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Cardiac ion channel current modulation by the CFTR inhibitor GlyH-101

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ABSTRACT

The role in the heart of the cardiac isoform of the cystic fibrosis transmembrane conductance regulator (CFTR), which underlies a protein kinase A-dependent Cl^- current ($I_{Cl.PKA}$) in cardiomyocytes, remains unclear. The identification of a CFTR-selective inhibitor would provide an important tool for the investigation of the contribution of CFTR to cardiac electrophysiology. GlyH-101 is a glycine hydrazide that has recently been shown to block CFTR channels but its effects on cardiomyocytes are unknown. Here the action of GlyH-101 on cardiac $I_{Cl.PKA}$ and on other ion currents has been established. Whole-cell patch-clamp recordings were made from rabbit isolated ventricular myocytes. GlyH-101 blocked $I_{Cl.PKA}$ in a concentration- and voltage-dependent fashion (IC_{50} at +100 mV = 0.3 ± 1.5 μM and at −100 mV = 5.1 ± 1.3 μM). Woodhull analysis suggested that GlyH-101 blocks the open pore of cardiac CFTR channels at an electrical distance of 0.15 ± 0.03 from the external membrane surface. A concentration of GlyH-101 maximally effective against $I_{Cl.PKA}$ (30 μM) was tested on other cardiac ion currents. Inward current at −120 mV, comprised predominantly of the inward-rectifier background K^+ current, I_{K1}, was reduced by ~43% (n = 5). Under selective recording conditions, the Na^+ current (I_{Na}) was markedly inhibited by GlyH-101 over the entire voltage range (with a fractional block at −40 mV of ~82%; n = 8). GlyH-101 also produced a voltage-dependent inhibition of L-type Ca^{2+} channel current ($I_{Ca,L}$); fractional block at +10 mV of ~49% and of ~28% at −10 mV; n = 11, with a ~−3 mV shift in the voltage-dependence of $I_{Ca,L}$ activation. Thus, this study demonstrates for the first time that GlyH-101 blocks cardiac $I_{Cl.PKA}$ channels in a similar fashion to that reported for recombinant CFTR. However, inhibition of other cardiac conductances may limit its use as a CFTR-selective blocker in the heart.

1. Introduction

Electrical activity in the heart depends on the combined effects of diverse ion channels and transporters. Various anion conductances have been identified that may contribute to cardiac physiology and pathophysiology [1,2]: Ca^{2+}-activated Cl^- current [3–5], swelling-activated Cl^- current [6–8], anionic background current [9,10] and cAMP/PKA-activated Cl^- current ($I_{Cl.PKA}$) [11–13]. The reversal potential (E_{Cl}) for Cl^- in cardiomyocytes likely lies between ~−60 and −40 mV [1]. In principle, therefore, Cl^- channel activation can influence cardiac cell excitability and modulate action potential duration (APD), contributing inward, depolarizing current negative to E_{Cl} and outward, repolarizing current positive to E_{Cl} (for reviews see: [1,2]). $I_{Cl.PKA}$ is of particular interest as potentially it may contribute to autonomic regulation of cardiac activity [1,2]: sympathetic activation of $I_{Cl.PKA}$ may act to offset the effects of β-adrenergic stimulation of L-type calcium current ($I_{Ca,L}$) and thereby contribute to rate-dependent shortening of APD [14]. Channels underlying $I_{Cl.PKA}$ are believed to be mediated by the cystic fibrosis transmembrane conductance regulator protein (CFTR: [15–19]).

Recent simulation work has supported a role for increased CFTR-mediated $I_{Cl.PKA}$ in abbreviating ventricular APD, whilst being suggestive also of a concomitant indirect increase in $I_{Ca,L}$ and calcium transient (and hence indirect positive inotropic effect), due to a decrease in action potential plateau potential and hence altered driving force for Ca^{2+} ion entry [20]. However, a significant barrier to developing an experimentally-based understanding of the role(s) of $I_{Cl.PKA}$ in modulating cardiac electrophysiology is the absence of highly selective pharmacological inhibitors that can be used for *in vitro* or *in vivo* experimentation on adult hearts or myocytes [21]. The use of genetically modified mice may offer some insights. For example, CFTR overexpression has recently been reported to predispose towards stress-related sudden death, conduction abnormalities and ventricular tachycardia [22,23]. However, the very high murine heart rate and greatly abbreviated ventricular action potential, lacking the high plateau phase present in other species, makes genetically modified mice of limited value in understanding the role(s) and contribution of CFTR-mediated $I_{Cl.PKA}$ in the process of action potential repolarization. Consequently, the identification of potent pharmacological CFTR

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inhibitors devoid of effects on other cardiac ion channels would represent a significant advance in allowing the physiological and pathological roles of cardiac $I_{Cl.PKA}$ to be better understood. In recent years, a water-soluble glycine hydrazide-based inhibitor of CFTR, GlyH-101, has been identified that inhibits CFTR channels via pore occlusion, apparently from the external surface [24]. Whilst this compound has been found to be effective at inhibiting CFTR activity in different epithelial cell types (e.g. [24–29]), its actions on cardiac $I_{Cl.PKA}$ and other major cardiac ion channel conductances have not hitherto been reported. This study identifies and characterizes for the first time voltage-dependent inhibition of native ventricular $I_{Cl.PKA}$ by GlyH-101 and reports its effects on a range of other major cardiac ionic currents.

2. Methods

Cardiac myocytes from the right ventricular free wall were isolated from Langendorff-perfused male New Zealand White rabbit hearts using a method described previously [30]. All procedures were approved by the Ethics Committee of University of Bristol and conformed to the UK Animals (Scientific Procedures) Act, 1986. After isolation, cells were stored in Kraft–Brühe (KB) medium at 4 °C until use [31].

2.1. Electrophysiological recording and data acquisition

Data acquisition and recording methods were as those reported previously [32]. Whole-cell patch-clamp recordings were made at 37 °C with the exception of $I_{Na,}$ for which room temperature (RT) was used. Experimental solutions were made using de-ionised water (Milli-Q, Millipore). Cells were superfused with normal Tyrode's solution containing (in mM): 140 NaCl, 5 HEPES, 10 D-glucose, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1 $BaCl_2$, pH 7.45 with NaOH.

Forskolin and nitrendipine (Sigma–Aldrich, USA) were made up as 10 mM stock solutions in ethanol and used at a final concentration of 10 μM in external solutions. GlyH-101 (Calbiochem, USA) was made up as a 100 mM stock solution in dimethylsulfoxide (DMSO).

$I_{Cl.PKA}$ was recorded as reported previously using a Ca^{2+}, K^+-free external solution containing 1 mM $CdCl_2$ and activated using 10 μM forskolin [18]. Appropriate concentrations of GlyH-101 were applied in the presence of forskolin.

"Signature currents" were recorded as described previously using a K^+-based internal solution [33–35]. Fast Na^+ current (I_{Na}) recordings were made at RT using equal concentrations of internal and external Na^+ (10 mM) and external solutions containing the L- and T-type Ca^{2+} current blockers, nitrendipine (10 μM) and Ni^{2+} (50 μM) [36]. L-type Ca^{2+} channel current ($I_{Ca,L}$) recordings were made using a K^+-based internal solution with external Ba^{2+} as the charge carrier [37].

2.2. Data analysis and presentation

Data were analyzed using Igor Pro (WaveMetrics, Inc., USA), Excel 2007 and GraphPad Prism software. Data are presented as mean ± standard error of the mean (SEM), '*n*' values refer to numbers of cells for recordings (typically from ⩾two hearts). Statistical comparisons were made using a Student's paired *t* test and one- or two-way repeated measures (RM) ANOVA. $P < 0.05$ was considered to be statistically significant.

The fractional block of forskolin-activated $I_{Cl.PKA}$ by GlyH-101 was calculated as the fraction of the current in the presence of GlyH-101 to the current activated by forskolin as follows:

$$\text{Fractional block} = \frac{I_{\text{forskolin}} - I_{\text{GlyH-101}}}{I_{\text{forskolin}-I_{\text{control}}}}, \quad (1)$$

where the $I_{control}$, $I_{forskolin}$, and $I_{GlyH\text{-}101}$ represent currents in the presence of control, forskolin and GlyH-101 with forskolin, respectively.

The half-maximal inhibitory concentration (IC_{50}) of GlyH-101 was calculated by plotting the mean ± SEM fractional block of $I_{Cl.PKA}$ against the GlyH-101 concentration and fitting the data with a logistic equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{(1 + 10\text{^}((\text{LogIC}_{50} - X)\text{HillSlope}))} \quad (2)$$

where *Y*, Top and Bottom represent the response, maximal and minimum response to the drug respectively; *X* represents the logarithm of [GlyH-101] (μM).

The fractional electrical distance from the external membrane surface at which GlyH-101 blocked $I_{Cl.PKA}$ channels, denoted by δ [38], was calculated by fitting the Woodhull equation to the voltage-dependent fractional block by 3 μM GlyH-101:

$$\text{Fractional block} = 1 - \frac{\text{IC}_{50}}{\text{IC}_{50} + [\text{GlyH} - 101]_{\text{outside}} exp((-z\delta \text{FVm})/(\text{RT}))} \quad (3)$$

where IC_{50} is the IC_{50} of GlyH-101 at 0 mV, *z* is the valency (−1) of GlyH-101 at pH 7.4 [24], V_m = membrane potential; *F*, *R* and *T* have their usual meanings.

The fractional block for I_{Na} and $I_{Ca,L}$ were calculated as follows:

$$\text{Fractional block of} I_{Na} \text{ or } I_{Ca,L} = 1 - \frac{I_{\text{GlyH-101}}}{I_{\text{control}}} \quad (4)$$

The *I–V* relationships of I_{Na} and $I_{Ca,L}$ were fitted with a modified Boltzmann equation:

$$I_{Na} \text{ or } I_{Ca,L} = \frac{G_{max}(V_m - V_{rev})}{1 + \exp\left(\frac{V_{0.5} - V_m}{\text{slope}}\right)} \quad (5)$$

where G_{max} is the maximum conductance, V_m the membrane potential, V_{rev} is the reversal potential and $V_{0.5}$ the membrane potential at which half-maximal activation of either of these currents occur.

3. Results and discussion

3.1. Effects of GlyH-101 on ventricular $I_{Cl.PKA}$

Fig. 1A shows a representative time-course of $I_{Cl.PKA}$ activation at +100 and −100 mV by 10 μM forskolin and with added GlyH-101 (30 μM). Under $I_{Cl.PKA}$-selective conditions, forskolin rapidly activated current at both negative and positive voltages (e.g. forskolin increased the control current by 318 ± 43% at +100 mV and at −100 mV the increase was 161 ± 31% in 36 cells). The forskolin-activated current reversed at −28.6 ± 1.1 mV ($n = 36$). Subsequent superfusion of GlyH-101 in the presence of forskolin led to a rapid attenuation of forskolin-activated current at both voltages, an effect that was reversible on washout of GlyH-101. Fig. 1B shows mean current–voltage (*I–V*) relations for the 10 μM forskolin-activated difference currents and the 30 μM GlyH-101-sensitive currents; both traces are identical across the range of voltages tested, indicative of complete inhibition of $I_{Cl.PKA}$. The actions of GlyH-101 on $I_{Cl.PKA}$ were then tested at a range of concentrations between 0.3 and 30 μM and the concentration-dependence of fractional block at +100 mV and −100 mV is shown in Fig. 1C. At +100 mV, the IC_{50} was 0.3 ± 1.5 μM (with a Hill slope of 0.5), whilst at −100 mV the compound was less potent, with an IC_{50} of 5.1 ± 1.3 μM (a Hill slope of 0.8) – more than ten-fold the value at +100 mV. This is comparable to the situation for epithelial I_{CFTR}, for which the reported K_i values for GlyH-101 inhibition were also markedly lower at positive than at negative voltages (1.4 μM at +60 mV and 5.6 μM at −60 mV) [24]. GlyH-101 exists as a monovalent anion across a range of pH values

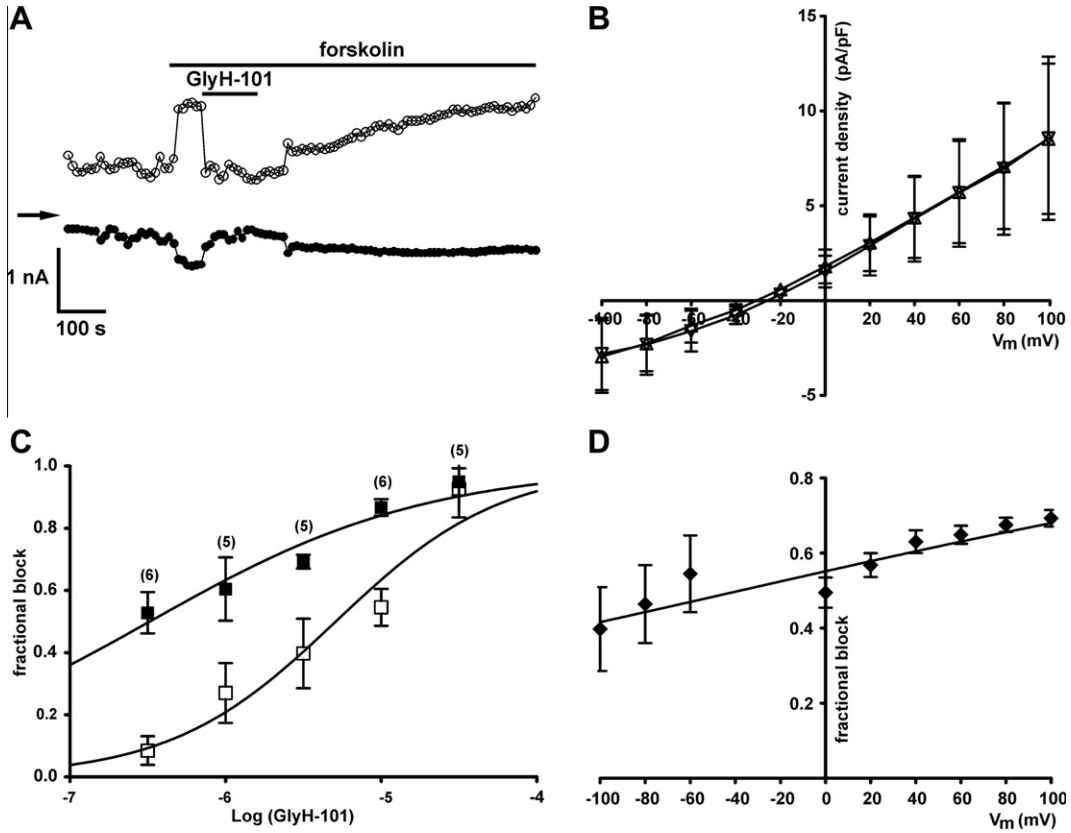

Fig. 1. Effect of GlyH-101 on forskolin activated $I_{Cl.PKA}$. (A) Representative time course of an experiment with currents sampled at +100 mV (open circles) and −100 mV (filled circles) during saw-tooth voltage-ramps from a holding potential of 0 mV to +100 and −100 mV (0.2 V/s, 1/10 s); arrow indicates zero current level and the solid bars at the top indicate application of 10 μM forskolin and 30 μM GlyH-101. (B) Mean current–voltage (*I–V*) relationships sampled during the descending phase of the ramp. Forskolin-activated difference currents (open upward triangles), and the 30 μM GlyH-101-senstive difference currents (open downward triangles) ($n = 5$). (**C**) concentration- response relationship of the effect of GlyH-101 on 10 μM forskolin-activated $I_{Cl.PKA}$. Concentration–response relations are shown at +100 mV (filled squares) and −100 mV (open squares). The '*n*' numbers at each respective concentration are shown in parentheses. Solid lines represent fits to Eq. (2). (D) Fractional block of forskolin-activated $I_{Cl.PKA}$ by 3 μM GlyH-101 is plotted against the test potential (filled diamonds). The solid line represents a fit to Eq. (3). Data points at −40 mV and −20 mV were excluded as the small current size close to the $I_{Cl.PKA}$ reversal potential made accurate determination of fractional block at these voltages difficult.

encompassing the physiological range and its inhibition of I_{CFTR} has been reported to exhibit features of a pore-blocker [24]. Transmembrane voltage may therefore influence access of the compound to its binding site on CFTR channels. In order to investigate this for cardiac $I_{Cl.PKA}$ we conducted Woodhull analysis [38,39]. The voltage-dependence of fractional block values of $I_{Cl.PKA}$ by 3 μM GlyH-101 was plotted (Fig. 1D) and the data fitted to Eq. (3). This yielded a δ value of 0.15 ± 0.03, suggesting that the binding site for GlyH-101 within the cardiac CFTR channel senses ~15% of the transmembrane field with respect to the external surface. A binding site towards the channel exterior is consistent with both the observed voltage-dependence of inhibition and the rapid onset of block seen when the compound was applied by external superfusion.

3.2. Effects of GlyH-101 on other cardiac conductances

In order to gain insight into the propensity, or otherwise, for GlyH-101 to interact with channels mediating other cardiac ionic currents, "signature currents" [33,34], were elicited using an ascending voltage ramp protocol: from −80 mV, membrane potential was stepped to −120 mV, followed by a ramp to +70 mV (ramp velocity of 1.6 V/s; stimulation frequency of 0.33 Hz). This protocol enables a range of currents rapidly to be surveyed [33,34]. Fig. 2A(i) and A(ii) show representative signature currents in control and in the presence of 30 μM GlyH-101 (the minimum concentration of those studied that inhibited $I_{Cl.PKA}$ at both positive and negative voltages). The inward current at the start of the ramp (mediated predominantly by inward-rectifier K^+ current, I_{K1} [33,34]) exhibited a modest decrease in amplitude in the presence of GlyH-101, whilst current at the upper end of the voltage ramp was little affected. The large and rapid inward current during the protocol represents the rapid sodium current, I_{Na}, and this was markedly inhibited by GlyH-101. The smaller inward deflection during the protocol, positive to the I_{Na} deflection, is mediated by L-type Ca^{2+} current ($I_{Ca,L}$): effects on this of GlyH-101 were unclear using this protocol. Fig. 2B(i), B(ii) and B(iii) quantitate the effects of GlyH-101 respectively on current at the start of the ramp, on the I_{Na} deflection and at the end of the ramp; these indicate statistically significant effects of the compound on I_{K1} and I_{Na}.

The signature current protocol is suitable for rapid screening of drug-effects on cardiac ion currents and for identifying components likely to be susceptible to drug-modulation [33,34]. However, it is not ideal for accurate quantitation of drug-effects, particularly for rapid, voltage-dependent currents. In order to assess actions of GlyH-101 on I_{Na} more accurately, selective recordings were made using previously validated recording conditions [40,41]. Fig. 3A(i) and A(ii) show representative recordings of I_{Na} elicited by step depolarization from −140 to −40 mV. Consistent with the data shown in Fig. 2, I_{Na} was very substantially inhibited by 30 μM GlyH-101. In order to investigate the voltage-dependence of this effect, command pulses were applied from −140 mV to a range of test potentials and the amplitude of the resulting I_{Na} measured. Fig. 3B shows the mean *I–V* relations for I_{Na}, with marked reduction of the current by GlyH-101 across the

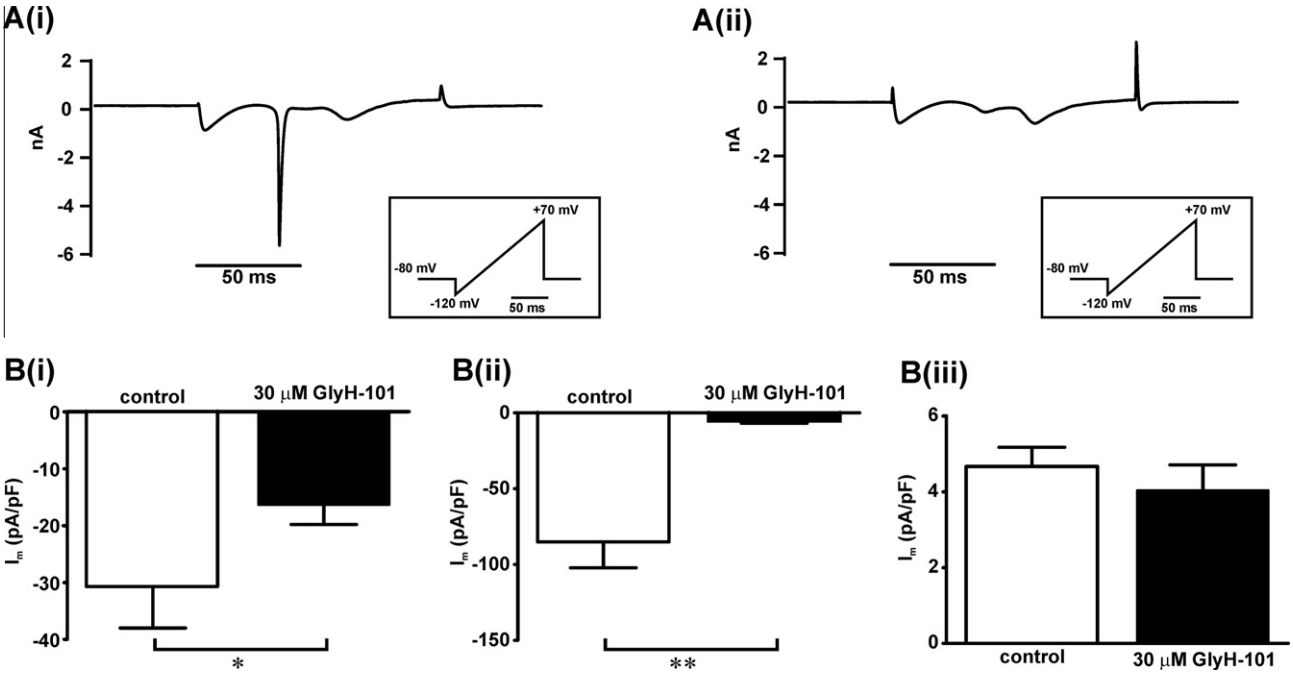

Fig. 2. Effects of 30 μM GlyH-101 on signature currents. (A) Representative net whole-cell signature current in control conditions (i) and in presence of 30 μM GlyH-101 (ii); insets show the voltage ramp used. (B) Mean data from five cells showing the effect of 30 μM GlyH-101 on the I_{K1} (i), the I_{Na} (ii) and the end of ramp outward currents (iii). $^{*}P < 0.05$, $^{**}P < 0.01$ (paired *t*-test).

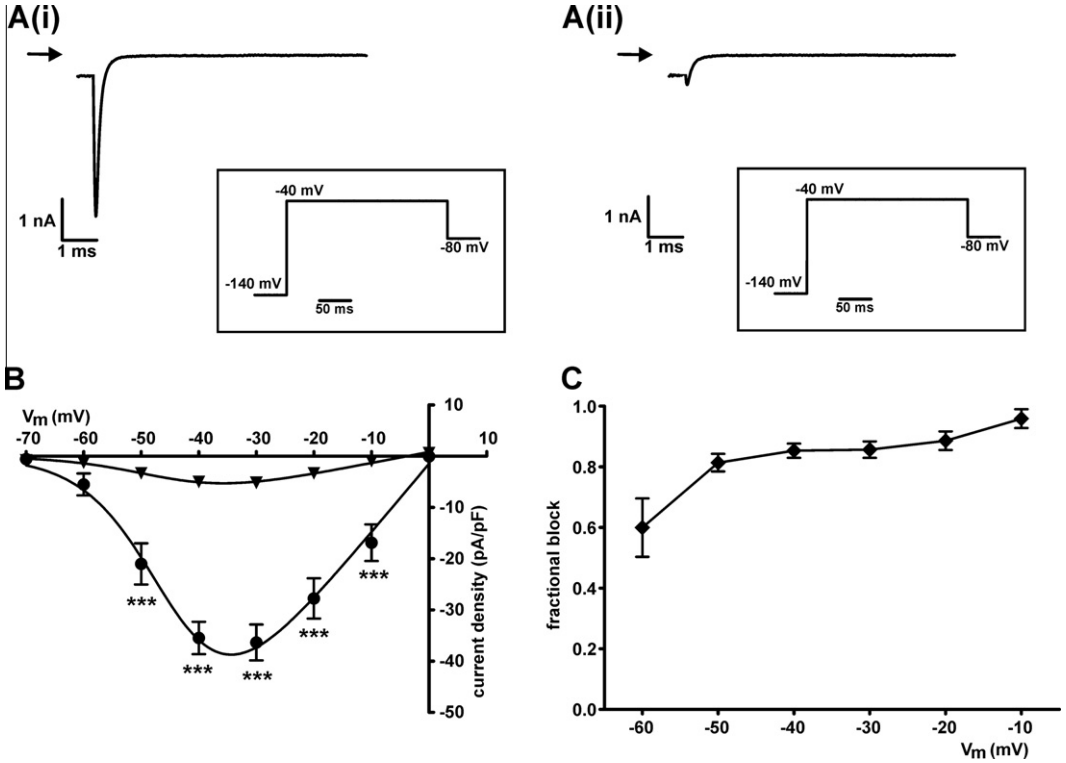

Fig. 3. Effect of 30 μM GlyH-101 on I_{Na}. (A) Representative net whole-cell currents in control conditions (i) and with GlyH-101 (ii); voltage protocol shown in inset and capacitive transients have been omitted for clarity. (B) Mean *I–V* relationships for peak I_{Na} in control conditions (filled circles) and in the presence of 30 μM GlyH-101 (filled downward triangles) ($n = 8$). Solid lines represent fits to Eq. (5). $^{***}P < 0.001$; two-way RM ANOVA. (**C**) Voltage-dependence of fractional block by 30 μM GlyH-101 ($P < 0.001$, one-way RM ANOVA; $n = 8$).

entire voltage range. The $V_{0.5}$ and k values for I_{Na} activation were -44.7 ± 3.6 mV and 7.6 ± 1.9, respectively in the presence of GlyH-101 compared to values of -44.1 ± 2.1 mV and 6.6 ± 1.2 in control ($n = 8$); thus whilst GlyH-101 reduced the I_{Na} magnitude, it did not significantly affect I_{Na} activation parameters. However, GlyH-101 block of I_{Na} was voltage-dependent (Fig. 3C).

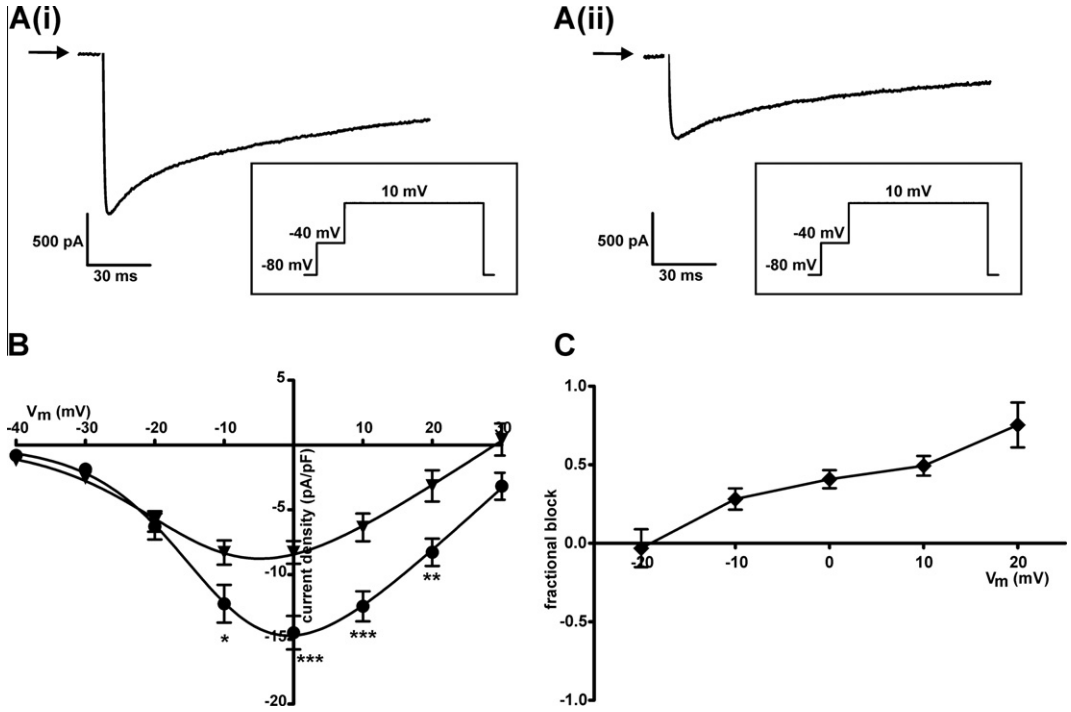

Fig. 4. 30 μM GlyH-101 and L-type Ca^{2+} current. (A) Representative net whole-cell currents in control conditions (i) and in the presence of 30 μM GlyH-101; (ii) voltage protocol shown in inset; capacitive transients have been omitted for clarity. (B) Mean *I–V* relationships for the peak $I_{Ca,L}$ in control (filled circles) and with GlyH-101 (filled downward triangles) ($n = 11$). Solid lines represent fits to Eq. (5). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$; two-way RM ANOVA. (**C**) Voltage-dependence of fractional block by 30 μM GlyH-101 ($P < 0.0001$, one-way RM ANOVA; $n = 11$).

As it was unclear from using the signature current protocol in Fig. 2 whether or not GlyH-101 modulated $I_{Ca,L}$, conventional voltage-step command experiments were conducted to determine the actions of the drug on L-type channels, using external Ba^{2+} ions as the charge carrier. Fig. 4A(i) and A(ii) show representative records of $I_{Ca,L}$ elicited by step depolarization from −40 mV to +10 mV. Peak current was markedly reduced by 30 μM GlyH-101, although the effect was not so pronounced as for I_{Na} (Fig. 3). In order to investigate the voltage-dependence of this effect, the $I_{Ca,L}$-voltage relations in the control and presence of GlyH-101 were plotted (Fig. 4B). Currents were significantly inhibited between −10 mV and +20 mV, but at more negative voltages a small apparent increase in current was seen. The $V_{0.5}$ and k values of $I_{Ca,L}$ were −11.0 ± 2.3 mV and 7.2 ± 1.2, respectively in control and in the presence of GlyH-101, −14.1 ± 3.8 mV and 8.6 ± 1.8. GlyH-101 block of $I_{Ca,L}$ was voltage-dependent ($P < 0.0001$; Fig. 4D). Thus, GlyH-101 appears to have inhibited $I_{Ca,L}$ with a trend towards a left-ward shift in voltage-dependent activation, which may account for the apparent lack of inhibition/augmentation at negative voltages in the range examined.

3.3. Results of the present study: context and implications

To our best knowledge this is the first study to have characterised the effects of GlyH-101 on native cardiac CFTR current and to have determined its effects on a range of other cardiac ionic conductances. Results of our study using rabbit ventricular myocytes are consistent with findings from studies with epithelial preparations that GlyH-101 inhibits $I_{Cl.PKA}$ in a voltage dependent manner [24,29]. GlyH-101 was more potent and effective against cardiac CFTR current than against the human atrial anionic background current, which was only partially inhibited at a concentration of 30 mM [9]. Furthermore it also inhibits inward-rectifier I_{K1} and $I_{Ca,L}$, an effect much less marked in comparison to its profound blockade of I_{Na}. It has been shown that the cardiac CFTR isoform is an alternative splice variant although it shares >95% homology with the human epithelial CFTR [42]; the results of our study suggest that the cardiac CFTR retains sensitivity to GlyH-101.

Our results are concordant with the notion that GlyH-101 interacts directly with the CFTR protein and induces a pore block, binding towards the external face of the channel and gaining access rapidly on external application [43]. However, it is not entirely selective for $I_{Cl.PKA}$ alone at a concentration that is effective at blocking both the inward and the outward CFTR currents, which might limit its use as a selective agent. Nevertheless, it is feasible that lower concentrations of this compound may have some value for blocking selectively the outward $I_{Cl.PKA}$. Small molecule CFTR inhibitors, including glycine hydrazides, may have utility against diarrhoea and polycystic kidney disease [24,44]. However, whilst GlyH-101 might be effective against the latter if applied directly; caution would be warranted in its systemic use as, quite aside from any effects on lung epithelia, our data indicate a potential not only to modify cardiac CFTR but also cardiac cationic conductances.

Acknowledgments

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