffeine on hERG currents could indicate either rapid open state block or closed state block. To distinguish between these possibilities we used the envelope of tails voltage protocol, which allows the amount of block to be measured as pulse duration is increased (e.g. Suessbrich et al., 1997; Teschmacher et al., 1999). If caffeine is an open channel blocker, then inhibition will increase with progressively longer depolarisations. Voltage pulses to 0 mV from a holding potential of –100 mV were applied with durations of between 20 – 400 ms. Tail currents were elicited by a pulse to –50 mV for 60 ms. The protocol was carried out in control conditions, and repeated after 5 mM caffeine had been applied for 2 minutes. Mean results for tail currents normalised to control following the 400 ms pulse are shown in figure 3A. Caffeine decreased hERG current amplitudes in a pulse duration manner. The differences in current amplitudes before and during caffeine application were measured and a mean percentage inhibition calculated and plotted against test pulse duration. Caffeine caused only a 2.7 ± 1.1 % block with a 20 ms test pulse, which increased to 42.5 ± 0.8% after 400 ms, with a mean time constant for onset of current inhibition (τ_{on}) of 95.8 ± 21.0 ms (Figure 3C). Similar experiments with an IC_{50} concentration (10 nM) of cisapride, a well-known open channel blocker, demonstrated inhibition accumulated with channel activation, with a τ_{on} of 383 ms (Walker et al., 1999). Thus

caffeine, like cisapride and many other long QT compounds, is an open channel blocker of hERG channels.

Recovery from caffeine block was also highly state and pulse duration dependent. Figure 3D shows mean results from experiments to monitor recovery from block after 2 minutes of caffeine washout. The holding potential during washout was –90 mV to keep channels in the closed state. Recovery from block was assessed with either 150 or 250 ms duration pulses to +40 mV. With short 150 ms pulses there was no increase of current with the first depolarisation following the caffeine washout period and no further increase of current with repetitive pulsing. This suggests caffeine remained bound to the closed channels upon washout and 150 ms pulses were too short for significant amounts of recovery from block. However, with 250 ms duration pulses, the current amplitude with the first depolarisation following washout was larger and there was further recovery (to 86.4 ± 3.4% of control) with subsequent depolarisations. These results are consistent with caffeine binding within the inner cavity and becoming trapped by closure of the activation gate. Recovery from block requires pulse durations of greater than 150 ms for unbinding and channel opening to occur.

The above results suggest the binding site for caffeine is likely to be within the inner cavity, behind the activation gate of the hERG channel. The drug binding site within the inner cavity is formed by amino acid residues from the inner helices (S6) pore helices. In particular, two aromatic residues on S6, Phe656 and Tyr652, are important for high affinity block by nearly all hERG channel blockers that have been tested so far. Mutation of both residues to alanine dramatically reduces block (reviewed by Sanguinetti and Mitcheson, 2005; Recanatini et al., 2005). To test if caffeine also binds within the inner cavity, inhibition of Y652A and F656A hERG by

caffeine was measured in *Xenopus* oocytes. This is an expression system that has been routinely used for investigating structural determinants of hERG channel block. WT currents were recorded by pulsing to 0 mV for 5 seconds from a holding potential of –90 mV. Tail currents were elicited by pulsing to –70 mV for 400 ms. This protocol was repeated every 6 s, so that channels were in the open state for most of the time. Attenuation of current in oocytes by 5 mM caffeine was similar to that seen in HEK cells (rapid and fully reversible), although percentage block was reduced to 77.8 ± 2.4% of control (n=7, Figure 4A). Reduction of block in oocytes compared to mammalian cells has been frequently reported and is likely to be due to accumulation of the drug in the lipophilic yolk of oocytes. Y652A currents showed a significantly reduced sensitivity to caffeine, with peak tail current during caffeine application only 92.6 ± 1.4% of control current (n=9, Figure 4C). F656A hERG currents were recorded with similar voltage protocols except that tail currents were measured at –140 mV to elicit larger amplitude currents (Mitcheson et al., 2000). F656A channels also showed a significantly lowered sensitivity to block by caffeine. Peak tail current during caffeine application was 93.6 ± 1.4% of control (n=9, Figure 4D).

Thus, both F656A and Y652A decrease hERG current inhibition by caffeine providing further evidence that caffeine exerts its effects on hERG currents by open state binding to residues in the inner cavity causing block rather than via a mechanism involving second messenger modulation of channel activity.

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Discussion

In this series of experiments we have shown caffeine to rapidly and reversibly attenuate hERG channels expressed in both HEK293 cells and *Xenopus* oocytes. This attenuation was not accompanied by any changes to the voltage dependence of activation or inactivation.

Caffeine releases calcium into the cytosol from the ryanodine sensitive calcium stores (Rousseau et al., 1988) and also increases cAMP levels (Butcher and Sutherland, 1962; Lindaman et al., 2002). Caffeine attenuated hERG channel current when calcium was both elevated by ionomycin (data not shown) and when buffered to low concentrations with BAPTA. Inhibition of PKC failed to alter attenuation of hERG channel currents by caffeine and caffeine continued to reduce hERG current when cAMP was raised with forskolin. Thus, caffeine does not exert its effects by calcium or cAMP mediated pathways. Instead, our results suggest that caffeine directly blocks the hERG channel. Caffeine had rapid onset and recovery kinetics, and inhibition was highly state dependent. Inhibition increased progressively as pulse duration was lengthened and channels became activated. Recovery from block was also more complete with longer duration test pulses. These results indicate that block in the open and/or inactivated state is the mechanism by which caffeine suppresses hERG currents.

Inhibition of WT channels expressed in HEK293 cells and *Xenopus* oocytes was very similar, despite the gross differences in size and subcellular organisation of the two cell types. This provides further evidence that caffeine has a direct rather than modulatory effect on hERG channels. To locate the binding site of caffeine, we used two mutants of the hERG channel, F656A and Y652A, that are known to reduce the sensitivity of hERG to a large number of long QT-inducing drugs that are open

channel blockers. Both are aromatic residues that face into the inner cavity of HERG, and can mediate hydrophobic and electrostatic interactions with structurally diverse compounds. The π electron faces of aromatic residues can mediate π-stacking interactions with aromatic moieties on compounds, and cation-π interactions with charged amines. We found that replacing either the Phe656 or Tyr652 residues with the small amino acid alanine, reduced the sensitivity of the channel to caffeine. This suggests caffeine interacts with both residues in the WT channel, and therefore binds within the inner cavity of the channel.

While investigating if the caffeine response was mediated by PKC, we found inhibition of HERG by caffeine to be slower in the presence of the PKC inhibitor bis-1. We, and others (Thomas et al., 2004) have shown bis-1 to directly inhibit HERG current at concentrations higher than 300 nM. Therefore, 300 nM bis-1, although not blocking the channel, may be competing with caffeine and partially obstructing caffeine entry into the HERG channel, leading to a slower block of the channel. Recovery of caffeine inhibition with washout in the presence of bis-1 was also slower, and may also be due to bis-1 hindering caffeine exit from the channel.

Caffeine is an alkaloid. The core of the molecule is a heterocyclic purine that is able to interact with aromatic residues on proteins. Caffeine can bind to the purine inhibitor site on glycogen phosphorylase A (Tsitsanou et al., 2000). This binding occurs by π-stacking interactions in which caffeine intercalates between two aromatic rings, Phe285 and Tyr613. Kapuscinski et al. (2002) found that caffeine binds to aromatic mutagens, such as quinacrine mustard, and reduces their pharmacological activity. Thus, caffeine may act as a protective agent in some cancer types. The interaction between caffeine and the aromatic agents was again due to π stacking interactions. Caffeine can decrease the effective concentrations of polycyclic

aromatic agents, but not aliphatic agents (Piosik et al., 2003). These three studies, although not carried out on ion channels, demonstrate caffeines ability to interact with aromatic residues. Our results are consistent with π-stacking interactions between caffeine and the Phe656 and Tyr652 residues on HERG, although other interactions with inner cavity residues may also take place.

Several studies have shown that caffeine can inhibit ion channels. The rapid, reversible effects of caffeine shown in our study concur with results of Barros et al (1996), who showed caffeine to cause a reversible increase of action potential firing frequency along with a depolarisation of membrane potential in GH_3 cells, a rat anterior pituitary cell line. They were unable to mimic the effects of caffeine with forskolin and isobutylmethylxanthine (IBMX), which both raise cAMP levels. The effects of caffeine could not be inhibited when cells were incubated with ryanodine or thapsigargain, to deplete intracellular calcium stores, or by buffering cytosolic calcium with EGTA dialysed into cells via the patch pipette. However, caffeine significantly inhibited an inwardly rectifying potassium current endogenously expressed in these cells. It has since been shown that GH_3 cells express channels with sequence homology to hERG that are blocked by astemizole (Barros et al., 1997). Thus, it is likely that the caffeine sensitive current is an endogenously expressed rat ERG channel.

Other membrane currents reported to be inhibited by caffeine include I_{K1} and I_{to} (Chorvatova and Hussein, 2003). In rat ventricular mycocytes, caffeine partially inhibited the inward rectifier current I_{K1} in a concentration dependent manner. Caffeine also inhibited I_{to} without altering the voltage and time dependence of activation or inactivation and in a fully reversible manner. The same study also showed 10 mM caffeine to partially inhibit the delayed rectifier I_K although selectivity

for the rapid or slow components was not established. $GABA_A$ current in hippocampal slices is also significantly inhibited by caffeine (Taketo et al., 2004), in a manner that could not be inhibited by calcium store depletion, or inclusion of EGTA or BAPTA in the pipette solution, and could not be mimicked by ryanodine or forskolin, indicating the inhibition of current may be due to direct block of channels. Therefore, several studies now suggest that ion channel block needs to be taken into consideration when using caffeine as a calcium-release tool, and that it is not specific for ryanodine receptors. At present, caffeine is widely used to investigate calcium-dependent transduction pathways in cellular preparations. For example, it is frequently used to study excitation-contraction coupling in cardiac myocyte preparations (e.g. Seidler et al., 2003; Huang et al., 2005; Gómez et al., 2004). Many studies use caffeine at concentrations higher than 5 mM, which we found to profoundly inhibit hERG currents. Thus, caffeine may not only elicit calcium release, but it may also inhibit I_{Kr}, I_{to} and I_{K1} to significantly alter action potential repolarisation in non-voltage clamped cells.

hERG is expressed in a variety of tissues in addition to cardiac myocytes, including some cancer cells (e.g. Cherubini et al., 2000) and neuronal tissues (e.g. Papa et al., 2003). Our results indicate that care should be taken with interpretation of results when using caffeine in hERG expressing excitable cells.

Is there a physiological relevance to caffeine inhibition of hERG channels? Caffeine is a common dietary constituent in many of today's cultures. However, it is unlikely to be a long QT syndrome-inducing drug since it only produces a significant inhibition of hERG channel current when used in milli-molar concentrations. A study carried out in 1982 showed the average plasma concentration of caffeine in non-smoking males was 10.9 $\mu M\ l^{-1}$ (Smith et al., 1982), a concentration that would not

significantly block hERG. It seems extremely unlikely that caffeine would cause any significant change to the ECG as a result of blocking the hERG channel.

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Footnotes

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Figure Legends

Figure 1

Characterisation of the effects of caffeine on hERG channel currents

A) Representative hERG currents measured from HEK293 cells at 0 mV with tail currents evoked by a repolarising pulse to –50 mV, before (control) and during 5 mM caffeine application. B) hERG peak tail currents at –50 mV were normalised to control conditions and mean results (± S.E.M.) plotted against time to show the time course of the response to 5 mM caffeine. C) Representative hERG currents measured using a standard I-V protocol (inset), before and during caffeine application. For clarity, only currents measured at -40, -20 and +20 mV are shown. D) Activation curves before and during caffeine application. Peak tail currents were normalised to the maximum current in each solution and plotted against test pulse potential. Solid lines are fits to data with Boltzmann functions. The voltage dependence of hERG activation was not shifted upon caffeine application. E) Concentration-response relationship of caffeine on hERG tail currents. $n \geq 4$ for each point.

Figure 2

Caffeine does not act on hERG currents through a intracellular calciu, PKC or cAMP mediated pathway

A) Typical calcium responses to caffeine in the absence or presence of 50 μM BAPTA-AM. BAPTA-AM was incubated with cells for 20 mins along with the Fura-2. The calcium response to caffeine was completely abolished in cells loaded with BAPTA-AM. B) Representative hERG current response to application of 5 mM caffeine in cells dialysed with pipette solutions containing 5 mM BAPTA. Peak tail current amplitudes were normalised to control and plotted against time. BAPTA was

dialysed into the cell for 10 minutes prior to application of caffeine. C) hERG current response to second application of caffeine under the same experimental conditions as in (B). D) Representative hERG current response to application of 5 mM caffeine after 3 mins of 300 nM bis-1. Peak tail currents were normalised to control and plotted against time. E) Representative hERG tail current reponse to application of 5 mM caffeine after application of 40 μM forskolin to raise cAMP. Forskolin was applied until currents had decreased to a steady level, before co-application with caffeine. F) Mean effects (± S.E.M.) of caffeine on hERG tail currents in the presence of BAPTA, bis-1 and forskolin ($n \geq 5$ for each point). There was no significant difference in the response to caffeine in each experimental condition.

Figure 3

Caffeine is an open channel blocker of hERG

A) Envelope of tails voltage protocol. Tail currents were measured upon repolarisation to –50 mV following pre-pulses to 0 mV. Pre-pulse durations started at 20 ms and increased to 400 ms in 20 ms increments. . B) Normalised mean (± S.E.M.) current amplitudes measured using the envelope of tails protocol before and during application of 5 mM caffeine. Peak tail current amplitudes were normalised to maximum in control conditions and plotted as a function of pre-pulse duration. C) The percentage inhibition of hERG currents by caffeine was plotted as a function of the pre-pulse duration and fit with a single exponential function. The mean time constant for onset of block was 95.8 ± 21.0 ms. D) Trapping of caffeine by closure of the activation gate. Cells were pulsed from a holding potential of –100 mV to +40 mV for either 150 or 250 ms in control solution before switching to caffeine. Once steady state block had been attained, pulsing was stopped and caffeine was washed off

for 2 minutes while channels were closed. Recovery from block was assessed with 150 or 250 ms pulses to +40 mV. Data are plotted as mean, normalised to control peak tail currents at each time point.

Figure 4

Characterisation of the caffeine binding site within the hERG channel

A) Representative WT hERG tail currents recorded in *Xenopus* oocytes before and during application of 5 mM caffeine. Tail currents were measured at –70 mV following 5 s test pulses to 0 mV. B) Time course of the response to caffeine in oocytes. Representative hERG peak tail currents were normalised to control and plotted against time. C and D) Caffeine block is abolished by inner cavity mutants Y652A (C) and F656A (D). C) Representative Y652A hERG tail currents at –70 mV. D) Representative F656A hERG tail currents at –140 mV. Dashed lines in panels A,C and D indicate zero current level. Voltage protocols are shown insets to A and C and boxes indicate where tail currents have been plotted.

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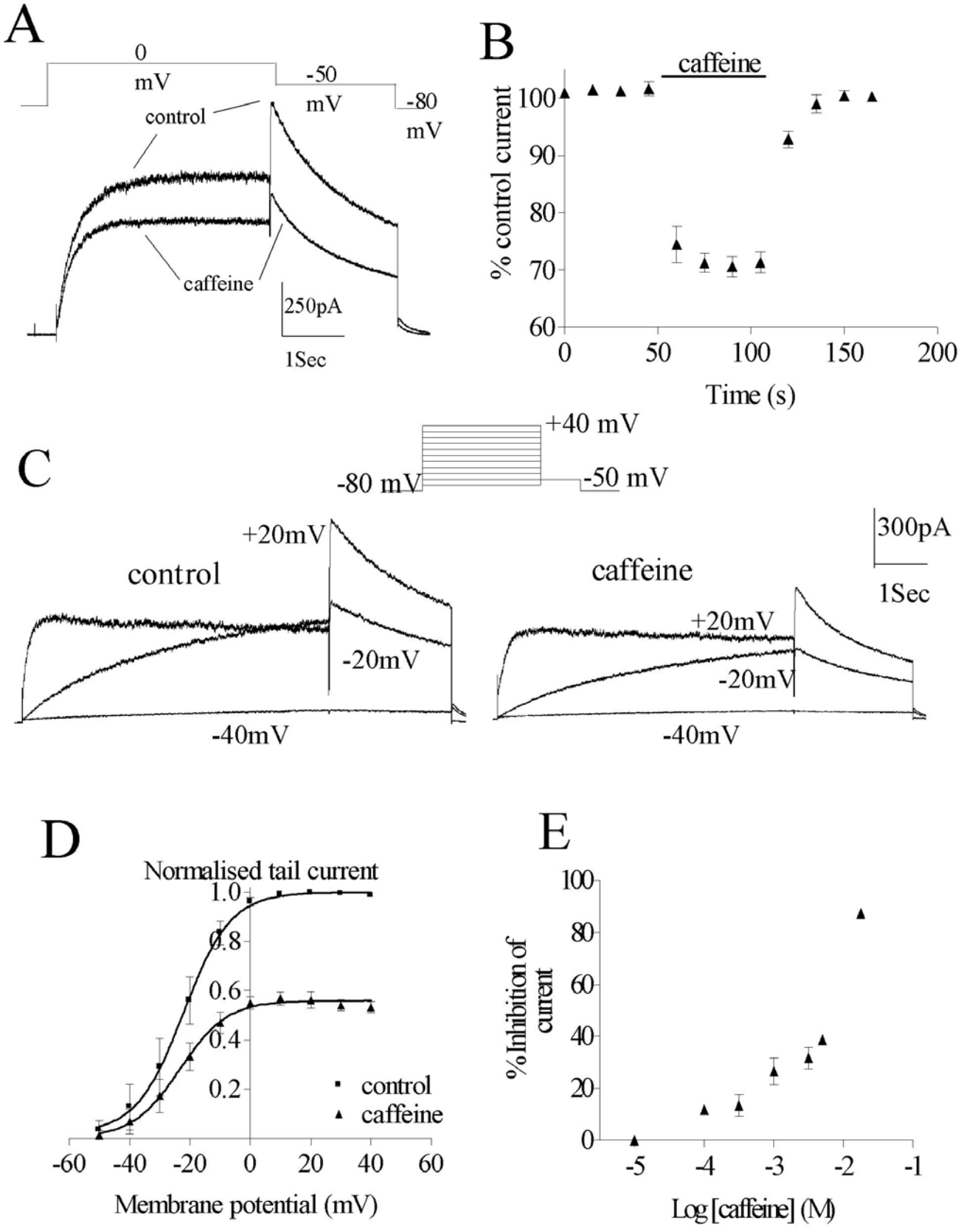

FIGURE 1

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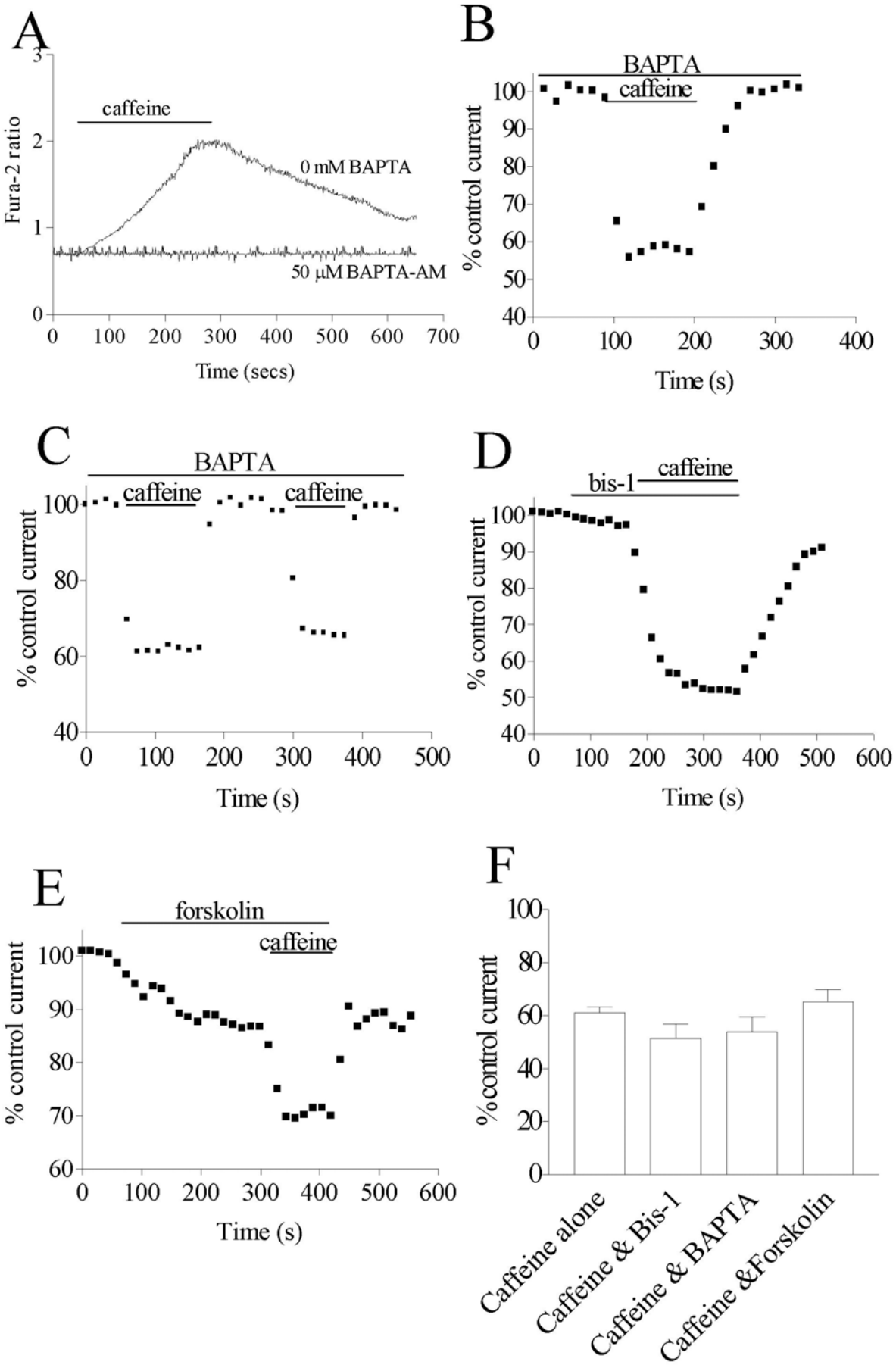

FIGURE 2

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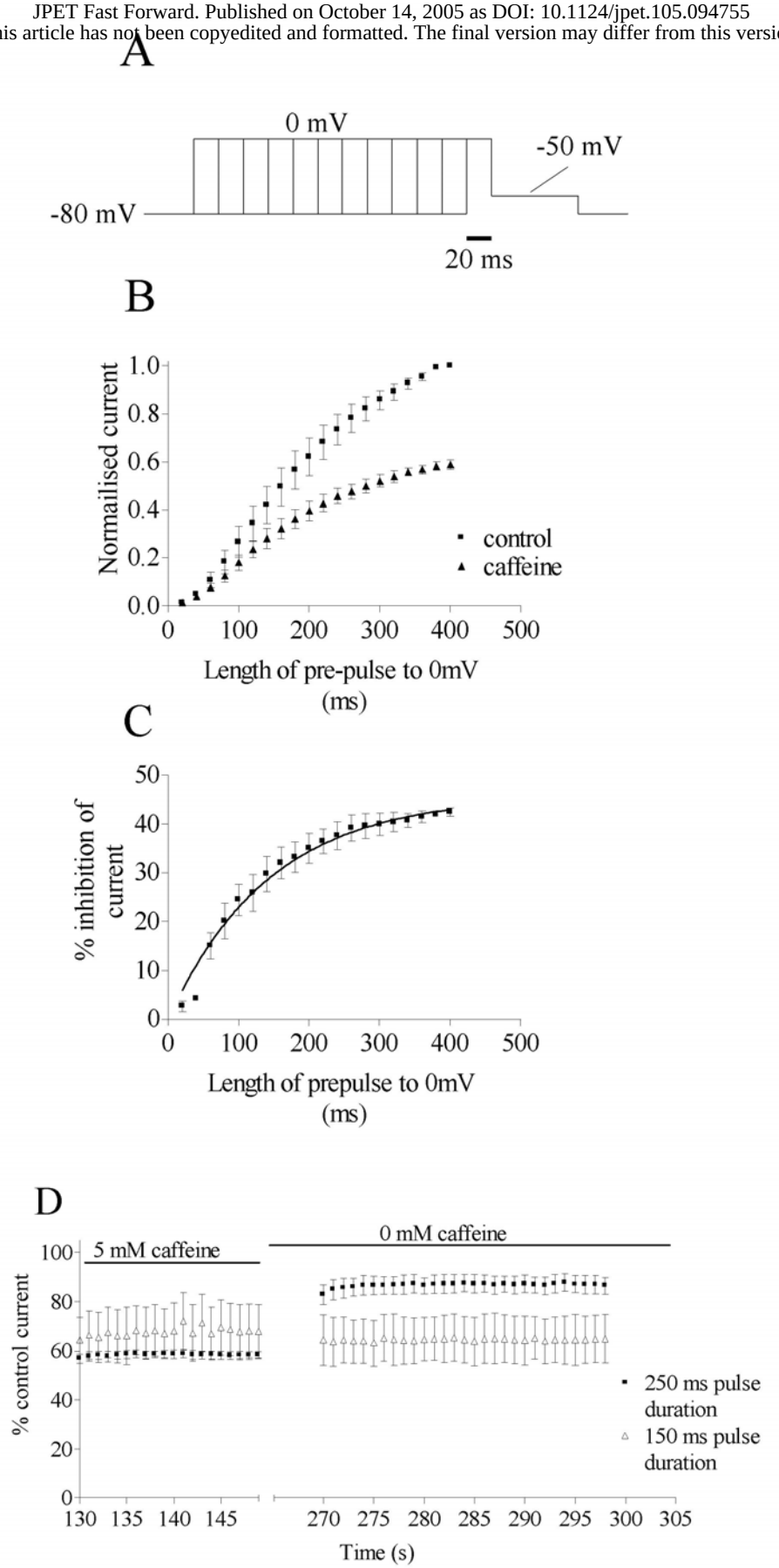

FIGURE 3

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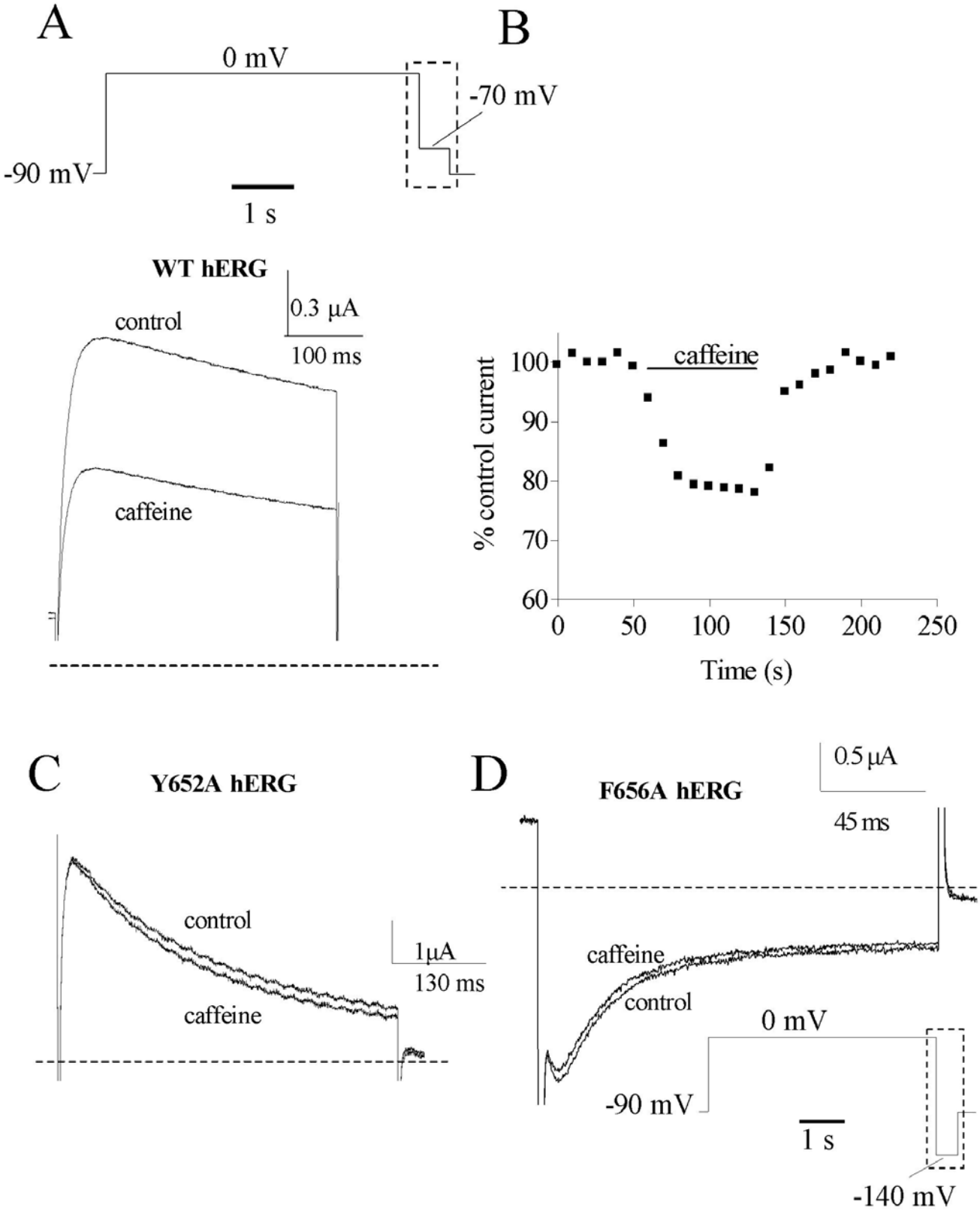

FIGURE 4