Cellular modelling: experiments and simulation to develop a physiological model of G-protein control of muscarinic K⁺ channels in mammalian atrial cells

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The first model of G-protein–KACh channel interaction was developed 14 years ago and then expanded to include both the receptor–G-protein cycle and G-protein–KACh channel interaction. The G-protein–KACh channel interaction used the Monod–Wyman–Changeux allosteric model with the idea that one KACh channel is composed of four subunits, each of which binds one active G-protein subunit (Gbg). The receptor–G-protein cycle used a previous model to account for the steady-state relationship between KACh current and intracellular guanosine-5-triphosphate at various extracellular concentrations of acetylcholine (ACh). However, simulations of the activation and deactivation of KACh current upon ACh application or removal were much slower than experimental results. This clearly indicates some essential elements were absent from the model. We recently found that regulators of G-protein signalling are involved in the control of KACh channel activity. They are responsible for the voltage-dependent relaxation behaviour of KACh channels. Based on this finding, we have improved the receptor–G-protein cycle model to reproduce the relaxation behaviour. With this modification, the activation and deactivation of KACh current in the model are much faster and now fall within physiological ranges.

Keywords: G-protein; GIRK channel; relaxation; heart; atria; computational model

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1. Introduction

G-protein-coupled receptors (GPCR) make up a large part of the human genome, and they and the G-protein intracellular signalling pathways are involved in the regulation of the cardiovascular system. Defects in the expression and regulation of GPCRs and/or G-proteins underlie various cardiovascular defects and diseases (Smith & Luttrell 2006; Alemany et al. 2007), contributing to heart failure (Meij 1996; Feldman et al. 2008) and cardiac hypertrophy (Akhter et al. 1998; Sakata et al. 1998; Barry et al. 2008). The G-protein signalling pathways are therefore regarded as therapy targets for cardiovascular diseases and their characteristics need to be understood quantitatively (Alemany et al. 2007). Mathematical models of G-protein signalling can play an important role in developing quantitative understanding of these signalling pathways (Thomsen et al. 1988; Mackay 1990; Felber et al. 1996; Mosser et al. 2002; Hao et al. 2003; Saucerman et al. 2003; Yi et al. 2003; Zhong et al. 2003; Suh et al. 2004; Katanaev & Chornomorets 2007; Linderman 2009). In the heart, models of sympathetic control of voltage-dependent inactivation in the L-type Ca^{2+} channel (Faber & Rudy 2007; Findlay et al. 2008) and a biochemical pathway model for β-adrenergic receptor stimulation (Saucerman et al. 2003) have been developed. Models of β-adrenergic signalling in the rat ventricular myocyte (Saucerman et al. 2003) and modulation of the M-current based upon KCNQ2/KCNQ3 channel subunits expressed in a cell line (Suh et al. 2004) were formulated from biochemical assays. To date there have been few models of parasympathetic control (Hosoya et al. 1996; Demir et al. 1999; Hosoya & Kurachi 1999). In this article, we present the most recent developments of our model of acetylcholine (ACh) activation of cardiac muscarinic K+ (K_{ACCh}) channels. The activation of the K_{ACCh} channel is mediated by the βγ subunit (G_{βγ}) of PTX-sensitive G-proteins (G_K) coupled to m_2 or A_1 purinergic receptors in the cardiac cell membrane (Kurachi et al. 1986, 1989; Logothetis et al. 1987, 1988). Thus, the model is a composite involving the receptor–G-protein cycle and the interaction between the G-protein and K_{ACCh} channel.

K_{ACCh} channels are activated by intracellular guanosine-5′-triphosphate (GTP) in a highly positive cooperative manner at any given concentration of extracellular ACh ([ACh]_0) and as the concentration of ACh is increased, both the apparent potency and efficacy of GTP are also increased (figure 1a, b; Kurachi et al. 1990; Ito et al. 1991). This may reflect properties of the G_K protein–K_{ACCh} channel interaction which can be accounted for by the Monod–Wyman–Changeux (MWC) allosteric model with the idea that one K_{ACCh} channel is composed of at least four functionally identical subunits, each of which binds one G_{Kβγ} (figure 1c; Hosoya et al. 1996). For the receptor–G-protein cycle we adopted the model of a receptor-mediated trimeric G-protein reaction cycle developed by Thomsen et al. (1988) (figure 1d(i)). We combined the two models to construct an integrated model of ACh activation of K_{ACCh} channels (Hosoya & Kurachi 1999) which reproduced the steady-state relationship between K_{ACCh} channel activity and GTP at various [ACh]_0 (figure 1b).

In isolated atrial myocytes, ACh (0.1 μM) evokes the K_{ACCh} current with a half time for activation of 500 ms (Ishii et al. 2001). Upon removal of ACh (1.1 μM) from the bath, the current deactivates with a half time of 3.7 s (figure 1e; Kurachi et al. 1987). The integrated model (Hosoya & Kurachi 1999) could not accurately
reproduce this temporal behaviour of the $K_{\text{ACH}}$ current, rather the half time for activation was approximately 1 min and that for deactivation was approximately 2.5 min (figure 1f(i, ii); Kurachi & Ishii 2004). Clearly, the model was incomplete.

The $K_{\text{ACH}}$ current exhibits strong inward rectification which is caused by $\text{Mg}^{2+}$ and polyamines blocking outwardly flowing current. Upon hyperpolarization from a holding potential of $-40 \text{ mV}$, the current increases in two distinct phases: instantaneous and slow time-dependent phases. The initial instantaneous increase is due to unblocking of $\text{Mg}^{2+}$ and polyamines. The following gradual current increase, which is called ‘relaxation’, reflects a slow recovery from the reduction of the product of $K_{\text{ACH}}$ channel number and $K_{\text{ACH}}$ channel open probability ($NP_o$) that occurs at depolarized potentials. Recently, we have found that regulators of G-protein signalling (RGSs) are involved in this phenomenon (Ishii et al. 2001, 2002). RGS proteins stimulate the GTPase activity of the $\alpha$ subunit of $G_K$ proteins ($G_{\text{K$\alpha$}}$) and decrease $G_K$ protein activation (Hepler 1999; Ross & Wilkie 2000). In the resting state this action of an RGS protein is inhibited by the binding of PtdIns(3,4,5)P$_3$. A $\text{Ca}^{2+}$/calmodulin (CaM) complex, which is formed following depolarization-induced $\text{Ca}^{2+}$-influx across the cardiac cell membrane, displaces PtdIns(3,4,5)P$_3$ and relieves the inhibition. The activated RGS proteins decrease the activated $G_K$ proteins and thus $K_{\text{ACH}}$ channel activity at depolarized potentials. This series of experimental results strongly suggest that relaxation directly reflects $G_K$ protein activity coupling to the $K_{\text{ACH}}$ channel. Thus, by analysing the relaxation behaviour of the $K_{\text{ACH}}$ current, the physiological kinetics of the receptor-mediated $G_K$ reaction cycle can be obtained. Here we incorporate these ideas into the model of ACh activation of the $K_{\text{ACH}}$ channel and we present the model in this paper.

2. Material and methods

(a) Modelling

The model consists of two parts: an allosteric model representing the interaction between $G_{K_{\text{BG}}}$ and $K_{\text{ACH}}$ channels, and a G-protein cycle model representing the interaction between muscarinic receptors and $G_K$ proteins (figure 1c,d(ii)). The parameters were searched and verified by comparing the model and experimental results (see §3 and appendix A for details).

(b) Electrophysiological measurements

Experiments were performed in accordance with the guidelines for the use of laboratory animals of Osaka University. The experimental protocol used here is explained in Ishii et al. (2002). In brief, single atrial myocytes were enzymatically isolated from hearts removed from adult male Wister-Kyoto rats. The $K_{\text{ACH}}$ currents evoked by various concentrations of ACh were recorded in the whole-cell configuration of the patch-clamp technique. Other experimental data, such as single channel current recording, were taken from published papers (Kurachi et al. 1986; Ito et al. 1991).
3. Results

(a) The MWC allosteric model applied to $G_K$ protein–$K_{AC}$ channel interaction

Cardiac $K_{AC}$ channels are activated directly by $G_{B\gamma}$ proteins (Logothetis et al. 1987, 1988; Kurachi et al. 1989). Here, we used the MWC allosteric model (Monod et al. 1965) to simulate the interaction between $G_{B\gamma}$ and $K_{AC}$ channels.
G-protein model of cardiac GIRK channel

Figure 1. (Opposite.) Previous experimental and simulation studies on K\textsubscript{ACCh} channels. (a) Examples of inside-out patch experiments obtained from guinea pig atrial myocytes. The concentration of acetylcholine ([ACh]) in the pipette was 0 or 1 μM as indicated. The bars above each trace indicate the periods of application of the various concentrations of GTP and 10 μM GTP\textsubscript{gS} to the internal side of the patch membrane. The holding potential was −80 mV. Note that a 3- to 10-fold increase in GTP concentration resulted in a dramatic increase in channel open probability (NP\textsubscript{o}) of K\textsubscript{ACCh} channels, indicating the existence of a highly positive cooperative process. (b) The experimental relationship between the concentration of GTP and the NP\textsubscript{o} of K\textsubscript{ACCh} channels normalized to the maximum NP\textsubscript{o} induced by 10 μM GTP\textsubscript{gS} in each patch (symbols and bars are mean ± s.d.), shown with simulated results obtained with the previous integrated model (Hosoya & Kurachi 1999) of the Monod–Wyman–Changeux (MWC) allosteric model and the Thomsen–Jaquez–Neubig model (lines). [ACh] = 10\(^{-6}\) M (filled circles), 10\(^{-7}\) M (open circles), 10\(^{-8}\) M (squares), 0 M (triangles). (c) Schematic of the MWC allosteric model. In this scheme, each K\textsubscript{ACCh} channel is assumed to be an oligomer composed of four identical subunits (i.e. n = 4). Each subunit is in either the available (A) or the unavailable (U) state, represented by circles and squares, respectively. Each subunit in the A or U state binds with one dissociated G-protein βγ subunit (filled circles) independently of other subunits, with microscopic dissociation constants \(K_A\) or \(K_U\), respectively. In this model, all subunits in the same oligomer must change their conformations simultaneously. Therefore, the channel can be either A\textsubscript{4} or U\textsubscript{4}. A\textsubscript{4} and U\textsubscript{4} are in equilibrium through an allosteric constant \(L\). (d) Models for receptor–G-protein interaction. (i) Thomsen–Jaquez–Neubig model for receptor–G-protein interaction in the previous model (Hosoya & Kurachi 1999). A, acetylcholine; R, muscarinic m\textsubscript{2}-receptor; G, G-protein. (ii) The modified model for receptor–G-protein interaction used in the present study. AR-G is removed and \(k_6\) parameter has been modified. (e) The experimental time courses of activation and deactivation phases of 1.1 μM ACh-induced K\textsubscript{G} channel currents. (f) The simulated time courses of (i) activation and (ii) deactivation phases of 1 μM ACh-induced K\textsubscript{G} channel currents in the presence of various concentrations of RGS. The RGS protein action was assumed to be voltage-independent in this prior simulation study (Kurachi & Ishii 2004). RGS accelerated the time course of deactivation but did not affect the activation time course. RGS at high concentrations suppressed maximal channel activity (a,b). Reproduced and modified from (a) Ito et al. (1991), (b) Hosoya & Kurachi (1999), (e) Kurachi et al. (1987) and (f) Kurachi & Ishii (2004).

In similar ways to Hosoya et al. (1996) and Hosoya & Kurachi (1999). In the present study we re-fitted the three parameters of the MWC model to improve the accuracy of the allosteric model. Corey & Clapham (2001) assayed biochemically the profile of G\textsubscript{βγ} subunit binding to K\textsubscript{ACCh} channels (GIRK4). They showed that from one to four G\textsubscript{βγ} subunits actually bind to one K\textsubscript{ACCh} channel (figure 2a, exp.). By comparing the G\textsubscript{βγ}-binding profile in the actual assay and that estimated by the MWC model, we examined the validity of the parameters that had been used in the previous studies (Hosoya et al. 1996; Hosoya & Kurachi 1999; figure 2a). The concentration-dependent activation of the K\textsubscript{ACCh} channel by G\textsubscript{βγ} and GTP in inside-out patches can be reproduced only with allosteric models (Hosoya et al. 1996) and since we now know that a K\textsubscript{ACCh} channel is a tetramer of Kir3.x protein subunits (Corey & Clapham 2001) an allosteric mechanism most probably underlies the activation process. In the MWC model a K\textsubscript{ACCh} channel consists of four protein units (Kir3.x; figure 1c). All of the Kir units in a given channel are defined as being in the same state, either tense (T) or relaxed (R), and change their state together (concerted transition). One G\textsubscript{βγ}
Figure 2. Gβγ protein binding to GIRK channels. (a) The densitometry profile of membranes containing GIRK4 with Gβγ. Experimental data (exp.) was obtained by densitometry from fig. 3d of Corey & Clapham (2001; n, the estimated number of Gβγ proteins per channel). Lines represent the densitometry profiles calculated with the parameter sets from the present study (fitting), Hosoya et al. (1996) and Hosoya & Kurachi (1999). (b) The concentration–response relationship between Gβγ proteins and KACH channel opening. The fractions of KACH channels in the open state were calculated with the parameter sets from the present study (solid line), Hosoya et al. (1996) (dotted line), and Hosoya & Kurachi (1999) (dot-dash line). Experimental data (circles) from Hosoya et al. (1996) were normalized for Gβγ concentration.

protein equilibrates with one Kir protein in either tense or relaxed states with separate equilibrium constants defined as $K_T$ for tense and $K_R$ for relaxed. A tetramer without any Gβγ equilibrates between tense and relaxed states according to the equilibrium constant $L$. Channels in the relaxed state are considered as available to open with fast gating kinetics (equation (A1) in appendix A, $N_P^0$), and channels in the tense state are considered to be unavailable (see Hosoya et al. 1996 for details).

In the previous studies simulation with the MWC model for Gβγ–KACH channel interaction assumed that the majority of the KACH channels would bind with four Gβγ subunits, and the numbers of channels binding one, two, or three Gβγ subunits were practically negligible (figure 2a; Hosoya et al. 1996; Hosoya & Kurachi 1999). However, the biochemical assay showed that KACH channels binding one, two, three and four Gβγ represent, respectively, approximately 10, 10, 40 and 40 per cent of the total channel population (figure 2a, exp.). Therefore, the parameters used for the MWC model in the previous studies were clearly invalid. In the present study, therefore, the parameters $K_T$, $K_R$ and $L$ (table 1) were re-calculated with the simplex method to reproduce the densitometry profiles of membranes containing GIRK4 preincubated with Gβγ. With the new parameters, the MWC model for the Gβγ–KACH channel interaction well reproduced the biochemical experimental results (figure 2a, fitting).

Figure 2b shows the relationships between the KACH channel $N_P^0$ and $[G_{Kβγ}]$ calculated by using the MWC model with the parameter sets used in the previous studies and the present study. The $[G_{Kβγ}]$ values were normalized at the half maximum of the KACH channel activity. Although $L$ values in the three sets were quite different, all of the relationships exhibited the positive cooperative effect of
Table 1. Parameters of the allosteric and G-protein cycle models.

<table>
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<th>parameter</th>
<th>value</th>
<th>unit</th>
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<td>$L$</td>
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<td>$K_R$</td>
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<td>$K_T$</td>
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<td>$k_1$</td>
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<td>$k_{-1}$</td>
<td>0.167</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$1.80 \times 10^{-2}$</td>
<td>[G–GDP]$^{-1}$</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>0.1</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>$k_{3,4}$</td>
<td>$2.86 \times 10^{3}$</td>
<td>s$^{-1}$ M$^{-1}$</td>
</tr>
<tr>
<td>$k_{-4,-3}$</td>
<td>$6.8 \times 10^{-4}$</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>$k_5$</td>
<td>10</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>$k_6$</td>
<td>$1.13\left(1 + \frac{1.30}{1 + \exp(-0.042(V + 25))}\right)$</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>$[R]_{\text{total}}$</td>
<td>$1.87 \times 10^{-3}$</td>
<td>M</td>
</tr>
<tr>
<td>$[G]<em>{\text{total}}/[R]</em>{\text{total}}$</td>
<td>30</td>
<td>dimensionless</td>
</tr>
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</table>

$[G_{K_{bg}}]$ on the $K_{ACh}$ channel $N\Pi_0$, and the relationship obtained by using the new parameters agrees well with the experimental data. We therefore used the new set of parameters for the MWC model in the following studies.

(b) The G-protein cycle model

The other part of the ACh activation of cardiac $K_{ACh}$ channel model is the $G_K$ protein cycle which produces $G_{K_{bg}}$ subunits. At first, we tested two G-protein cycle models: the Thomsen–Jaquez–Neubig model (Thomsen et al. 1988; figure 1d(i)) and the Mackay model (Mackay 1990) to see if they could reproduce various characteristics of ACh activation of the $K_{ACh}$ channel. However, after considerable trial and error, we found that neither model was satisfactory when we tried to reproduce at various $[ACh]_0$ not only the steady-state relationship between GTPi and $K_{ACh}$ channel activity, but also the relationship between the membrane potential and the steady state $N\Pi_0$ of $K_{ACh}$ channels and the voltage-dependent relaxation behaviour. In the present study, we modified the Thomsen–Jaquez–Neubig model. This modified $G_K$-protein cycle model (figure 1d(ii)) is a model of chemical reaction kinetics consisting of concentrations and reaction rate constants ($k$). AR-G was removed and the $k_6$ parameter was modified from the original Thomsen–Jaquez–Neubig model. Six values of concentration ($[R]$, $[AR]$, $[ARG–GDP]$, $[ARG–GTP]$, $[G–GTP]$ and $[G–GDP]$ for ACh (A), $m_2$-receptor (R), $G_K$ protein (G)) in the G-protein cycle model are updated at each calculation step with six ordinary differential equations (equations (A2)–(A7) in appendix A). In the previous studies all of the values of rate constants were fixed (figure 1d(i)). A novel aspect of the modified model (figure 1d(ii)) in the present study is that we have incorporated the concept of regulation of G-protein signalling (RGS). RGS proteins regulate G-protein signalling by accelerating GTP
hydrolysis, and they may be involved in the development of certain cardiovascular pathologies (Wieland & Mittmann 2003; Semplicini et al. 2006; Wieland et al. 2007; Hendriks-Balk et al. 2008). Modulation of GTP hydrolysis by RGS proteins also underlies a voltage- and time-dependent character of the K\(_{ACh}\) current in atrial myocytes known as ‘relaxation’ (figure 3b; Inanobe et al. 2001; Ishii et al. 2001, 2002). Relaxation of the K\(_{ACh}\) current reflects an increasing suppression of channel open probability during depolarization and a gradual recovery during hyperpolarization. The time course of these phenomena depends upon voltage-dependent Ca\(^{2+}\) influx and Ca\(^{2+}\)/CaM modulation of RGS protein activity (Ishii et al. 2001).

Therefore, GTPase activity of G\(_K\) in atrial myocytes can be modulated by RGS proteins in a voltage-dependent manner (Ishii et al. 2001). In the modified G\(_K\)-cycle model used in the present study, the action of RGS proteins is expressed by the new parameter \(k_6\). The value of each rate constant was fixed, except for \(k_6\) (table 1). Parameter \(k_6\) is defined as a function of membrane voltage so that depolarization of the membrane potential can accelerate GTPase activity (equation (A8) in appendix A). The rate constants, the total concentration of the receptor ([R]\(_{\text{total}}\)) and a conversion multiplier between inside-out and whole cell recording (\(r\); see §3a) were obtained by the simplex method searching for the best fit of the following three properties at various [ACh]\(_0\): (i) the steady-state relationships between K\(_{ACh}\) channel open probability (\(N_{Po}\)) and GTP\(_i\), (ii) the voltage dependence of K\(_{ACh}\) channel \(N_{Po}\), and (iii) the relaxation time constants at various membrane potentials. We set the value of GTP\(_i\) to 100 μM in the whole cell current simulation because this concentration was used in the internal pipette solution in whole cell recording. The [G\(_{Kbg}\)] generated by the G\(_K\) protein cycle model was taken into the allosteric model and then the K\(_{ACh}\) channel \(N_{Po}\) was calculated by using equation (A1).

\(\text{(c) Analysis of simulations with the integrated model}\)

The improved MWC allosteric model for G\(_{Kbg}\)–K\(_{ACh}\) channel interaction and the modified G\(_K\) protein cycle model were combined into a new integrated model for ACh activation of K\(_{ACh}\) channels. The properties of the K\(_{ACh}\) current in the integrated model were examined as follows.

(i) The steady-state relationship between K\(_{ACh}\) channel activity and GTP\(_i\)

Since intracellular GTP was not changed in whole cell recording, experimental inside-out patch data for the effect of [GTP] in the presence of different [ACh]\(_0\) were taken from Ito et al. (1991), converted to that representing whole cell recording and shown as symbols in figure 3a. Both GTP and ACh increased K\(_{ACh}\) channel activity in a concentration-dependent manner. GTP\(_i\) activated the K\(_{ACh}\) channel in excised membrane patches much more potently than in whole cell recording. This is probably because in the inside-out patch experiments the internal solution contained high chloride which interferes with the GTPase activity of G\(_a\) and increases the sensitivity to GTP by shifting the GTP-relative \(N_{Po}\) curve by 100-fold (Nakajima et al. 1992, fig. 4). Therefore, we multiplied the concentration of GTP in excised membrane patch data by 100 to reproduce the GTP\(_i\)-dependence of whole cell K\(_{ACh}\) currents. Also in the inside-out patch experiments with the high chloride internal solution, GTP\(_i\) induced significant K\(_{ACh}\) channel activity in the absence of external ACh (figure 3a, Phil. Trans. R. Soc. A (2010)
Figure 3. Voltage-dependent relaxation of $K_{ACH}$ current. (a) The relationship between $K_{ACH}$ channel open probability ($NP_o$) and GTP concentration in the presence of different concentrations of ACh. Symbols represent data from Ito et al. (1991) and lines represent data obtained from the new model. See text for further details. (b) Voltage-clamp single cell current recordings of voltage-dependent relaxation. Single rat atrial cells were subjected to the voltage-clamp protocol inset. Experiments were conducted in the presence of two different concentrations of ACh$_0$ and 3 μM intracellular GTP$_g\gamma$S. (c) (i) Normalized $K_{ACH}$ current relaxation traces for 0.1 and 0.3 μM ACh. (ii) Equivalent currents simulated by the model. (iii) Time constants of $K_{ACH}$ current relaxation. Symbols represent experimental data and lines represent the results from model simulation. (d) The relationship between the relative $K_{ACH}$ channel availability ($NP_o$) and prepulse voltage. Experimental data (dots) and simulated results (lines) are shown for ACh concentrations of 0.1 and 0.3 μM.

open diamonds; Nakajima et al. 1992, fig. 7). In whole cell recordings there was no detectable $K_{ACH}$ current in the absence of ACh, since in the absence of ACh the G-protein cycle should not be activated at all. Since the basal $K_{ACH}$ current, presumably due to the high chloride, is difficult to separate from the inside-out experimental data, only in this calculation we instead increased the [ACh] in experimental data by an equivalent of 0.01 μM to reproduce the experimental response to GTP in the absence of ACh (figure 3a, open diamonds) and then multiplied [ACh] by a fitted parameter of a multiplier $r$ (1.44) to represent a lower sensitivity to ACh in whole cell recording. With these conversions, the model quantitatively reproduced the experimental data for the relationships between $K_{ACH}$ channel activity and GTP$_i$ at various [ACh]$_0$ (figure 3a, lines).
(ii) Voltage and time dependence of the $K_{\text{ACh}}$ current

Voltage- and time-dependent characteristics of the $K_{\text{ACh}}$ current are revealed by the phenomenon of relaxation. The experimental relaxation behaviour of $K_{\text{ACh}}$ current at various $[\text{ACh}]_0$ is shown in figure 3b. The preconditioning voltage steps from $-100 \text{ mV}$ to $+40 \text{ mV}$ (in 10 mV increments) were applied for 1 s and followed by a test voltage step (2 s in duration) to $-100 \text{ mV}$. During the test voltage step relaxation is the slow increase in $K_{\text{ACh}}$ current which is observed following a prior instantaneous depolarization (figure 3b). This represents the recovery from a time- and voltage-dependent reduction of $K_{\text{ACh}}$ current which results from $Ca^{2+}$ entry and $Ca^{2+}$-CaM stimulation of RGS proteins which accelerate the GTPase activity of the GPCR (Ishii et al. 2001, 2002). One characteristic of relaxation is voltage dependence: more of the $K_{\text{ACh}}$ current shows relaxation as the prepulse is depolarized. Another characteristic is that relaxation is reduced as $[\text{ACh}]_0$ is increased. GTP$\gamma$S, a non-hydrolysable GTP analogue, also abolished the relaxation behaviour. The time-dependent current change in GTP$\gamma$S-loaded atrial cells represented the intrinsic gating of $K_{\text{ACh}}$ channels.

To extract the character of relaxation, the experimental currents showing relaxation with 0.1 and 0.3 $\mu$M ACh were normalized to that evoked by GTP$\gamma$S (figure 3c(i)). The equivalent simulated $K_{\text{ACh}}$ currents using the improved integrated model are shown in figure 3c(ii). The relaxation is generated by the voltage-dependent facilitation of GTPase activity of $G_K$ due to RGSs. Therefore, we incorporated this process in $k_6$ of the $G_K$ protein cycle model by formulating $k_6$ as a function of the membrane potential (table 1, $k_6$; see §2 for

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Figure 4. Time-dependent development of $K_{\text{ACh}}$ current relaxation. (a) Voltage-clamp single cell current recordings from rat atrial cells obtained during a voltage step to $-100 \text{ mV}$ following a prepulse voltage step to $+40 \text{ mV}$ of variable duration (22–1306 ms) in cells exposed to either three different concentrations of ACh$_0$ or 3 $\mu$M intracellular GTP$\gamma$S ((i) 0.1 $\mu$M, (ii) 0.3 $\mu$M, (iii) 3 $\mu$M, (iv) GTP$\gamma$S). (b) (i,ii) Superimposed traces of relaxation currents recorded in the presence of two concentrations of ACh following prepulses of different durations. These currents have been normalized to data recorded in the presence of intracellular GTP$\gamma$S. (iii,iv) Equivalent currents generated by the model.
The model correctly reproduced the voltage-dependent slow increase in experimental current relaxation (figure 3c(ii)). The time constant of relaxation in the model was also calculated and compared with the experimental data (figure 3c(iii)). The time constants of relaxation in simulated data did not show clear voltage dependence and coincided with the experimental data well except at $-60$ and $-80$ mV in $0.3 \mu$M ACh where the amplitudes of the relaxation were too small to be accurately assessed.

Figure 3d shows the voltage dependence of the $K_{ACh}$ channel relative $NP_o$ in the presence of 0.1 and 0.3 $\mu$M $[ACh]_0$. The symbols correspond to the experimental results and the lines are the results calculated from the integrated model. As $[ACh]_0$ was decreased, the $K_{ACh}$ channel relative $NP_o$ decreased especially at depolarized potentials because of the modulated action of RGS proteins represented by the voltage-dependent $k_6$. The integrated model developed in this study can therefore quantitatively account for the voltage- and ACh-dependence of the $K_{ACh}$ current relaxation behaviour.

Figure 4a shows experimental data examining the effects of prepulse (+40 mV) duration on the relaxation behaviour of the $K_{ACh}$ current at $-100$ mV. As $[ACh]_0$ was increased, the instantaneous component increased and the time-dependent relaxation component became smaller. This may indicate that the effects of RGS proteins are attenuated as $[ACh]_0$ is increased. At each $[ACh]_0$, as the duration of prepulse was prolonged, the instantaneous component decreased and the relaxation component increased. This implies that the effect of the RGS protein action develops in a time-dependent manner during the prepulse, because the instantaneous component reflects the $K_{ACh}$ channel $NP_o$ at the end of the prepulse. In GTPγS-loaded atrial cells, the prepulse duration did not affect the $K_{ACh}$ current. This is consistent with the idea that the effect of prepulse duration on $K_{ACh}$ current is mediated by the voltage- and time-dependent action of RGS proteins.

In figure 4b(i)(ii), the temporal characteristics were extracted from the experimental data by normalizing relative to the data obtained with GTPγS. The normalized currents clearly demonstrated the prepulse duration-dependent development of relaxation (figure 4b, dotted lines). The model correctly reproduces the experimental results (figure 4b(iii)(iv)). Both the model and experimental results also showed that the alterations of the instantaneous and relaxation components depend on $[ACh]_0$. Therefore, the model successfully replicates the characteristics of $K_{ACh}$ channel current relaxation.

(iii) The temporal and steady-state behaviour of ACh-induction of $K_{ACh}$ current

Figure 5a shows the temporal behaviour of the ACh-induced $K_{ACh}$ channel current simulated with the improved integrated model. When ACh was applied, the $K_{ACh}$ current was induced with a time constant of 2–8s. Upon removal of ACh from the external solution, the $K_{ACh}$ current was deactivated with a time constant of 6–12s. These values are similar to those in physiological activation and deactivation and much smaller than those calculated with the previous models which do not take the voltage-dependent RGS action into account. In the simulated $K_{ACh}$ currents, differences in the activation time course at different membrane potentials were not obvious, while those in the deactivation time
course were evident. The deactivation of $K_{ACh}$ current was faster at depolarized potentials than at hyperpolarized potentials. This phenomenon agrees with experimental results (half deactivation times of 6.3 ms at 0 mV and 15.7 ms at $-80$ mV; Ishii et al. 2001).

In figure 5b, the $[ACh]_0$ dependence of the steady state $K_{ACh}$ current was calculated with the improved integrated model at various membrane potentials. The experimental data at $-53$ mV were taken from Kurachi et al. (1987; figure 5b, circles). Experimentally, as $[ACh]_0$ was increased, the whole cell $K_{ACh}$ current was augmented in a concentration-dependent manner. The concentration–response relationship in the model was similar to the experimental data except at intermediate ACh concentrations (figure 5b, line $-53$ mV). Because the quasi-steady-state current was measured approximately 1 min after the onset of ACh application, the experimental data contain the effect of short-term desensitization. This may explain at least in part the difference between experimental data and the simulation data with the new model. The model results also suggest a novel possibility that the membrane potential affects the steady-state relationship between $K_{ACh}$ current and $[ACh]_0$: as the membrane is more depolarized, the sensitivity of the $K_{ACh}$ channel to ACh decreases. This possibility should be examined experimentally.

Figure 5. The relationship between $K_{ACh}$ channel open probability and the concentrations of GTP and ACh obtained by the improved integral model. (a) (i) The simulated time courses of activation and deactivation phases of 0.1, 0.3 and 3.0 μM ACh-induced K$_G$ channel currents and G$\beta\gamma$ on steps of various membrane voltages. (ii) Simulated time constants for activation and deactivation phases. (b) The relationship between the whole cell $K_{ACh}$ current and ACh concentration. Symbols represent data from Kurachi et al. (1987), lines represent data obtained from the model at the indicated voltages. See text for further details.
This study presents the further development of a mathematical model to simulate the kinetics of G\textsubscript{K}-protein signalling and the K\textsubscript{ACH} current in the heart. This integrated model reproduced major characteristics of the K\textsubscript{ACH} current, including steady-state relationships and temporal behaviour. Two experimental results played essential roles in the improvement of this model: (i) the biochemical assay of G\textsubscript{br} binding to K\textsubscript{ACH} channels and (ii) the discovery of the involvement of RGSs in the control of the K\textsubscript{ACH} channel activity.

Using these two key experimental advances, we improved the integrated model for ACh activation of the K\textsubscript{ACH} channel (Hosoya \textit{et al}. 1996; Hosoya \& Kurachi 1999). Now with the new integrated model, we can reproduce the fast onset and offset of the K\textsubscript{ACH} current upon application and removal of ACh. The calculated time constants for activation and deactivation are close to the physiological experimental data. However, we recognize that the activation of K\textsubscript{ACH} current in experiments is yet faster than that of the model. We currently hypothesize that this may be related to the high affinity and low affinity properties of muscarinic receptors.

The deactivation time course of the simulated K\textsubscript{ACH} current is quite in the physiological range. In simulation, as the membrane is depolarized, the deactivation becomes faster. This is also consistent with experimental observations reported in Ishii \textit{et al}. (2001), and may be due to the higher GTPase accelerating activity of RGS proteins at depolarized potentials.

The simulated steady-state relationship between the K\textsubscript{ACH} current and [ACh]\textsubscript{0} is shown in figure 5b. As the membrane is depolarized, the relationship shifts to the right and the maximal K\textsubscript{ACH} channel activity is attenuated. This is also due to the higher activity of RGS proteins at depolarized potentials. But such an effect of the membrane potential on the relationship has not been yet reported \textit{in vitro} and should be examined experimentally.

Models of processes associated with G-protein intracellular signalling may have practical applications. For example, an estimated 50 per cent of current pharmaceuticals target GPCR (Howard \textit{et al}. 2001) and models may be useful tools to examine drug effects on G-protein signalling. The contributions of G-protein signalling to atrial fibrillation (AF) may be a good example. Hirose \textit{et al}. showed that while G\alpha\textsubscript{q-TG} mice activate the IP3 and DAG pathways and other intracellular signalling pathways to promote cellular hypertrophy, the over-expression of DAG kinase isoform DGK\zeta in the hearts of these mice prevented the generation of atrial arrhythmias including AF (Hirose \textit{et al}. 2009). This suggests that therapies targeting G\alpha\textsubscript{q} and/or DAG-mediated signalling may prevent atrial arrhythmogenic remodelling and models of the appropriate signalling pathways in the atria may help to examine theoretical possibilities. The K\textsubscript{ACH} current has been proposed to contribute to AF. Dobrev \textit{et al}. (2005) and Cha \textit{et al}. (2006) suggest that a constitutively active K\textsubscript{ACH} current contributes to enhanced basal K\textsuperscript{+} conductance and action potential shortening in chronic AF and blockade of the K\textsubscript{ACH} current has become a new therapeutic target in AF. K\textsubscript{ACH} channel blockers are under development and among them tertiapin and NIP-151 show effectiveness in terminating AF (Hashimoto \textit{et al}. 2006, 2008). Differential phosphorylation-dependent regulation of the ion channel might underlie this constitutively active K\textsubscript{ACH} current (Voigt \textit{et al}. 2007). It will be interesting to use our model to
investigate possible sources of constitutive $K_{ACh}$ channel activity—activation of $K_{ACh}$ channels by GTP in the absence of ACh (Ito et al. 1991) and the loss of Ca$^{2+}$–CaM regulation of RGS proteins might prove to be attractive targets for further investigation. In which context it is interesting to note that knock-out of the RGS4 protein abolished desensitization of the response to ACh in the SA node in mice (Cifelli et al. 2008). A major limitation of the model presented here is that it cannot reproduce desensitization, and this after considerable effort. This may be because the model consists of linear systems subjected to a simple input (ACh concentration) which cannot reproduce a complex response such as desensitization. Further investigation of the function of RGS proteins may contribute to the understanding of sinus bradycardia.

Another limitation is that the experimental data used to develop the model were from different species (rat, guinea pig). Though the different expression levels of ion channels between species lead to different action potential forms, the characteristics of $K_{ACh}$ channels in mammalian atrial myocytes do not seem to be so different and the species difference needs to be noted but it should not cause significant problems in the present modelling study which focuses on ion channel level phenomena.

In conclusion, progress in experimental research, such as the assay of $G_{\beta\gamma}$ binding to the $K_{ACh}$ channels and the discovery of RGS proteins’ action upon the $K_{ACh}$ current, was essential for the development of this mathematical model for ACh activation of the $K_{ACh}$ current. The simulation analyses raise further questions concerning the physiological behaviour of the $K_{ACh}$ current, which indicates the necessity of new experiments. Experiments and simulation thus complement each other for the further understanding of physiological systems.

Appendix A

(a) Allosteric model

The $G_{K\beta\gamma}$ generated by the $G_K$ protein cycle model was taken into the allosteric model and then the channel availability was calculated with the following equation:

$$NP_0 = \frac{(1 + L(K_R/K_T)^4)(1 + ([G_{\beta\gamma}]/K_R)^4)}{L(1 + (K_R/K_T)([G_{\beta\gamma}]/K_R)^4 + (1 + ([G_{\beta\gamma}]/K_R))^4).} \quad (A \ 1)$$

(b) G-protein cycle model

In the $G_K$-protein cycle model (figure 1d(ii)), six values of concentrations in the G-protein cycle are calculated with the following six ordinary differential
G-protein model of cardiac GIRK channel

Equations:

\[
\frac{d[R]}{dt} = -k_1[A][R] + k_{-1}[AR],
\]

\[
\frac{d[AR]}{dt} = k_1[A][R] + k_{-2}[ARG-GDP] + k_5[ARG-GTP] - k_{-1}[AR] - k_2[AR][G-GDP],
\]

\[
\frac{d[ARG-GDP]}{dt} = -k_{-2}[ARG-GDP] + k_2[AR][G-GDP] - k_{3,4}[ARG-GDP][GTP] + k_{-4,3}[ARG-GTP][GDP],
\]

\[
\frac{d[ARG-GTP]}{dt} = k_{3,4}[ARG-GDP][GTP] - k_{-4,3}[ARG-GTP][GDP] - k_5[ARG-GTP],
\]

\[
\frac{d[G-GTP]}{dt} = k_5[ARG-GTP] - k_6[G-GTP],
\]

\[
\frac{d[G-GDP]}{dt} = k_6[G-GTP] + k_{-2}[ARG-GDP] - k_2[AR][G-GDP],
\]

where A is ACh, R is m2-receptor, G is G_K protein, and k are reaction rate constants. The value of each rate constant was fixed, except for \(k_6\) (table 1). Since GTPase activity of G_K in atrial myocytes can be modulated by RGS proteins in a voltage-dependent manner (Ishii et al. 2001), \(k_6\) is defined as a function of membrane voltage as follows:

\[
k_6 = 1.13 \left(1 + \frac{1.30}{1 + e^{(-0.042(V+25))}}\right),
\]

where V is the membrane potential.

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