G$\beta\gamma$ Binding Increases the Open Time of I_{KACh}: Kinetic Evidence for Multiple G$\beta\gamma$ Binding Sites

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ABSTRACT I_{KACh} is an inwardly rectifying potassium channel that plays an important role in the regulation of mammalian heart rate. I_{KACh} is activated by direct interaction with G$\beta\gamma$ subunits of pertussis toxin-sensitive heterotrimeric G-proteins. The stoichiometry of the G$\beta\gamma$/channel complex is currently unknown, and kinetic analysis of the channel behavior has led to conflicting conclusions. Here, we analyze the kinetics of the native I_{KACh} channel in inside-out cardiomyocyte patches activated directly by G$\beta\gamma$. We conclude that the channel has at least two open states and that binding of G$\beta\gamma$ prolongs its mean open time duration. These findings imply the existence of at least two binding sites on the channel complex for G$\beta\gamma$. We also show that the duration of the channel opening is negatively correlated with the duration of subsequent channel closing, which further constrains the possible kinetic models. A simple qualitative model describing the kinetic behavior of I_{KACh} is presented.

INTRODUCTION

I_{KACh} is an inwardly rectifying potassium channel present in heart atria and sinus node. I_{KACh} channel open probability is increased by binding of acetylcholine (ACh) to cardiac muscarinic M_2 receptors and subsequent dissociation of pertussis toxin-sensitive heterotrimeric G-proteins (Wickman and Clapham, 1995; Ackerman and Clapham, 1997). G$\beta\gamma$ binds directly to the channel to elicit its activation (Krapivinsky et al., 1995b; Logothetis et al., 1987; Wickman et al., 1994). The native channel is a heteromultimer, consisting of GIRK1 and GIRK4 protein subunits (Krapivinsky et al., 1995a). Experiments in mice with targeted disruption of the *Girk4* gene confirmed the essential role of this channel in the vagal regulation of heart rate (Wickman et al., 1998).

The first report on the single channel properties of I_{KACh} (Sakmann et al., 1983) noted that the histogram of open time durations could be fitted with a single exponential (~1 ms mean open time) and that the channel exhibited bursting behavior, implying the existence of one open and at least two closed states. Others have reported similar findings (Kurachi et al., 1986; Logothetis et al., 1987). However, in inside-out patches from rat atrial myocytes, channel openings lasting >20 ms are not uncommon, though they should be very infrequent if the channel has a single open state of 1 ms mean open time (~1 in 4×10^8 channel open events would be predicted to last 20 ms or longer given that the proportion of channels of mean duration τ_o that stay open for time t or longer is described by $e^{-t/\tau o}$). Data obtained from frog sinus venosus suggest complex kinetics for I_{KACh} in the cell-attached configuration with at least three open states (the longest open state with mean open time of 4.8–12.3 ms) and multiple bursting modes (Ivanova-Nikolova and Breitwieser, 1997; see also Kubo et al., 1993), while other groups have used spectral analysis to conclude that guinea pig I_{KACh} has a single open state (Hosoya et al., 1996). Experiments involving ATP addition to the cytosolic patch surface indicate that phosphorylation of the channel may substantially prolong its mean open time (Kim, 1991).

In order to eliminate potential complicating effects occurring during signal transduction from the M_2 receptor to the channel (e.g., receptor and channel phosphorylation, intracellular Na^+ concentration changes; Sui et al., 1996), we investigated the kinetics of I_{KACh} in inside-out patches activated directly with G$\beta\gamma$. Assuming that the kinetics of the channel can be described by a finite-state Markovian model and that the channels in the patch are mutually independent, we report here that 1) at least two open states exist, even in the absence of ATP; 2) the duration of the open intervals is negatively correlated with the duration of the subsequent closed intervals, imposing further constraints on the possible kinetic models; and 3) the increase in Np_o in slowly activating patches, caused by locally increasing G$\beta\gamma$ concentration, is accompanied by increased duration of the mean open time. This is not a nonspecific effect of exposure of the cytosolic patch surface to the bath solution, since inhibition of I_{KACh} activity with Gα-GDP leads to a decrease in mean open time duration. As channel activity in the absence of G$\beta\gamma$ is negligible, this suggests that there are at least two G$\beta\gamma$ binding sites on I_{KACh}.

MATERIALS AND METHODS

Neonatal rat atrial cell culture

Atrial cells were isolated from 10 to 15 newborn rats (1–2 days old). Briefly, the animals were decapitated and the atria minced in Hanks'

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solution (Sigma, St. Louis, MO). The tissue was subsequently digested with 0.3 mg/ml trypsin (Gibco, Gaithersburg, MD) and 0.25 mg/ml collagenase (Worthington, Lakewood, NJ) in Hanks' solution at 37°C for 10 min. The tissue was then centrifuged briefly and supernatant discarded. The pellet was resuspended in trypsin/collagenase solution again and the procedure repeated. Supernatants from the third to the sixth rounds of digestion were separately mixed with trypsin inhibitor solution (DMEM; Gibco) with 20% horse serum (Gibco), filtered through Sweenex filter and centrifuged for 2 min at 1000 rpm. The supernatant was discarded and the pellet resuspended in 2 ml culture medium [DMEM with 10% fetal bovine serum and 0.1% penicillin/streptomycin (Sigma)]. All electrophysiological experiments were performed within 48 h of cell isolation.

Electrophysiology

The pipettes for single channel recordings were pulled from KG-12 glass (Garner, Claremont, CA) on a P-80 Flaming/Brown puller (Sutter Instrument Company, Novato, CA) and coated with HIPEC R-6101 "sylgard" (Dow Corning Corporation, Midland, MI). The pipette resistance was 2–5 MΩ after fire-polishing when filled with K-5 solution (KCl 118.5 mM, KOH/EGTA 5 mM, $MgCl_2$ 2 mM, KOH/HEPES 10 mM, with pH adjusted to 7.2). Single channel recordings were performed in the inside-out configuration, in symmetrical K-5. Excised patches were activated with Gβγ (purified from bovine brain as described by Sternweis and Robishaw, 1984; Krapivinsky et al., 1995b) added to the bath for a final concentration of 20 nM. The holding potential was −80 mV in all cases. The currents were amplified using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) filtered at 5 kHz (4-pole low-pass Bessel filter; 10–90% rise time 70 μs) and stored on a VCR tape. The signal was then digitally sampled at 50 kHz frequency (Digidata 1200, Axon Instruments) and stored on a computer hard disk using pCLAMP6 (Axon Instruments) software.

Single channel analysis

For data analysis, idealized traces were created using a half-threshold crossing algorithm. The dead time was set to 0.25 ms except in the experiments used for correlation measurements, where it was set to 0.1 ms (Colquhoun and Sigworth, 1995a). Exponential fitting of the open time histogram was performed using the maximum likelihood estimate (MLE) method (Colquhoun and Sigworth, 1995b). The fit limits used were 0.8–30 ms, so that the lower limit of fit was > 3δ, assuring that the number of exponential terms is not affected by imposition of dead time ($\delta = 250\ \mu s$; Hawkes et al., 1992).

Data from nine inside-out patches activated with 20 nM Gβγ were used to compare mean open time at low and high levels of channel activity. We used patches that activated slowly (i.e., it was possible to select two data segments lasting at least 10 s and separated by at least 30 s, where the Np_o of the high-activity segment was at least 10 times that of the low-activity segment). In 10 patches, the channel was first activated with 10 nM Gβγ and subsequently suppressed by adding increasing concentrations of Gα-GDP (either purified from bovine brain or recombinant myristoylated $G\alpha_{i1}$; Wickman et al., 1994). Data segments were compared from patches before and after addition of Gα-GDP that led to at least a 100-fold decrease in Np_o (range 30–100 nM). In one case, the channel activity was completely abolished after increasing the Gα-GDP concentration by 100 nM and was partially restored by adding 30 nM Gβγ. Custom-written software (TurboPascal, Borland International) was used to calculate the mean open time of the idealized recording, as given by

$$T = \left(\sum_{i} L_i t_i\right)/N \tag{1}$$

where t_i is the total time the patch spent at level L_i during the recording and N is the total number of channel openings in the recording. Data were corrected for Np_o as described in Appendix B. Custom-written software was also used to calculate the correlation between open and closed times.

In five inside-out patches exhibiting relatively steady activity (20 nM Gβγ) with few multiple openings, time intervals from a channel opening to its closing were paired with time intervals from this channel closing to opening of any channel in the patch. Spearman's correlation coefficient between these two variables was then separately determined for each patch (see Appendix A for detailed discussion of the methods employed for analysis of correlation between an open time and subsequent closed time durations). Mean open times from low- and high-activity segments were compared using a paired *t*-test for both activation with Gβγ and suppression by Gα-GDP; $p < 0.05$ was considered statistically significant.

RESULTS

One problem complicating the kinetic analysis of rodent atrial I_{KACh} is the difficulty of obtaining patches containing only a single channel. Most patches contain many double and triple openings when fully activated. If multiple channels are open simultaneously, errors occur in exponential fitting of open time distributions, since level 1 interval duration does not correspond to the open time duration. We analyzed data from five inside-out patches exhibiting low levels of channel activity elicited by 20 nM Gβγ. Each recording lasted at least 1 min and exhibited no double openings. These patches did contain multiple channels since multiple openings appeared later upon gradual activation, but the absence of double openings during a long continuous segment ensures that the distribution of open times was not distorted. We pooled the data from these patches and compared the fits with one and two exponentials using the MLE method (Fig. 1 *B*). The best single exponential fit was obtained for $\tau = 1.6$ ms, but this clearly underestimated the frequency of long channel openings (>5 ms). The best fit with two exponentials was obtained for $\tau_1 = 1.2$ ms and $\tau_2 = 7.2$ ms (with corresponding weights $a_1 = 0.965$ and $a_2 = 0.035$). The biexponential fit described the data significantly better than the single exponential fit: the logarithm likelihood ratio (LLR) was 17.63 ($p < 0.005$; $2 \cdot \mathrm{LLR}$ should have a χ^2 distribution with 2 degrees of freedom if the one exponential fit were valid; Rao, 1973). It appears that in these low-activity patches, most openings corresponded to an open state with 1–2 ms duration, but a small proportion of channel events had an open time > 5 ms.

To obtain more information on the channel kinetics, we analyzed the relationship between the duration of the open interval and the duration of the subsequent closed interval in five inside-out patches activated with 20 nM Gβγ. Each patch displayed a steady level of activity with few double openings. All kinetic models with a single open state and many models with multiple open states would predict no correlation between the duration of adjacent open and closed intervals (Colquhoun and Hawkes, 1995). Spearman's correlation coefficients between open and subsequent closed intervals were calculated and statistically significant negative values were obtained for all five patches (range −0.09 to −0.16), indicating that short closed intervals tended to be preceded by long open intervals, and vice versa

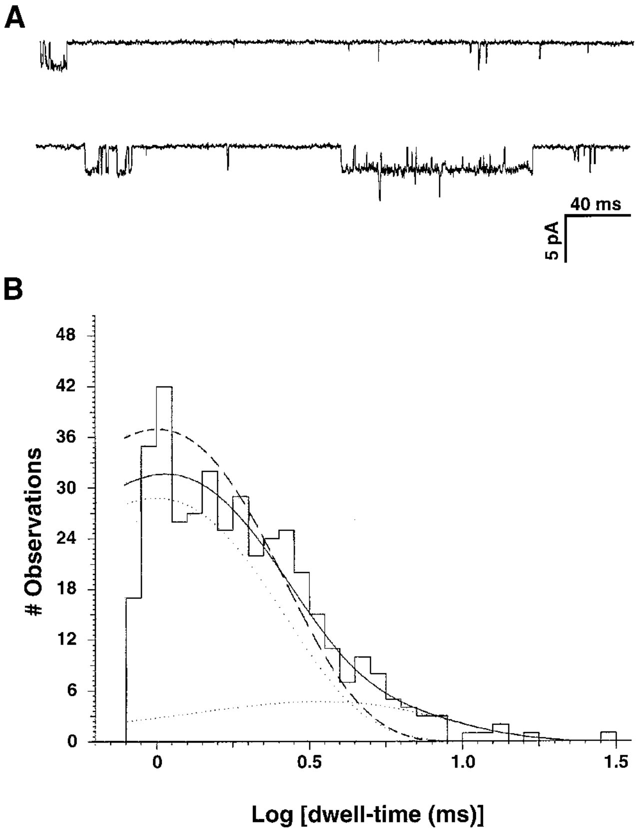

FIGURE 1 (*A*) Continuous single channel recording from an inside-out patch in which I_{KACh} has been activated by 20 nM G$\beta\gamma$. The recording illustrates that the openings forming a burst tend to be longer than the isolated openings, resulting in a negative correlation between the durations of an open interval and the subsequent closed interval. (*B*) Histogram of open interval durations obtained from five inside-out patches activated with 20 nM G$\beta\gamma$. The recordings contained no double openings. The dashed line represents the maximum likelihood estimate (MLE) fit by a single exponential. This systematically underestimates the frequency of long openings. The solid line indicates the MLE fit with two exponential components (which are themselves indicated by the dotted lines). The fit limits are 0.8–30 ms. The lower limit of the fit is >3× longer than the dead time (δ), assuring that the number of components is not affected by imposition of dead time ($\delta = 250$ μs; Hawkes et al., 1992).

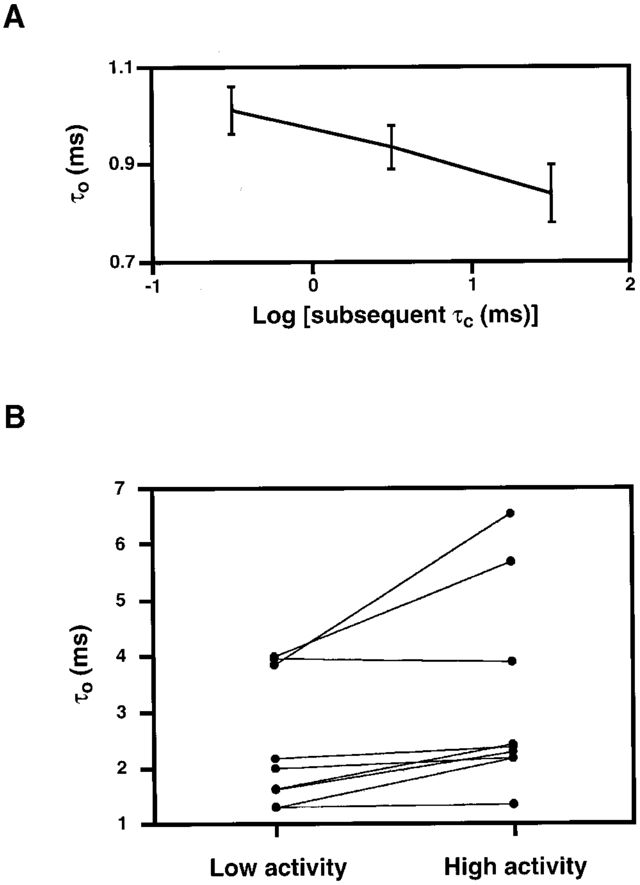

FIGURE 2 (*A*) Relationship between open intervals (τ_o) and subsequent duration of stay at level 0 (τ_c) from a single inside-out patch activated by 20 nM G$\beta\gamma$. The τ_c intervals (all channels closed) have been sorted into three logarithmic bins. For each bin, the mean duration of the preceding open interval was calculated. The error bars indicate the standard error of the mean. The total number of paired interval intervals in this patch was 930 (the Spearman correlation coefficient was −0.09). In this and the other four patches, long channel openings tended to be followed by short closings. (*B*) Mean open time calculated from nine inside-out patches that activated slowly after addition of 20 nM G$\beta\gamma$. In all cases, the low-activity and high-activity segments were separated by at least 30 s and differed at least 10-fold in Np_o. The mean open time increased in eight of nine patches.

(Table 1, Fig. 2 *A*). This confirms the qualitative impression that openings forming a burst (which are by definition associated with short closed times) are longer than isolated openings (Fig. 1 *A*). This correlation implies that the channel has multiple open and closed states, and rules out several simple kinetic schemes. For example, all kinetic schemes in which deletion of a single kinetic state disconnects the sets of open and closed channel states are excluded from consideration (Colquhoun and Hawkes, 1987; see Appendix A for detailed discussion).

TABLE 1 Parameters of recordings used for correlation analysis

Patch	Openings	Double openings	Spearman correlation coefficient	$p<$
1	266	0	−0.146	0.05
2	386	3	−0.144	0.01
3	321	0	−0.158	0.01
4	629	0	−0.105	0.01
5	930	1	−0.090	0.01

Having established the existence of multiple open states of I_{KACh} in inside-out patches, we decided to determine whether G$\beta\gamma$ shifts the occupancy between the open states. We analyzed data from nine inside-out patches with channels that activated slowly (see Materials and Methods) after addition of 20 nM G$\beta\gamma$. The time interval between addition of G$\beta\gamma$ to the bath and full I_{KACh} activation is variable and ranges from ~1 s to a few minutes, probably reflecting variable diffusion distances and the precise geometry of patch/pipette system. Average open time duration was calculated from low-activity and high-activity segments of each patch as the arithmetic mean. In eight of nine patches, higher channel activity was accompanied by an increase in mean open time (2.4 vs. 3.2 ms, $p < 0.05$; Fig. 2 *B*). One possible explanation of the data is that the gradual local increase in Gβγ concentration leading to slow patch activation also caused the channel to spend more time in the open state(s) with longer open times. Alternatively, the prolongation of the mean open time might be unrelated to an increase in Gβγ concentration and could simply reflect longer exposure of the patch to the bath (e.g., allowing time for channel dephosphorylation). To discriminate between these two possibilities, we measured mean open time in 10 inside-out patches that had been activated with 10 nM Gβγ. Channel activity was subsequently decreased by adding increasing concentrations of Gα-GDP (Fig. 3 *A*). Mean open time was measured before and after the Gα-GDP dose that caused a major decrease (>100-fold) in channel activity (30–100 nM final concentration). Gα-GDP added to the bath presumably bound Gβγ, decreasing the concentration of free Gβγ that bound and activated the channel. Addition of Gα-GDP led to a decrease in mean open time (2.4 vs. 1.7 ms, $p < 0.05$), indicating that the concentration of free Gβγ and not the duration of exposure of the cytosolic patch surface to the bath solution determined the mean open time (Appendix B). In Fig. 3, *B* and *C*, Np_o and mean τ_o from one patch were plotted simultaneously to demonstrate that they decreased in parallel after Gα-GDP application.

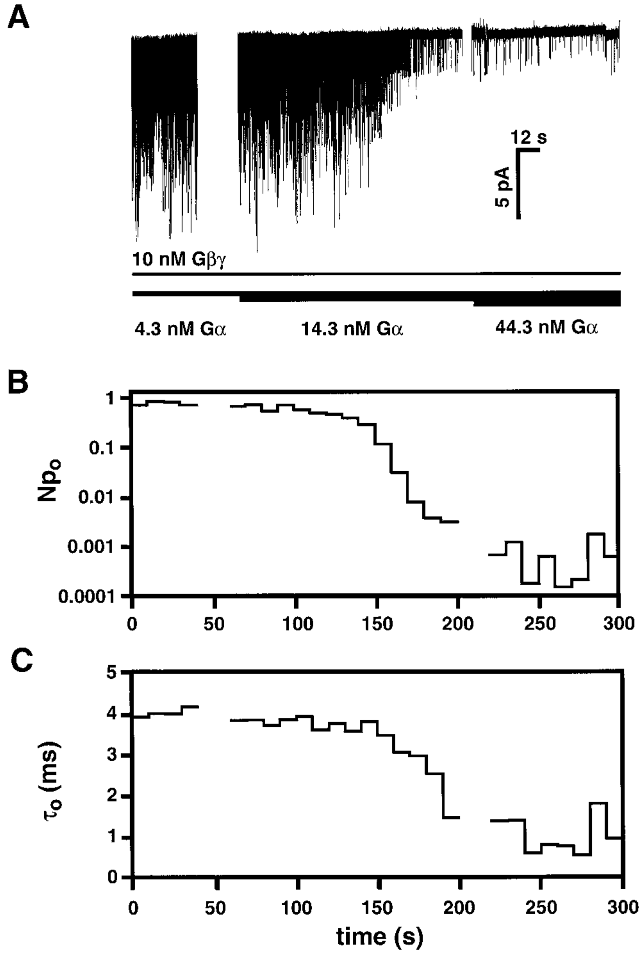

FIGURE 3 Comparison of Np_o and mean open time from an inside-out patch activated with 10 nM Gβγ. Increasing amounts of Gα-GDP were added to suppress the channel activity. (*A*) In the single channel recording, the channel activity decreased markedly when Gα-GDP concentration was increased from 4.3 nM to 14.3 nM, and even more when the Gα-GDP concentration was increased to 44.3 nM. The electrical artifacts caused by the addition of substances to the bath have been deleted from the recording. (*B*) Np_o calculated from 10-s data segments from the same patch decreased ~1000-fold. (*C*) Mean open time calculated from the same 10-s segments and corrected for Np_o is shown in the bottom panel. A gradual decrease in Np_o is accompanied by a parallel decrease in τ_o.

DISCUSSION

The data presented here provide strong evidence for the existence of at least two open states for rat I_{KACh} in Gβγ-activated inside-out patches from atrial myocytes. This result is largely consistent with the data from cell-attached recordings from frog (Ivanova-Nikolova and Breitwieser, 1997) and contradicts the conclusion reached by spectral analysis of I_{KACh} current in inside-out patches from guinea-pig (Hosoya et al., 1996). The most likely explanation for this discrepancy is the higher sensitivity of direct MLE fitting of the open time histograms for detection of multiple channel states. It has been shown that application of ATP to the cytosolic surface of an inside-out patch increases the mean open time duration (Kim, 1991) and that alkaline phosphatase can reverse this effect. This suggests that the mean open time of the phosphorylated channel is longer than that of the unphosphorylated channel. While no exogenous phosphate donor was present in any of our experiments, it is possible that the considerable variation between mean open time of different patches was a result of different levels of phosphorylation that persist after patch excision. However, the prolongation of open time seen with addition of Gβγ cannot be explained by channel phosphorylation. If Gβγ activated a membrane-bound kinase, a phosphate donor would be required for channel phosphorylation. Alternatively, prolonged exposure of the cytosolic patch surface to the bath solution could result in mean open time prolongation unrelated to Gβγ (dephosphorylation would be an example), but such a mechanism cannot account for the observed decrease in open time after Gα-GDP application.

We hypothesize that Gβγ binding shifts the channel to longer open state(s). Since channel activity is negligible in the absence of Gβγ, this would require the presence of at least two binding sites for Gβγ. A channel with only one binding site can have multiple open states, but if we assume that the channel does not open in the absence of Gβγ, then the equilibrium between these open states (all of which would have one Gβγ molecule bound) and their respective mean open times would not depend on Gβγ concentration. According to our model, only one site would be occupied at low Gβγ concentrations, leading to an increase in the frequency of opening compared to the basal state. At higher concentrations, another site would bind G$\beta\gamma$, resulting in a further increase in opening frequency as well as prolonging open time duration. This mechanism could partially account for the decreased mean τ_o seen in cell-attached patches in which channels desensitized rapidly (Kim, 1991). In our experimental conditions, the change in Np_o resulting from increased open time was small (30% increase in mean open time for a 20-fold increase in Np_o), confirming that increased frequency of channel openings accounts for most of the increase of I_{KACh} current upon activation by G$\beta\gamma$.

The stoichiometry of G$\beta\gamma$ binding to the channel is currently unknown. The dose-response curve of channel activation by G$\beta\gamma$ has a Hill coefficient between 1.2 and 3.1, depending on the exact protocol and type of G$\beta\gamma$ used (Ito et al., 1992; Krapivinsky et al., 1995b), suggesting that more than one G$\beta\gamma$ molecule binds to the heteromultimeric channel. However, in vitro binding of G$\beta\gamma$ to the channel subunits is best fitted by a Hill coefficient of 1, which would be consistent with 1:1 binding (Krapivinsky et al., 1995b). The data reported here provide independent support for multiple G$\beta\gamma$ binding sites on the intact channel complex. The difference in predicted binding stoichiometry may reflect the difference between binding to the native channel versus the solubilized complex.

A negative correlation between adjacent closed and open times has been reported before for the Ca^{2+}-activated K^+ channel and a Cl^- channel from a skeletal muscle (McManus et al., 1985). Such a correlation imposes further constraints on the kinetic schemes, ruling out certain simple mechanisms such as

$$\begin{tikzcd} C1 \arrow[r, leftrightarrow] & O1 \arrow[r, leftrightarrow] \arrow[d, shift right] & C2 \\ & O2 \arrow[u, shift right] & \end{tikzcd} \tag{2}$$

$$\begin{tikzcd} C1 \arrow[r, leftrightarrow] & C2 \arrow[r, leftrightarrow] \arrow[d, shift right] & O2 \\ & O1 \arrow[u, shift right] & \end{tikzcd} \tag{3}$$

$$C1 \leftrightarrow C2 \leftrightarrow O1 \leftrightarrow O2 \tag{4}$$

which could otherwise account for the existence of two exponential components in open time distribution as well as for channel bursting.

One simple kinetic scheme that could account for all the data presented here is analogous to that of the kinetic state model proposed for the nicotinic receptor (Colquhoun and Sakmann, 1985):

$$\begin{tikzcd} C0 \arrow[r, leftrightarrow, "G_{\beta\gamma}"] & C1 \arrow[r, leftrightarrow, "G_{\beta\gamma}"] \arrow[d, shift right] & C2 \arrow[d, shift right] \\ & O1 \arrow[u, shift right] & O2 \arrow[u, shift right] \end{tikzcd} \tag{5}$$

Here, the channel would be in a closed state C0 in the absence of G$\beta\gamma$. Binding of G$\beta\gamma$ would lead to transition to long closed state C1, linked to a short open state O1. Binding of another G$\beta\gamma$ molecule to the channel would lead to transition to short closed state C2, linked to a long open state O_2. If the transition between C1 and C2 is slow, such a scheme would result in negative correlations between the subsequent closed and open times and explain the longer mean open time at higher G$\beta\gamma$ concentrations. Based on this kinetic scheme and simplifying assumptions, O1 → C1 and O_2 → C2 rates can be roughly estimated at 1000/s and 200/s, respectively. Obviously, many other more complicated kinetic schemes are consistent with our data.

In summary, we conclude that at least two open states of I_{KACh} can be identified in excised patches activated with G$\beta\gamma$ even in the absence of ATP, and that high G$\beta\gamma$ concentrations prolong the mean open time of the channel, implying the existence of at least two channel binding sites for G$\beta\gamma$.

APPENDIX A

Correlation between open and closed time durations in patches containing multiple channels

Imposition of a nonzero dead time can introduce spurious correlations into certain kinetic models. In these models, the duration of open and subsequent closed interval are independent, but the duration of two consecutive closed intervals is not (Blatz and Magleby, 1989; Colquhoun and Hawkes, 1995). This problem does not arise for models in which transitions between closed and open states always involve the same open (or alternatively, the same closed) state. In such models, the durations of an open interval and any subsequent closed or open interval are independent (this follows from the Markovian assumption).

In an idealized patch recording containing a single channel only and starting in an open state, we denote the times at which open → closed transitions occur as ($Tc_1, \ldots, Tc_n$) and the times of closed → open transitions as ($To_1, \ldots, To_n$). Obviously, $Tc_1 < To_1 < Tc_2 < \ldots < To_n$. For $1 < i < n$, the duration of the *i*th opening in the idealized recording is Tc_i-To_{i-1}. For a given δ, this value only depends on the durations of openings and closing of the actual channel (i.e., before dead time imposition) before Tc_i (tc_k-to_{k-1}, to_{k-1}-tc_{k-1}, tc_{k-1}-to_{k-2}, . . . , where tc_1, to_1, . . . , to_{k-1}, tc_k denote the times of channel closing/opening before dead time imposition occurring before Tc_i). The observation of open → closed transition at time Tc_i in the idealized recording also implies that the channel is closed for at least δ (i.e., to_k-$tc_k \geq \delta$ and $tc_k + \delta = Tc_i$). The duration of the subsequent channel closing in the idealized recording will be To_i-Tc_i, which depends only on the sequence of closed/open channel times after Tc_i (i.e., to_k-tc_k, tc_{k+1}-to_k, to_{k+2}-tc_{k+1}, . . .). For the kinetic models discussed above (in which the transitions between closed and open states always involve the same open or the same closed state), the duration of channel opening or closing is independent of duration of any subsequent (or prior) channel opening/closing. Therefore, Tc_i-To_{i-1} and To_i-Tc_i are functions of independent variables, and are consequently themselves independent. Thus, imposition of dead time would not be expected to introduce spurious correlations into the models described above. Models (2), (3), and (4), which could otherwise account for multiple open states and for bursting behavior of I_{KACh}, fall into this category.

In a patch containing a single ion channel, correlation between closed time duration and subsequent open time duration imposes certain constraints on the kinetic scheme: it is necessary that the sets of open and closed states are connected in such a way that deletion of any single state does not lead to separation of the open and closed sets (Colquhoun and Hawkes, 1987). Again, the presence of multiple channels in the patch complicates the situation. If openings of two or more channels of the same amplitude overlap, it is not possible to determine the durations of individual openings unequivocally (see Fig. 4), and even if the channel activity is so low that there are no multiple openings in the patch recording, it is not possible to determine the closed time durations of individual channels

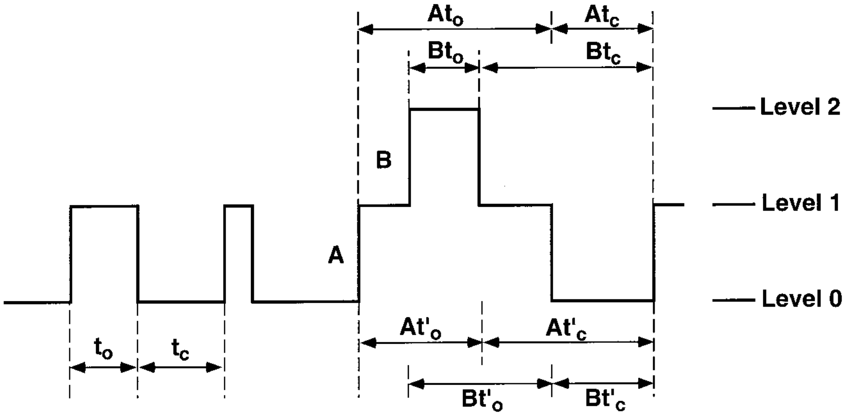

FIGURE 4 The method in which double openings have been handled for correlation analysis is illustrated. For single openings, t_o is an interval from opening of a channel to the closing of the same channel, and t_c is the interval from this channel closing to opening of any channel in the patch. These two values are independent if the duration of channel opening is independent of the duration of its subsequent closing for a given channel. In the case of double openings, channel B opens before channel A closes. The duration of the open interval is equivocal for both channels: if channel B closes before channel A, then channel A is open for At_o and closed for at least At_c, while channel B is open for Bt_o and closed for at least Bt_c. Alternatively, if channel A closes before channel B, then channel A is open for At'_o and closed for at least At'_c, while channel B is open for $Bt'o$ and closed for at least Bt'_c (note that $At_c = Bt'_c$, and $At'_c = Bt_c$). All possible combinations were analyzed for recordings with double openings.

unequivocally. The time a channel spends at level 0 (all channels closed) may correspond to a closed time of a given channel (interval from its closing to its opening), or it may be an interval from closing of channel A to the opening of channel B.

To analyze only the correlation between durations of stay at level 1, uninterrupted by another channel opening, and the subsequent durations of stay at level 0 is inappropriate. Longer openings are more likely to be interrupted by opening of another channel, and bias might be introduced by this procedure. To put it another way, excluding double openings implicitly assumes that while channel A is open, all other channels in the patch remain closed. Therefore, if a long channel opening is observed, all other channels in a patch have to be closed for a long time to be included in the analysis. This may affect the effective rate of opening of these channels: the duration of open time of channel A and time from closure of channel A to the opening of channel B no longer need to be independent. Spurious correlations might thus be introduced if double openings are omitted. This problem could be circumvented if only channel recordings without double openings could be analyzed. In this case, the duration of a channel's stay in an open state(s) is unequivocal without further assumptions.

If there are *n* independent channels in the patch ($K_1, \ldots, K_n$), we denote by G_1 the duration of an open time interval of K_1. Then the time interval H_i from the closing of channel K_1, which ends G_1 to the subsequent opening of any other channel (K_i, $i = 2, \ldots, n$) is independent of G_1. For the class of kinetic models discussed above (where open/closed transitions always involve the same open or the same closed state), the time interval from closing of K_1 to its subsequent opening (denoted H_1) is also independent of G_1. The time interval the channels in the patch spend at level 0 after closing of K_1 is $\min(H_1, \ldots, H_n)$. This is a function of *n* variables which are independent of G_1, and it is therefore itself independent of G_1. In other words, for the class of kinetic models discussed above, the patch current's duration of stay at level 1 should not be correlated with its subsequent duration of stay at level 0, even with multiple channels in the patch and after dead time introduction, provided there are no multiple openings in the recording.

As mentioned above, most patches from rodent atrial myocytes contain multiple I_{KACh} channels, and recordings with no double openings that nevertheless contain enough openings (several hundred) to analyze the correlation between open and closed times are difficult to obtain. However, it is possible to analyze recordings with a few double openings only. As in recordings with no double openings, the correlation between the duration of time interval between opening of channel A to closing of channel A and the subsequent time interval from the closing of channel A to opening of any channel in the patch can be analyzed. In patches with a few double openings (of . . . 0-1-2-1-0-1. . . type only), the duration of closed times is unequivocal, but two possible combinations of open times can produce the observed transitions for each double opening (Fig. 4). If, however, significant negative correlations are observed for all possible open/closed time combinations that can give rise to the pattern observed in a given recording, one can conclude that in that recording, the duration of the open state is not independent of the duration of the subsequent closed state. Since each double opening can result from two combinations of true open/closed times (Fig. 4), recordings with k double openings (of 0-1-2-1-0 pattern) can be interpreted in 2^k ways.

We evaluated recordings from five separate patches that showed relatively stable activity after activation with 20 nM Gβγ. We only evaluated patches with at least 250 openings containing <1% double openings of 0-1-2-1-0 type and no complex double openings (such, as e.g., 0-1-2-1-2-1-0). For patches with double openings, all open/closed interval pairs that could give rise to the pattern observed in a given recording were analyzed, and the least significant correlation coefficient was used. See Table 1 for details about the recordings used for correlation analysis.

APPENDIX B

Mean open time duration and channel activity

The method of mean open time measurement is considered in more detail here. The mean open time is determined by dividing the total area under the idealized recording curve by the total number of openings. In contrast to exponential fitting of open time durations, this can be precisely calculated from a recording with multiple simultaneously opened channels, provided that all channel openings are resolved. One potential concern is that with multiple channels in the patch, the imposition of finite dead time may lead to a loss of resolution of some channel openings that would be resolved in a patch containing a single channel only. This might disproportionately affect data segments with high channel activity, and result in spurious prolongation of the mean open time derived from these segments.

Imposition of a dead time δ leads to a loss of a resolution of an opening of channel K_1 (that would be resolved in an idealized recording with the same dead time, but containing only one channel), if and only if it is preceded or followed by a closing of another channel ($K_2, \ldots, K_n$) and the time interval between these two events is $<\delta$. Since the channels are assumed to be mutually independent, the proportion of unresolved events can be estimated as $2(n - 1)N\delta/(nT)$, where n is the number of channels in the patch, N is the number of openings before the resolution loss, and T is the duration of the recording. As the total number of channels in the patch is usually unknown, and the effect we are correcting for would bias the data in the direction we have observed, we chose to overestimate the number of missed openings and define $M = N(1 - 2\delta N/T)$, where N is the number of channel openings that would be resolved with only one channel in the patch and M is the number of resolved openings in a patch with multiple channels. Therefore, if $\alpha = 2\delta/T$, then $\alpha N^2 - N + M = 0$ and $N = (1 - \sqrt{1 - 4\alpha M})/2\alpha$. All data on τ_o duration changes in this paper have been corrected in this way, i.e., using N instead of M (number of actually resolved openings) in the denominator of formula (1). Therefore, this increase in open time would be expected to occur even in patches containing a single ion channel.

After dead time imposition, apparent open time could theoretically increase with G$\beta\gamma$ addition in a patch containing a single channel even if there were just a single open state. This could happen if, for example, the open state was connected to a short-lived closed state, the mean sojourn in which would be comparable to δ and whose occupancy would be increased by high G$\beta\gamma$ concentration. In such a case the true open interval would be unchanged, but since many short closings would be missed, the apparent open time would be prolonged as G$\beta\gamma$ concentration increased. At least two G$\beta\gamma$ binding sites would still be required to account for the data. There is no way of excluding this possibility apart from using very short δ, but the existence of multiple open states would still be mandated by the results of the correlation analysis and presence of multiple exponential components in patches with stable activity level. It is also conceivable that there might be, for example, two open states with the same true open time, but different "apparent" open times after dead time imposition. Our data would then indicate that the occupancy of "apparent long" open state increases with increasing G$\beta\gamma$ concentration.

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