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Acute desensitization of acetylcholine and endothelin-1 activated inward rectifier K^+ current in myocytes from the cardiac atrioventricular node

Stéphanie C.M. Choisy, Andrew F. James, Jules C. Hancox *

School of Physiology & Pharmacology and Cardiovascular Research Laboratories, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, UK

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ABSTRACT

The atrioventricular node (AVN) is a vital component of the pacemaker-conduction system of the heart, co-ordinating conduction of electrical excitation from cardiac atria to ventricles and acting as a secondary pacemaker. The electrical behaviour of the AVN is modulated by vagal activity *via* activation of muscarinic potassium current, I_{KACh}. However, it is not yet known if this response exhibits 'fade' or desensitization in the AVN, as established for the heart's primary pacemaker – the sinoatrial node. In this study, acute activation of I_{KACh} in rabbit single AVN cells was investigated using whole-cell patch clamp at 37 °C. 0.1–1 μM acetylcholine (ACh) rapidly activated a robust I_{KACh} in AVN myocytes during a descending voltage-ramp protocol. This response was inhibited by tertiapin-Q (TQ; 300 nM) and by the M2 muscarinic ACh receptor antagonist AFDX-116 (1 μM). During sustained ACh exposure the elicited I_{KACh} exhibited bi-exponential fade (τ_f of 2.0 s and τ_s 76.9 s at −120 mV; 1 μM ACh). 10 nM ET-1 elicited a current similar to I_{KACh}, which faded with a mono-exponential time-course (τ of 52.6 s at −120 mV). When ET-1 was applied following ACh, the ET-1 activated response was greatly attenuated, demonstrating that ACh could desensitize the response to ET-1. For neither ACh nor ET-1 was the rate of current fade dependent upon the initial response magnitude, which is inconsistent with K^+ flux mediated changes in electrochemical driving force as the underlying mechanism. Collectively, these findings demonstrate that TQ sensitive inwardly rectifying K^+ current in cardiac AVN cells, elicited by M2 muscarinic receptor or ET-1 receptor activation, exhibits fade due to rapid desensitization.

1. Introduction

The atrioventricular node (AVN) of the heart is situated at the junction between the right atrium and ventricle [1] and is normally the only route by which electrical excitation can pass from atria to ventricles [2]. During atrial fibrillation the slow conduction properties of the AVN limit impulse transmission to the ventricles, thereby affording them some protection from an excessively fast ventricular rate [2,3]. The AVN also possesses pacemaker properties and can take over ventricular pacing should the primary pacemaker, the sinoatrial node (SAN), fail [2,4]. The electrophysiological properties of the intact AVN depend on both the anatomy and electrophysiology of the region [2,5] and the cellular electrophysiology of different sub-regions of the AVN depends on the interplay between a range of ion channel currents [6–8].

Vagal stimulation or application of acetyl-choline (ACh) produces negative dromotropic and chronotropic effects on the AVN [2]. Activation of G-protein dependent, inwardly rectifying Kir3.1/3.4 channels is important to the cardiac actions of ACh [9,10]. Application of cholinergic agonists to small multicellular AVN preparations or to single AVN cells activates an inwardly rectifying K^+ current, I_{KACh} which decreases excitability [7,11–14], contributing to suppression of spontaneous activity [12,13] and, in perfused intact hearts, to AV conduction block [15]. In the SAN, I_{KACh} (likely carried by Kir3.1/3.4; [10]) contributes significantly to the negative chronotropic effect of ACh [16] and SAN I_{KACh} activation 'fades' in the continuous presence of ACh [17]. However, there is disagreement as to whether or not vagal responses of the AVN exhibit a 'fade' phenomenon. For example, in one study the negative dromotropic response of anaesthetised dogs to tonic vagal stimulation was reported to exhibit fade when the initial response to vagal stimulation was large [18], whilst in a separate study dromotropic effects of ACh in anaesthetised dogs were reported not to fade during maintained cholinergic activation [19]. On the other hand, vagal stimulation of rabbit isolated atrial preparations from which SAN and AVN responses were monitored simultaneously has been reported to produce membrane hyperpolarization that exhibited similar fade in both regions [20]. We have recently observed transient activation in isolated AVN myocytes of a tertiapin-Q sensitive inwardly rectifying I_{KACh}-like current by the peptide hormone ET-1 [21]. This raises the possibility that muscarinic activation of I_{KACh} in myocytes from the AVN is followed by fade/desensitization of the response. The present study was con-

* Corresponding author. Fax: +44 1173312288.
E-mail address: jules.hancox@bristol.ac.uk (J.C. Hancox).

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ducted to test this proposition, using an established single AVN cell preparation from the rabbit heart [13].

2. Methods

2.1. Rabbit AVN cell isolation

Male White New-Zealand rabbits (~2.0–3.5 kg) were killed in accordance with the United Kingdom Home Office Animals Scientific Procedures Act (1986). Their hearts were rapidly excised and cells isolated from the entire atrioventricular nodal (AVN) region as described previously [13,22,23]. Isolated AVN cells were stored in Kraft-Brühe "KB" solution [13,24] at 4 °C until use.

2.2. Electrophysiological recording

Cells were placed in an experimental chamber (0.5 ml) mounted on the stage of an inverted microscope (Nikon Diaphot) and superfused with a normal Tyrode's solution, containing (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5 and Glucose 10, (pH 7.4 with NaOH). ACh, ET-1 and other compounds were added to this solution. Patch-pipettes (Corning 7052 glass, AM Systems Inc., Sequim, WA, USA) were pulled using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA) and were filled with a pipette solution containing (in mM) [23,25]: KCl 110, NaCl 10, HEPES 10, $MgCl_2$ 0.4, and Glucose 5, K_2ATP dihydrate 5, GTP-Tris salt 0.5, BAPTA 5, pH 7.1 with KOH. Recordings were made using an Axopatch 1D amplifier (Axon Instruments; now Molecular Devices, Sunnyvale, CA, USA). Pipette resistance was typically <3 MΩ; series resistance was typically compensated by ~60–80%. Under voltage clamp, membrane potential was held at −40 mV as this value corresponds to the zero current potential for rabbit AVN cells (e.g. [13,26]). Electrophysiological protocols were generated and data recorded using Clampex 8 (Axon Instruments; CA, USA). Repetitive application of a descending voltage ramp protocol (shown in the left panel of Fig. 1A) was used to survey rapidly current between +20 and −120 mV. A brief (50 ms) step from −40 to +20 mV preceded the descending ramp in the voltage protocol. The initial depolarizing step phase of the protocol elicited an L-type calcium current ($I_{Ca,L}$), which was used as an independent marker of AVN cell responsiveness to ACh (and ET-1), as both receptor agonists inhibit AVN $I_{Ca,L}$ (e.g.[12,13,21]).

2.3. Solutions and chemicals

Cells were superfused with experimental solutions at 35–37 °C (checked regularly using a hand-held thermocouple). ACh, ET-1 and other compounds were applied externally to the cell under study using a home-built, rapid solution exchange device capable of exchanging superfusate in <1 s [27]. ACh (Sigma–Aldrich Company Ltd., Dorset, UK) was used at 0.1 and 1 μM from a stock solution (1 or 10 mM) made each experimental day in distilled, deionized water. ET-1 (Sigma–Aldrich Company Ltd., Dorset, UK) was used at 10 nM from a stock solution of 100 μM prepared in 0.1% acetic acid. Tertiapin-Q (Tocris Bioscience, Bristol, UK) was prepared in deionised water and used at a final concentration of 300 nM in Tyrode's solution. AFDX-116 (Ascent Scientific, Bristol) was used at 1 μM from a stock solution made in DMSO. All drug stocks except ACh were aliquoted and stored at −20 °C.

2.4. Data analysis

Data were analysed and graphical plots produced using Clampfit 10.2 software (Molecular Devices Sunnyvale, CA, USA), Microsoft Excel (2003) and GraphPad Prism (v5; GraphPad Software Inc., La Jolla, CA, USA). Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's *t*-test, one-way ANOVA with a Bonferroni post hoc test. Values of '*p*' less than 0.05 were taken as significant.

3. Results and discussion

One micromolar of ACh was employed to elicit I_{KACh} in this study, as this concentration has been established to produce a robust activation of AVN cell I_{KACh} [7], leading to rapid membrane potential hyperpolarisation and quiescence in AVN cell and tissue preparations [12,13]. Fig. 1A (right panel) shows representative currents during the descending ramp phase of the command protocol plotted against voltage, obtained in normal Tyrode's solution and immediately following rapid application of ACh. One micromolar of ACh increased current across the entire range of measured voltages. In ACh the net current during the ramp exhibited marked inward rectification and reversed slightly negative to −80 mV. Fig. 1B shows the mean current density–voltage (*I*–*V*) relation for the maximal ACh-activated current, I_{KACh} ($n = 6$). The mean reversal potential (E_{rev}) for this current was −85.5 ± 0.6 mV. In additional experiments, I_{KACh} was activated by a lower ACh concentration (0.1 μM); the current profile was similar to that produced by 1 μM ACh, but was of smaller magnitude. The M2 receptor is considered to be the predominant muscarinic receptor sub-type in mammalian heart [28]. When AVN cells were pre-treated with the M2 receptor inhibitor AFDX-116 (1 μM), the response to 1 μM ACh was largely abolished (Fig. 1C), demonstrating M2 receptor activation to be responsible for generation of AVN I_{KACh}. This is concordant with: (i) prior work on anaesthetised dogs in which AFDX-116 inhibited chronotropic and dromotropic responses to intracardiac vagal nerve stimulation [29]; (ii) the persistence of AV conduction block in response to intravenous ACh in mice deficient in M1-receptors [30], and (iii) presumed M2-receptor mediated conduction effects of propofol on guinea-pig hearts [31]. SAN I_{KACh} is sensitive to the bee venom toxin tertiapin [32,33]. To our knowledge, there are no similar data for AVN I_{KACh} *per se*, although ACh effects on guinea-pig atrio-ventricular conduction have been shown to be sensitive to tertiapin [15]. When we applied ACh to cells after exposure to tertiapin Q (300 nM), I_{KACh} was largely abolished (Fig. 1C). Collectively, the data in Fig. 1 demonstrate that rabbit AVN I_{KACh} is tertiapin-Q sensitive and involves ACh binding to M2 muscarinic receptors.

Fig. 2 shows the time-course of the effect of sustained exposure to 1 μM ACh. Fig. 2A and B show representative time-plots for current at two voltages (+20 and −120 mV) during the voltage ramp protocol. It is clear that at both voltages ACh application led to a maximal response within seconds of its application. However, in the maintained presence of ACh the current response declined over ~2 min, with an initial rapid decline followed by a slower second phase. In six experiments the peak response at −120 mV was −37.6 ± 5.1 pA/pF, whilst after 2 min of ACh application the response declined to an amplitude of −16.9 ± 0.8 pA/pF ($p < 0.01$). At +20 mV, the peak response amplitude was 21.0 ± 2.6 pA/pF, declining to 9.3 ± 1.1 pA/pF at 2 min of ACh application ($p < 0.01$). Thus, acute activation of AVN I_{KACh} exhibited rapid bi-phasic 'fade'. This was quantified by bi-exponential fitting of the decline of net current at each voltage; data from individual cells at each time-point were normalised to maximal current in ACh, and then pooled. Fits to the mean data are shown in Fig. 2C (at +20 mV) and 2D (at −120 mV). At −120 mV 62.2 ± 6.7% of the current decline was described by a time-constant (τ_f) of 2.0 s, whilst the remainder of the current decline was described by a time-constant (τ_s) of 76.9 s (see Fig. 2 legend for further numerical information). In contrast to the situation for I_{KACh}, $I_{Ca,L}$ elicited by the +20 mV

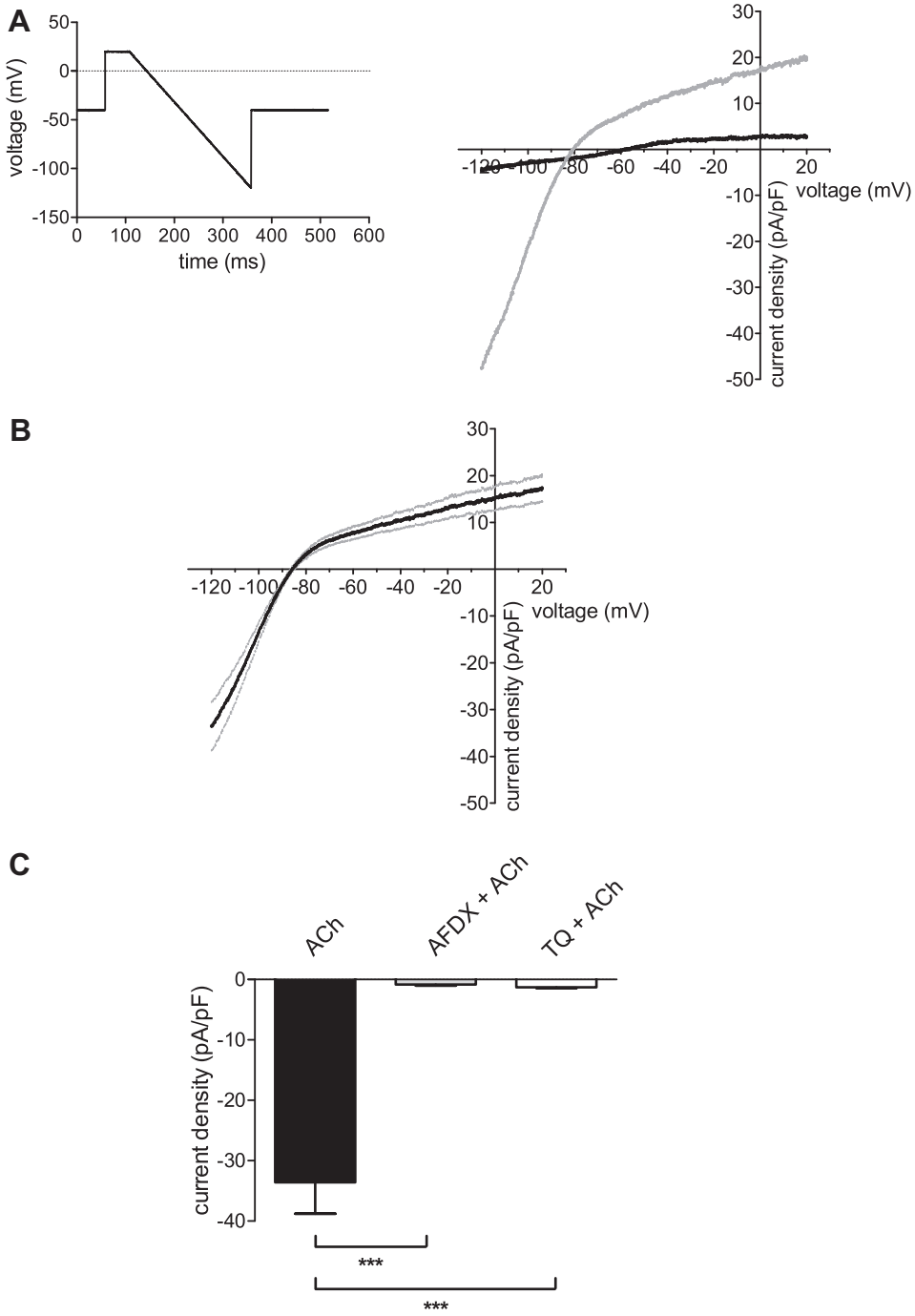

Fig. 1. Activation of AVN I_{KACh} and sensitivity to M2 receptor inhibition and tertiapin-Q (A) Left panel shows voltage protocol used: from a holding potential of −40 mV, a step to +20 mV for 50 ms preceded a descending voltage-ramp to −120 mV over 250 ms. The start-to-start interval between successive applications of the protocol was 2 s. Right panel shows representative currents elicited in control (normal Tyrode's) solution (black trace) and immediately following rapid superfusion of 1 μM ACh (grey trace). (B) Mean current–voltage (*I–V*) relation for current activated by 1 μM ACh ($n = 6$; obtained by digital subtraction of current in control from that in ACh and normalised to cell membrane capacitance). The black trace denotes the mean current values at each voltage, with grey traces indicating ±SEM values. (C) Mean current–density (pA/pF) of the maximal I_{KACh} at −120 mV (1 μM ACh sensitive current) in the presence of ACh alone (black bar; $n = 6$), with 1 μM of the M2 muscarinic receptor inhibitor AFDX-116 (grey bar; $n = 5$), and in the presence of 300 nM of the GIRK channel inhibitor tertiapin-Q (white bar; $n = 6$). Asterisks denote statistically significant differences (***$p < 0.001$).

voltage step phase of the voltage protocol decreased monotonically with 1 μM ACh application (not shown), declining by 41.7 ± 6.0% ($n = 6$) of its initial amplitude by ~2 min following the peak I_{KACh} response. The biphasic nature of the fade of AVN cell I_{KACh} in this study correlates qualitatively with a reported biphasic decline of hyperpolarising response of the rabbit intact AVN to trains of vagal nerve stimuli [20], although in absolute terms the time-course of the observed fade of the intact node membrane potential response was faster than that seen here [20]. Differences in time-course of response decline between that study and this one are likely to be accounted for by the different preparations used (intact tissue versus isolated cell) and mode and duration of vagal activation (vagal nerve stimulation versus sustained superfusion of ACh).

Recent data from experiments conducted using voltage-step protocols have shown that ET-1, acting through the ET_A receptor, transiently activates a tertiapin-Q sensitive K^+ current that resem-

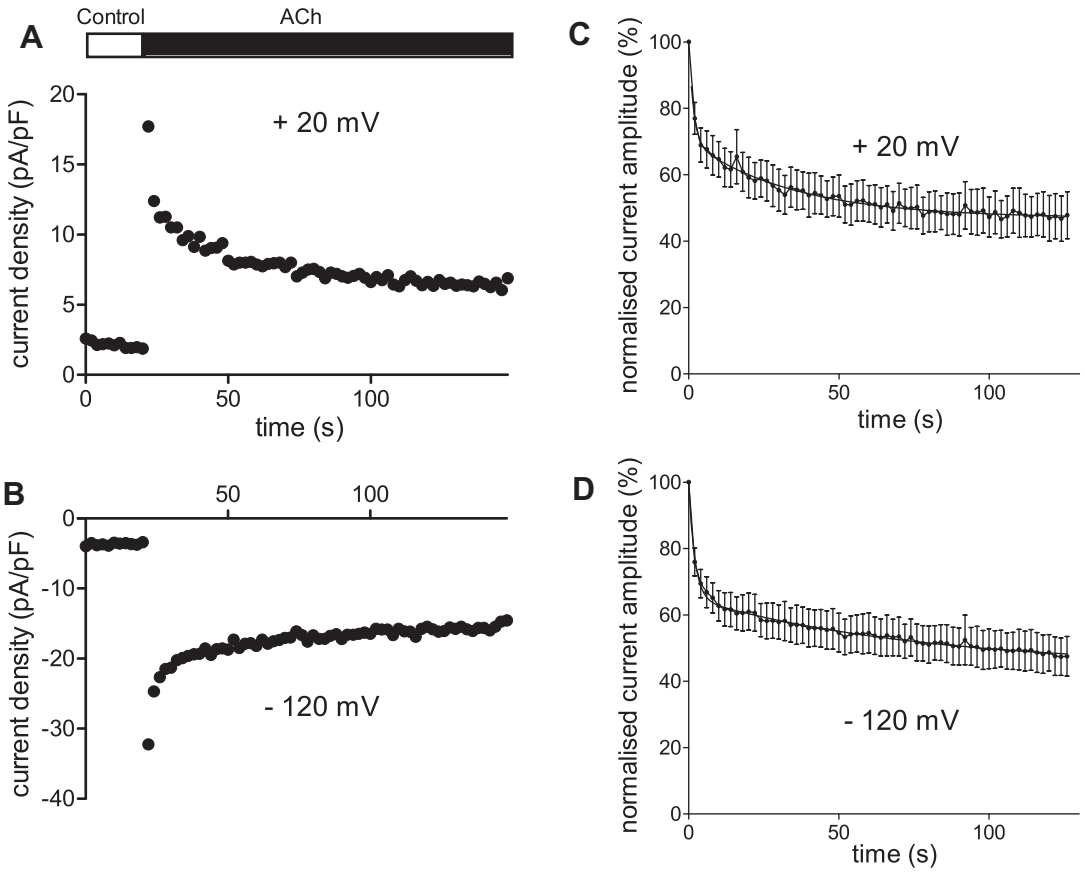

Fig. 2. Time-course of fade of the ACh response. (A and B) show representative continuous time-plots of current at +20 mV (A) and −120 mV (B) before and during exposure to 1 μM ACh. Time of ACh application is denoted by the horizontal bar in panel A. (C and D) show mean data-plots ($n = 6$) in which for each cell the net current amplitude was normalised to the maximum response in the presence of ACh at +20 mV (C) and −120 mV (D). Data were fitted by a two-phase decay equation. At +20 mV 56.1 ± 8.8% of the declining current was characterised by a fast rate of decline (K_{fast}) of 0.67 ± 0.42 s^{-1} (equivalent to a τ_f of 1.5 s) whilst the remainder of current decline was characterised by a slow rate of decline (K_{slow}) of 0.029 ± 0.012 s^{-1} (equivalent to a τ_s of 34.5 s). At −120 mV 62.2 ± 6.7% of current decline was characterised by a K_{fast} of 0.50 ± 0.20 s^{-1} ($p > 0.7$ versus +20 mV; equivalent to a τ_f of 2 s), whilst the remainder of current decline was characterised by a K_{slow} of 0.013 ± 0.011 s^{-1} ($p > 0.3$; equivalent to a τ_s of 76.9 s).

bles I_{KACh} [21]. Consequently, for comparison with our observations on I_{KACh}, we conducted experiments with ET-1 using the same voltage protocol used to study ACh. Fig. 3A shows the time-course of current activation by ET-1 at +20 and −120 mV during the voltage ramp protocol. At both voltages ET-1 application led to a maximal response within seconds of its application (mean *I–V* data for peak ET-1 activated current are shown in Fig. 3B, showing the response to be very similar to that to ACh (Fig. 1B), though of smaller magnitude). Also similar to ACh, in the maintained presence of ET-1 the response magnitude declined over a 2–3 min recording period. This is shown for an individual experiment in Fig. 3A and in Fig. 3C and D for mean data (normalised to maximal response amplitude as for ACh in Fig. 2). The rate of decline of the ET-1 response could be described satisfactorily by a single exponential function, with time-constant values of 55.5 and 52.6 s respectively at +20 and −120 mV. As reported recently [21], 10 nM ET-1 also produced a monotonic decrease in the amplitude $I_{Ca,L}$ elicited by the +20 mV voltage step (declining by 69.7 ± 3.3% ($n = 6$) at ~2 min of exposure).

Acute desensitization of I_{KACh} in rat atrial myocytes has been proposed to depend on K^+ ion flow and to arise due to an acute reduction in electrochemical driving force for K^+ ion flow rather than from changes to receptor-coupled signalling [34]. If such an explanation applies to AVN cells, then the magnitude of peak response to ACh (representing an index of K^+ flux) might be expected to determine the rate of subsequent current decline. Fig. 4A and B respectively show plots of the τ_f and τ_s of decline of ACh responses against the initial peak magnitude of the current response, incorporating data with both 0.1 and 1 μM ACh (respectively, open and filled symbols). There was no significant correlation between either time constant and the initial response amplitude (r of 0.0 for τ_f and −0.4 for τ_s, with respective p values of 1.0 and 0.2). Fig. 4C shows similar data for ET-1, demonstrating little correlation between fade time constant and initial response amplitude (r of 0.09, p of 0.9). Thus, regardless of the receptor system by which I_{KACh} was activated, there was no relation between the rate of fade and the magnitude of K^+ flux. In addition, were a change in the electrochemical driving force for K^+ flow to result from activation of AVN I_{KACh}, this might be expected to be reflected in a positive shift in the reversal potential for the current during continued application of the agonist. Fig. 4D shows plots of E_{rev} for the 1 μM ACh-activated current at the time-point of peak response to ACh and at 6 s after the initial peak (at which time-point any initial decline would have been attributable to the fast component of current fade) and after 2 min of ACh application (by which time-point both fast and slow fade would have occurred). The values obtained at the three time points did not differ significantly, indicating that statistically significant changes in electrochemical driving force (and thereby E_{rev}) had not occurred ($p > 0.1$). In this regard our results are similar to those from a prior study of guinea-pig atrial myocytes, in which biphasic fade of I_{KACh} occurred with no alteration in E_{rev} for the current [35]. Thus, it can be concluded that, in contrast to the mechanism proposed for rat atrial myocytes by Bender et al. [34], the fade of I_{KACh} in AVN cells does not depend

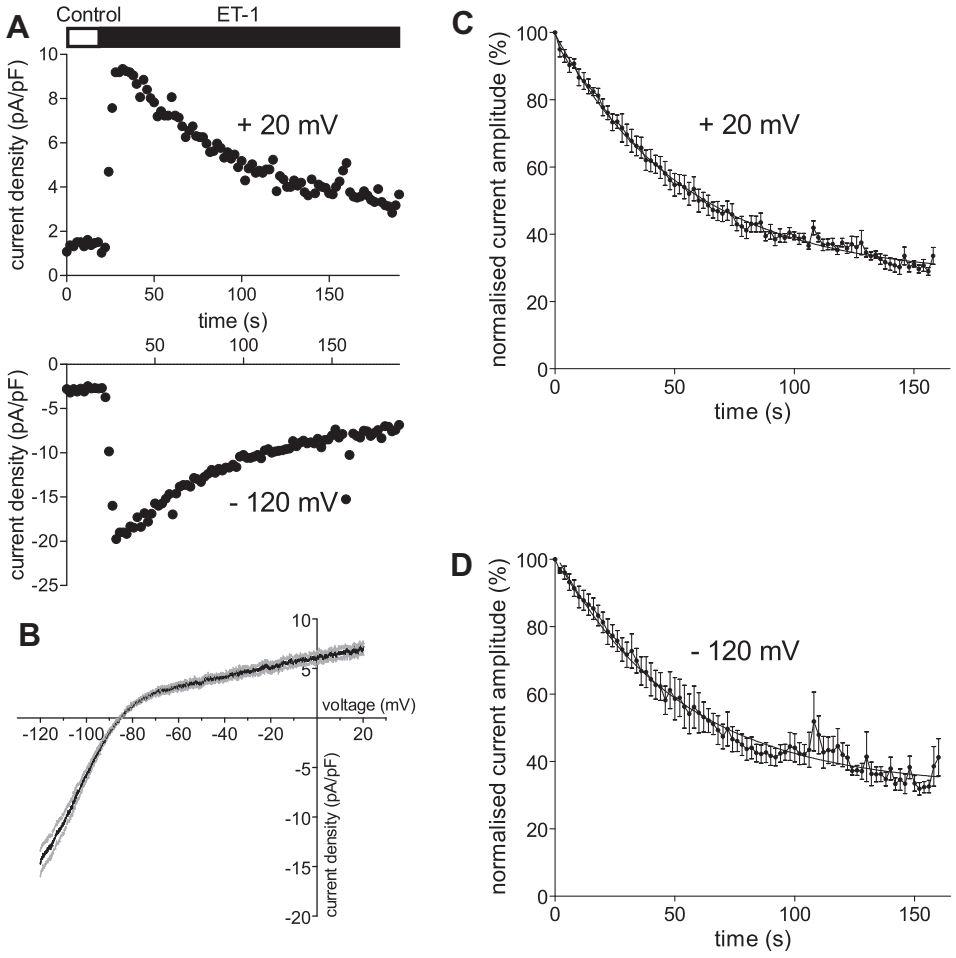

Fig. 3. Fade of ET-1 activated K^+ current. (A) shows representative continuous time-plots of current at +20 mV (upper panel) and −120 mV (lower panel) before and during exposure to 10 nM ET-1. Time of ET-1 application is denoted by the horizontal bar in upper panel. Voltage protocol same as Fig. 1A. (B) shows mean current density–voltage relation for ET-1 activated current ($n = 6$). (C, D) show mean data indicating time-course of decline of response to ET-1 at +20 mV (C) and −120 mV (D). Data were normalised as for ACh in Fig. 2. The fade in response exhibited monophasic decline with rate constants of 0.018 ± 0.001 s^{-1} and 0.019 ± 0.001 s^{-1} for +20 and −120 mV, respectively ($p > 0.4$; equivalent to τ values of 55.5 and 52.6 s).

on K^+ ion flow and represents genuine desensitization of the response to ACh.

In a final set of experiments, we investigated whether or not ACh was able to cause desensitization of the response to ET-1. In these experiments 1 μM ACh was applied and after the response had exhibited considerable fade (with at least two minutes of ACh exposure), ET-1 was then rapidly applied in the maintained presence of ACh. Fig. 4E shows representative traces of the ACh-sensitive current at the peak of the ACh response (black trace) and after 2 min in the presence of ACh (light grey trace). Also superimposed is the peak ET-1-sensitive current following subsequent ET-1 application (dark grey trace): it is notable that under these conditions relatively little additional current was activated by ET-1. Fig. 4F shows the mean current density at −120 mV of ACh-activated and ET-1 activated current (when each agonist was applied separately), together with the mean ET-1 activated current density for cells receiving prior ACh exposure. The small size of the ET-1 activated current following exposure to ACh in comparison to the response to ET-1 alone indicates that ACh and ET-1 responses are not simply additive. Furthermore, once the ACh response had faded, the affected channels appeared to be relatively unresponsive to ET-1. Thus, it can be concluded that ACh causes desensitization not only of the ACh-sensitive K^+ current but also of the ET-1-sensitive K^+ current (this was not the case for $I_{Ca,L}$, which was still able to respond to ET-1 with a 66.0 ± 8.0% ($n = 6$) decline, following prior ACh exposure (data not shown; not significantly different from extent of reduction without ACh pre-treatment; $p > 0.6$)). This observation suggests that despite working through different receptors, ET-1 and ACh share pathways of action downstream from the receptors and likely elicited their responses by acting on the same pool of K^+ channels; furthermore once the ACh response had faded, the affected channels appeared to be relatively unresponsive to ET-1. To our knowledge, this may constitute the first report for any region of the heart of cross-desensitization by ACh of the cardiac G-protein-dependent inwardly rectifying current activated by an agonist of a distinct receptor.

In conclusion, this study establishes that both M2 muscarinic receptor and ET-1 activated "I_{KACh}" in AVN cells exhibit marked fade during continuous exposure to ACh and ET-1. The two different receptor agonists appear to act on the same underlying K^+ channel pool. Acute fade of I_{KACh} is not explicable as a consequence of K^+ flux mediated alterations to electrochemical K^+ driving force. Rather it is likely to involve desensitization of the receptor/signalling pathway. Involvement of a receptor-linked mechanism is also consistent with differences in the time-course of fade of 10 nM ET-1 and 1 μM ACh responses; such differences would not be expected from a mechanism depending purely on K^+ ion flux. Muscarinic receptor-mediated inhibition of AVN basal $I_{Ca,L}$ has been proposed to involve suppression of cAMP synthesis [36] or nitric-oxide mod-

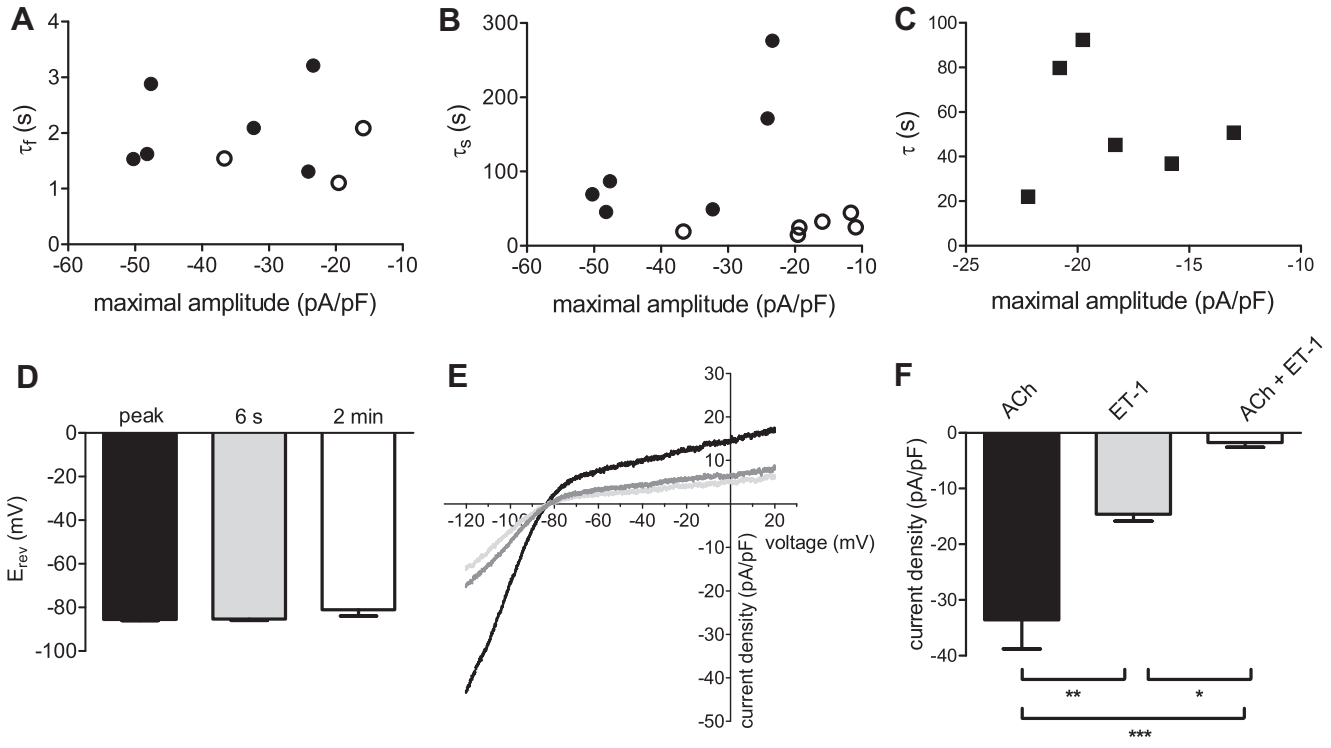

Fig. 4. Relationship between K^+ current response magnitude and decline rate, and effect of combined ACh and ET-1 application. (A and B) Plots of time constants of current fade (A: τ_{fast} (τ_f); B τ_{slow} (τ_s)) against magnitude of initial response to 1 μM ACh ($n = 6$; filled circles). Open circles show data for 0.1 μM ACh. For this concentration of ACh 3 cells exhibited biphasic decay and 3 cells monophasic decay; for the latter, the single τ values are plotted in B. (C) Plot of time constant of current fade against magnitude of initial response to 10 nM ET-1 ($n = 6$; filled circles). (A)–(C) time-course measured for responses at −120 mV. (D) Plot of E_{rev} for I_{KACh} activated by 1 μM ACh at the initial peak of the response (black bar), at 6 s following the maximal response (grey bar) and at 2 min following maximal response (open bar). There was no significant difference in E_{rev} values at the different time-points ($n = 6$; $p > 0.1$). (E and F) Effects of 10 nM ET-1 following prior exposure to 1 μM ACh. (E) shows representative currents (plotted as current density against voltage) for responses in the same cell to 1 μM ACh (I_{KACh}) measured as ACh-activated current at maximal response (black trace) and 2 min after the maximal response (light grey trace). The dark grey trace shows maximal current in 10 nM ET-1 (plotted as ET-1 activated current compared to control) following 2 min exposure to ACh. In the concomitant presence of ACh, ET-1 elicited little additional current. (F) Maximal current densities for current at −120 mV for ACh-activated current (black bar; $n = 6$), ET-1 (grey bar; $n = 6$,) and the ET-1 difference current (ET-1 minus ACh) when ET-1 was applied following fade of the ACh response. Asterisks denote statistical significance ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

ulation of cGMP-stimulated phosphodiesterase [14]; the lack of fade of the response of $I_{Ca,L}$ to ACh in the present study suggests that these pathways are unlikely to be involved in AVN I_{KACh} fade/desensitization. Cardiac muscarinic K^+ current desensitization has been suggested to involve mechanisms involving G protein-coupled receptor kinase (GRK2), β arrestin and M2 receptor internalisation (e.g. [37–40]). Further work is required to elucidate the contributions of such mechanisms to AVN cell I_{KACh} desensitization. However, the fact that in our experiments the inhibitory effects of ACh and ET-1 on $I_{Ca,L}$ were sustained in the same cells in which I_{KACh} fade occurred, suggests either that rapid receptor internalisation was not responsible for desensitization of the AVN cell K^+ current response in our experiments, or that the M2 receptors coupled to channels modulating $I_{Ca,L}$ and I_{KACh} reside in different pools. Rapid cross-desensitization of ET-1 activated K^+ current by prior ACh exposure is likely to occur down-stream of the ET-1 receptor itself, but could feasibly involve GRK2 linked desensitization of those ET_A receptors coupled to K^+ channels [41,42]. Fade of I_{KACh} may provide a mechanism for physiological adaptation of the AVN to cholinergic stimulation [20,43]. Further investigation of this phenomenon and of the mechanism(s) underlying desensitization is now warranted.

Acknowledgments

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