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Charge Displacements in a Single Potassium Ion Channel Macromolecule during Gating

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ABSTRACT Single ion channel currents can only provide indirect information on channel molecular events (except for timing). In contrast, the electric displacement currents associated with channel gating, termed gating currents, can provide direct information regarding the channel molecule's conformational changes. However, thus far gating currents have been measured only from ensembles of numerous stochastically activated channels and therefore the information they provide is limited. This work presents, for the first time, measurements of gating currents from a single channel molecule. Averaging close to 8000 pre-open currents, aligned to the single channel opening time, enabled the detection of single channel gating currents with a resolution of 2 electron charges. The measured charge displacements show: 1) a slow component, ~2 fA above baseline level, assumed to represent stochastic conformational changes, and 2) transients, the most significant of which occur 1.1 and 0.3 ms before channel opening. The transients most likely represent apparent deterministic stages in the gating process. The largest transient current peak was 5.1 ± 1.6 fA and the total equivalent charge transported across the membrane was 4.7 ± 2.5 electron charges. This data is unique also in that it presents monitoring of the behavior of a single, well-defined macromolecule.

INTRODUCTION

The investigation of the mechanisms of regulation of ionic flow through ion channels, a process called gating, is usually carried out by measuring either single channel or multichannel ("macroscopic") currents (Neher and Sakmann, 1976; Hodgkin and Huxley, 1952). Unfortunately, from these currents one cannot extract much information regarding the channel conformational changes, except for timing of some events that underlay gating. Therefore, the amount of information that can be extracted from them in regard to channel molecular events is limited. However, such information can be obtained, using similar techniques, from the intramolecular charge displacements associated with the channel conformational changes. These charge movements generate minute transient currents the characteristics of which are directly related to the underlying molecular processes (Almers, 1978; Crouzy and Sigworth, 1993; Armstrong, 1981; Conti and Stühmer, 1989; Schoppa et al., 1992; Perozo et al., 1992; Meves, 1990). To date only the sum of numerous individual displacement currents generated by many channels (macroscopic currents) was measured (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1974; Schoppa et al., 1992; Stühmer, 1992; Bezanilla et al., 1991) and is termed gating current. However, since the gating process, i.e., the transitions from one conformational state to another, are stochastic processes, the summation combines nonsynchronous charge displacement events. As a result, the amplitudes and relative timing of the various stages of the process are lost.

The obvious (but perhaps technically impossible) way to gain more insight into the behavior of polar molecules in general, and ion channel molecules in particular, is to measure the currents generated by charge displacements of a single molecule or channel. The amplitude of a single channel gating current, estimated on the basis of macroscopic gating currents (Schoppa et al., 1992) or gating currents noise analysis (Conti and Stühmer, 1989), is on the order of 5 fA ($5 \cdot 10^{-15}$A, 5 kHz bandwidth) and its duration is <25 μs. As the noise levels of available voltage clamp systems are ~100–200 fA RMS (5 kHz bandwidth), to measure the single channel gating currents it is essential to improve the signal-to-noise ratio by a factor of ~100. This can be achieved by time-locked averaging, i.e., by taking ~10,000 current trace segments, immediately preceding or following channel openings or closings, temporally aligning them to the time of channel state change, and averaging them. In this work we chose to measure the single channel gating currents that precede channel opening (Mika and Palti, 1994), because this period in time is free from channel ionic current noise and is not distorted by the system's response to the current changes associated with channel opening or closure.

MATERIALS AND METHODS

Single channel currents were recorded from *Shaker* H4-IR channels (Zagotta et al., 1989; Bezanilla et al., 1991; Iverson et al., 1988; Kamb et al., 1987) expressed in *Xenopus* oocytes (Stühmer, 1992) 1 to 2 days after the mRNA injection. Recordings were made from an isolated patch, in an outside-out configuration (Sakmann and Neher 1983; Hamill et al., 1981), using an Axopatch 1B amplifier (Axon Instruments Inc., Foster City, CA) with an IHS-1 integrating headstage. Each patch, held at −70 mV, was subjected to 1000–3000 250-ms-long depolarization steps to −50 mV. The pulse interval was 2–4 s and the integrator was reset before the beginning of each new pulse. No spontaneous resets of the integrator occurred during the depolarization pulses.

Currents were filtered at 2–5 kHz (4-pole Bessel filter) and sampled at 50–100 kHz at a 12-bit resolution. High gain was used to achieve a current

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resolution of 1–3 fA. Only experiments that met the following criteria were analyzed: seal resistance >50 GΩ, noise level <150 fA RMS, and close state (baseline) current drift <200 fA throughout the duration of the experiment. Channel openings were identified using the 1/2 amplitude threshold crossing method (Sakmann and Neher, 1983). Pre-open segments, at least 2 ms long, that satisfied the following criteria were aligned, with reference to opening time, and averaged: no openings were detected throughout the 2-ms period, and the current during that period did not deviate from the mean level by more than 440 fA.

We specifically chose to step the voltage to −50 mV as it enabled us to get the maximum number of events that are separated by time periods >2 ms. The time period of 2 ms was selected as it is about one order of magnitude higher than the system's rise time. The rationale for this selection is to avoid the effect of channel closing signals that may "contaminate" the results.

Channel alignment error was relatively small (~ ±60 μs). The error was estimated using the autocorrelation of channel threshold crossing point with other time points. The above conclusions were confirmed using simulation of channel openings superimposed on sampled noise. The high sampling rate (20–25 times the filter corner frequency), the steady baseline level, and the relatively good signal-to-noise ratio helped in reducing the alignment error. Pipette solution (mM): 80 KF, 35 KCl, 1 EGTA, and 10 HEPES (pH 7.4). External solution (mM): 125 KCl, 5 NaCl, 5 MgCl2, 5 HEPES, and 0.2% BSA (pH 7.4); temperature 23–24°C.

RESULTS

The solid line in Fig. 1 *A* gives the average of ~8000 pre-open currents (*B*) aligned to the opening time. This current trace is to be compared with the baseline level which includes background noise (dashed line). The last was obtained by averaging the same number of 2-ms long current segments, randomly selected from traces measured when the channel was probably in the inactivated state such that no openings were detected throughout the segments.

The average pre-open displacement current has the following two distinct characteristics: 1) Throughout the measurement period (2 ms) its average amplitude is maintained at ~2 fA above the noise level. It is difficult, at this stage, to estimate the temporal behavior of this relatively stable current component. However, we can speculate that this relatively steady current is the integral of multiple fast charge displacements of a stochastic temporal behavior. 2) The current has a number of relative rapid transient components (α, β, γ) well exceeding the background noise level. The peak of the highest current transient (β in Fig. 1) appears 1.1 ± 0.17 ms (3 experiments) before channel opening and its amplitude is 7.1 ± 1.6 fA above the average background noise level and 5.1 ± 1.6 fA above the steady mean current (Conti and Stühmer, 1989; Crouzy and Sigworth, 1993). The calculated amount of equivalent charge displaced across the membrane during this peak is 9.1 ± 3.2 electron charges when measured from noise level and 4.7 ± 2.5 electron charges when measured from steady averaged current. In view of these findings this transient which is locked in time with, rather than stochastically linked with, the channel opening time, may represent processes of, at least apparent, deterministic nature.

Because of the limited frequency response of voltage clamp systems, the actual duration of this transient, as well as of all others, is most likely shorter then the apparent 300 μs. A nonstationary autocorrelation function of the β peak, with respect to the current at other times (Fig. 2), confirms that its duration is limited by the system's frequency response.

The limited frequency response may also be responsible for another potential source of experimental error: the attenuation of signals generated by very short openings which become unresolved openings and can therefore be mistaken for gating currents. We eliminated the possibility of such errors by using ion concentrations in which the direction of the ionic currents is opposite to that of the gating currents.

Since the averaged current transients are minute, the following protocols were used to eliminate the possibility that they are due to some experimental error.

1. First we ruled out the possibility that the measurements result from distortion of the signal by the experimental system or the analysis method. A single channel like synthetic signal was generated by capacitive coupling of an external signal source to a pipette sealed to a Sylgard ball or to an uninjected oocyte membrane that did not contain channels. The source produced currents similar to single channels currents that occurred randomly in time and had a random opening duration. The resulting current was sampled and processed by the same techniques used for the processing of the single channel current measurements. The averaged synthetic pre-open trace did not contain any signals similar to those in the channel pre-open trace as can be seen in Fig. 3, where both currents are superposed. These results indicate that the pre-open gating transient current is not due to system or analysis artifacts.

2. To reveal any hidden periodicity in the signal that could bias the channel-triggered traces, an average of blank traces, triggered on noise peaks, was studied. The procedure used here resembles the alignment and averaging of the pre-open current, but instead of using the channel opening as the alignment point, a threshold was set at 200 fA. Noise peaks which crossed the threshold were aligned and the traces that proceeded them were averaged. The averaging was repeated twice using both positive and negative values as threshold. As can be seen in Fig. 4, except for the initial filtration effect that lasts 400 μs at most, no hidden periodicity can be detected and the noise behaves like the random non-triggered baseline noise.

3. We checked which transients are statistically significant signals, time-linked to channel opening, by means of the following "random selection" procedure: Sub-groups of 1000–2000 opening events were randomly selected from the entire set of 7692 openings, aligned and averaged. The process was repeated several hundred times, making sure that mutually exclusive subgroups are also selected. In such averaged current subgroups, transients which are uncorrelated with the opening would appear at different times (relative to opening time). In contrast, in each subgroup, events correlated with our time marker should show a similar signal at some specific time. As can be seen in Fig. 5, all β transients

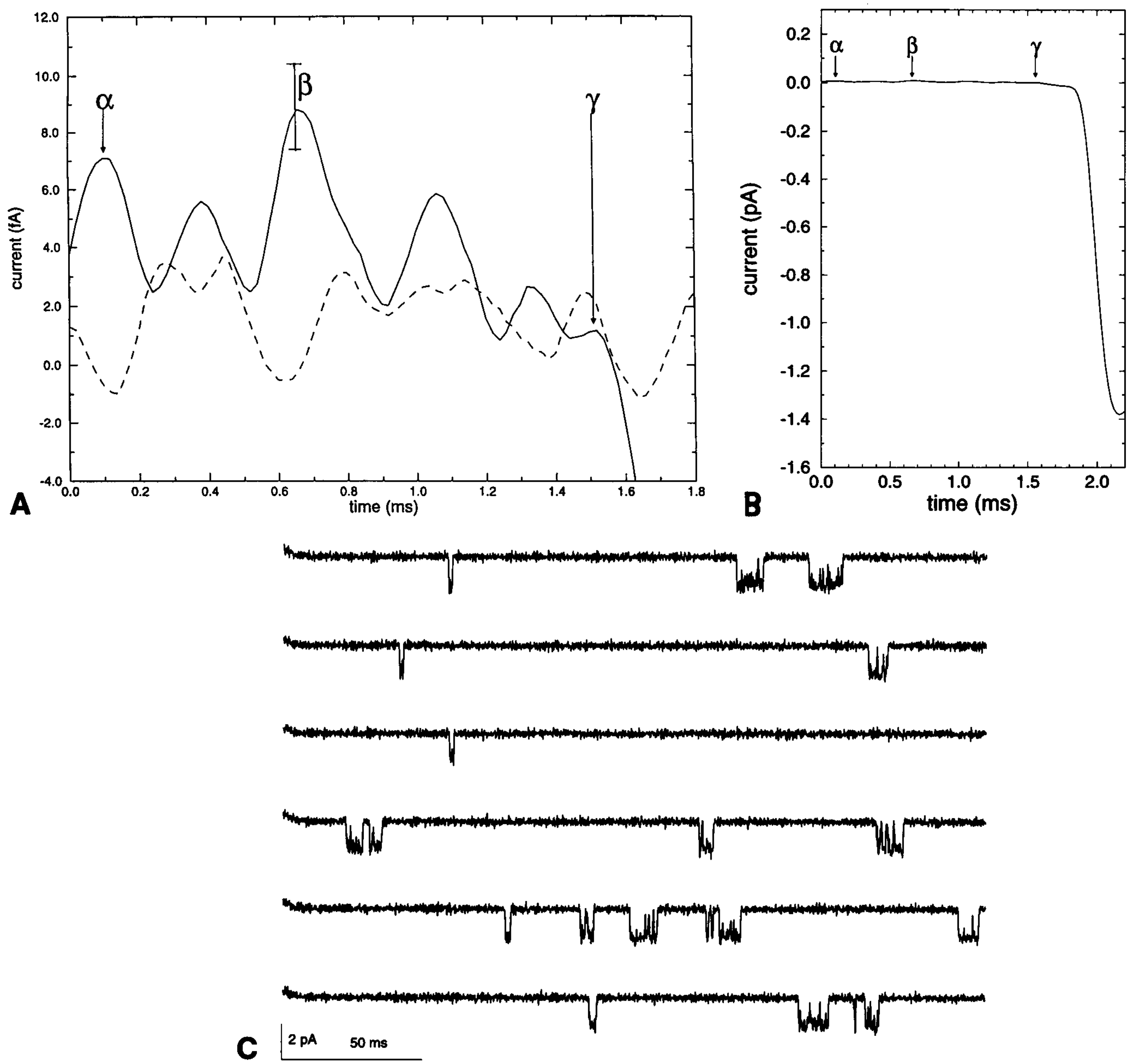


FIGURE 1 (*A*) Superposition of aligned and averaged pre-open single channel current segments and reference noise. (*Solid line*) The average of 7692 aligned pre-open segments (bar on the β peak = 1 SE). Errors at other time periods are are within 20% of the presented error bar). (*Dashed line*) Reference noise level obtained by averaging the same number of 2 ms current records randomly selected from traces in which no openings where detected. The amplitude of the pre-open average current is 2 fA above the noise level. The signal shows transients marked α, β, and γ. The β transient appears 1.1 ms before the channel opens, its peak is 5.1 ± 1.6 fA above the steady mean current and 7.1 ± 1.6 fA above the average background noise level. The total equivalent electric charges carried across the membrane, calculated by numerical integration of β, is 9.1 ± 3.2 charges when measured from noise level and 4.7 ± 2.5 charges when measured from steady current. Zero time corresponds to 2 ms before opening detection time. (*B*) Illustration of the amplitude of the aligned pre-open signals relative to the open channel current. The same average of 7692 segments depicted in (*A*) is plotted at a low gain. (*C*) Representative 250 ms long single channel traces. The signal was filtered by a 4-pole Bessel filter set at 2 kHz and sampled at 50 kHz.

superpose, indicating that they represent charge displacements temporally linked with the channel opening transition. A more quantitative estimate can be obtained by looking at the distribution of extrema along the current traces. The location, on the time axis, of maximums and minimums in each subgroup was determined by numerically differentiating the signal and detecting the location of maximums and minimums by derivative sign changes. At the time corresponding to the β transient, >80% of the subgroups have maximums and, more importantly, the number of minimums at the time of the peak is 0. Applying the same procedure to the background noise traces or to the synthetic signal produced mini-

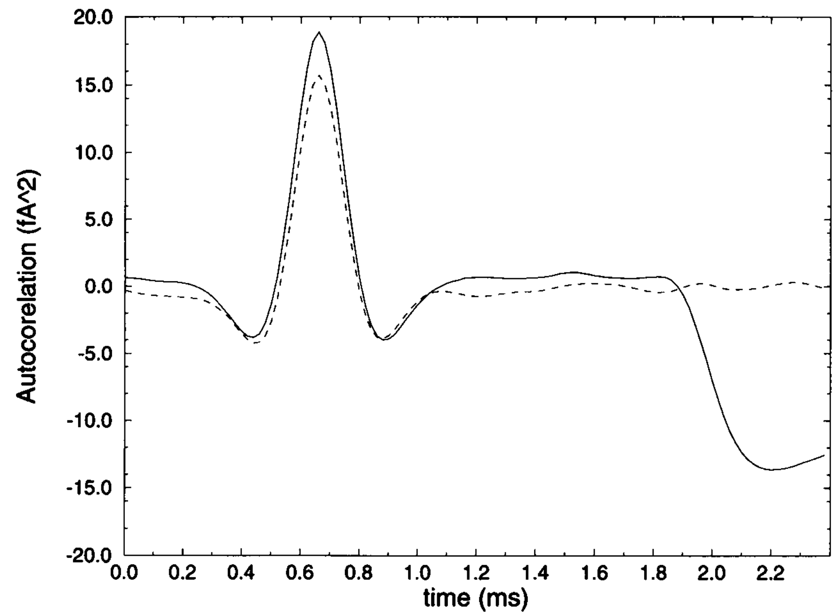


FIGURE 2 Autocorrelation function of the β peak. (*Solid line*) Autocorrelation of the β peak with respect to the current at other times. (*Dashed line*) Autocorrelation of a randomly selected time point in the noise data with respect to noise current at other times. The autocorrelation was calculated using the equation:

$$C(k) = \left(\frac{1}{N}\right) \cdot \sum_{i=1}^{N} (I_{\beta,i} - \bar{I}_{\beta}) \cdot (I_{k,i} - \bar{I}_{k}) \qquad (3)$$

where N is the number of traces (7692 in the presented case); $\bar{I}_{\beta}$ is the average current at the β peak; $\bar{I}_{k}$ is the average current at time point k; $I_{\beta,i}$ is the current at the β peak of current trace i; $I_{k,i}$ is the current at time point k of current trace i. The data are presented with the same time axis as that of the pre-open gating currents in Fig. 1 *A*. The noise autocorrelation is drawn such that it superposes the β peak. The time course of the two traces around the β peak is almost identical, leading to the conclusion that the time course of the β signal is fast compared with the system's frequency response. Note the high negative correlation between the β peak and channel opening.

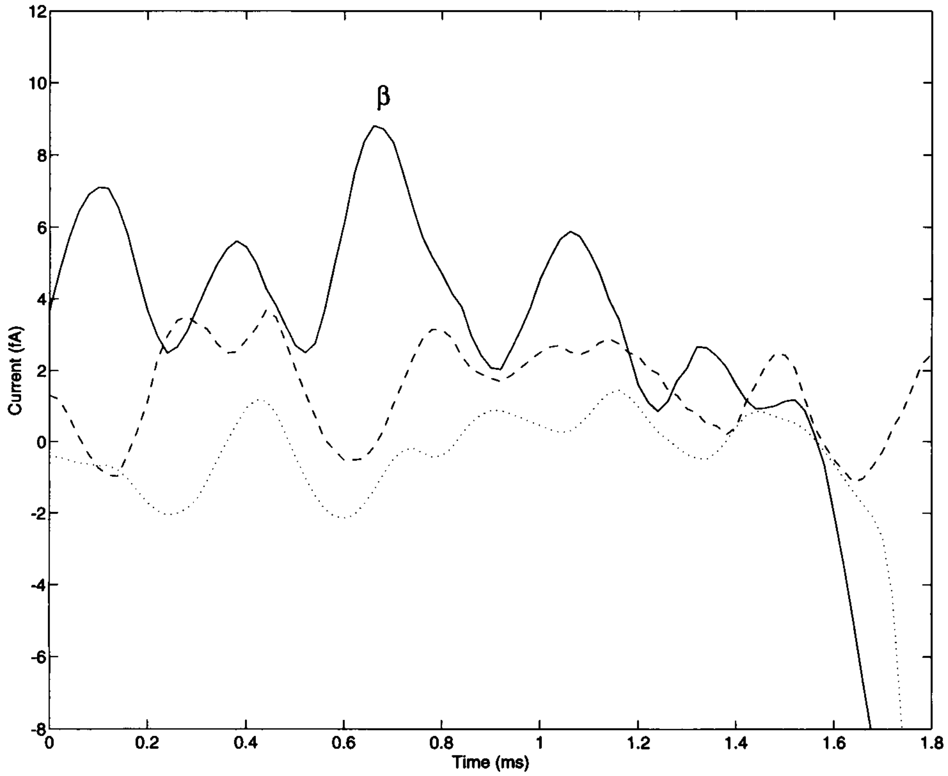


FIGURE 3 Superposition of aligned and averaged pre-open single channel current segments (*solid line*) and reference noise (*dashed line*) from Fig. 1, with an average of the same amount of synthetic single channel events generated by capacitive coupling of an external signal source to a pipette sealed to an uninjected oocyte membrane that did not contain channels (*dotted line*). The synthetic single channel events were sampled and processed by the same procedures used for the processing of the single channel current measurements. The pre-open signal of the synthetic single channel events behaves like the baseline background noise and does not contain peaks like the β signal. This confirms that the pre-open gating current peak is not the result of a system or analysis artifact.

mums in >10% of the subgroups at all points in time. These results strongly support the notion that the β transient is an event time-locked to channel opening.

4. Each time point of the pre-open gating currents was compared with the background noise to evaluate the probability that the two have different means. Analysis of variance was used with a null hypothesis that the two means are equal. The probability for each time point in the pre-open signal 'was calculated against each time point of baseline current. The mean results for each time point of the pre-open signal are plotted in Fig. 6. The probability that the β peak has the same mean as the noise is on the average 0.0022 (the maximum probability is 0.008). In other words, the probability that the β peak differs from the baseline level is >99%. At the two sides of the β peak, the probability rises steeply to values in the order of 0.1, indicating that the β peak is an abrupt change and not a mean level of a distribution. As to the other peaks in the pre-open current, it is difficult to decide

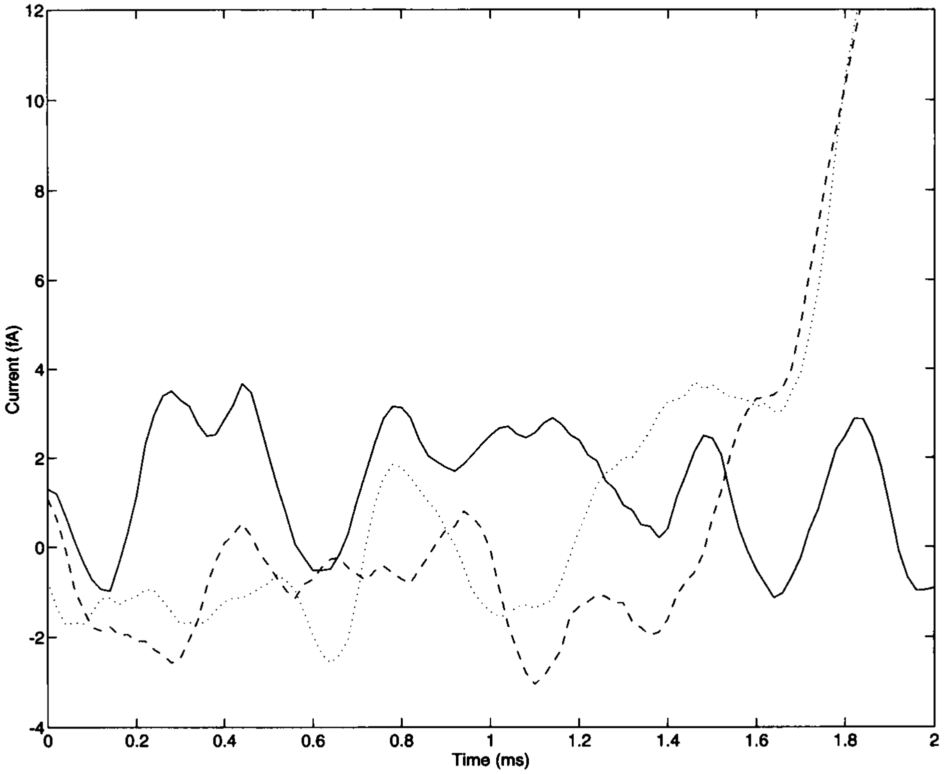


FIGURE 4 An average of 7692 blank traces triggered on noise peaks. The average was made from the same current traces that were used for the construction of the baseline in Fig. 1 *A*, with the difference that noise peaks were used as trigger points. Threshold peak value was 200 fA. The crossing of threshold by noise was detected and used for alignment and averaging. The procedure was repeated twice, using positive and negative thresholds. This procedure should reveal any hidden periodicity in the averaged signal. However, except for the initial filtering effect lasting 400 μs at most, no hidden periodicity can be detected. The averages obtained using the positive threshold (*dotted line*) and the negative threshold (*dashed line*) behave like the randomly selected baseline noise (*solid line*).

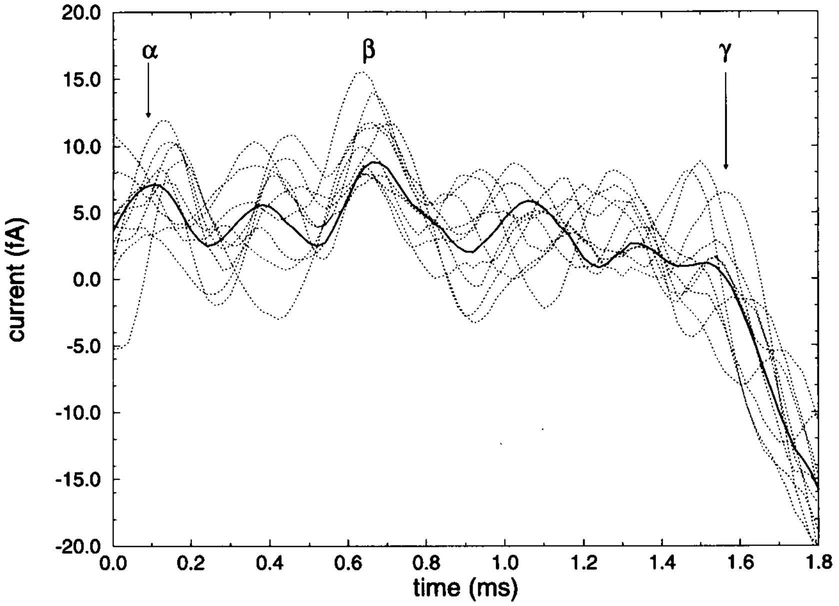


FIGURE 5 Averages of 2000 pre-open traces, randomly selected from the 7692 single channel current traces used to generate Fig. 1. (*Dashed line*) 10 averaged traces randomly selected from 256 such averages. (*Solid line*) The averaged 7692 pre-open traces, illustrated in Fig. 1. It is expected that in each subgroup, events correlated with our time marker would show a similar signal at some specific time. It can be seen that all subgroups have a transient, the peak of which coincides or is close to the β peak. The same is apparent for the very small γ peak (probably masked by unresolved opening currents). This behavior is expected from events time-linked to the channel opening, i.e., events of apparent deterministic nature. In contrast, the peaks between the β and γ peaks on the solid line coincide with both minimums and maximums of the averaged subgroups, indicating that these correspond to random fluctuations.

at the given signal-to-noise ratio. The low level of 0.01 at the beginning of the analyzed time, corresponding to the α peak, is close to channel closures, and may thus result from the filter overshot in response to channel closure. Although the slow current level can only be estimated at this stage, the probability that the β peak has a mean current value of 2 fA above the noise level was tested using the same statistical methods described above. The probability was computed to be 0.03, i.e., the hypothesis that the β peak is a fluctuation of the slow current component can be excluded with 97% degree of confidence.

5. To rule out the possibility that the referred transients result from fast undetectable openings (unresolved openings), the following procedure was used to decrease the possible error resulting from such events. The procedure is based on the assumption that only a small part of the current traces have unresolved openings at the specific time period, as opposed to gating currents that appear in most of the current

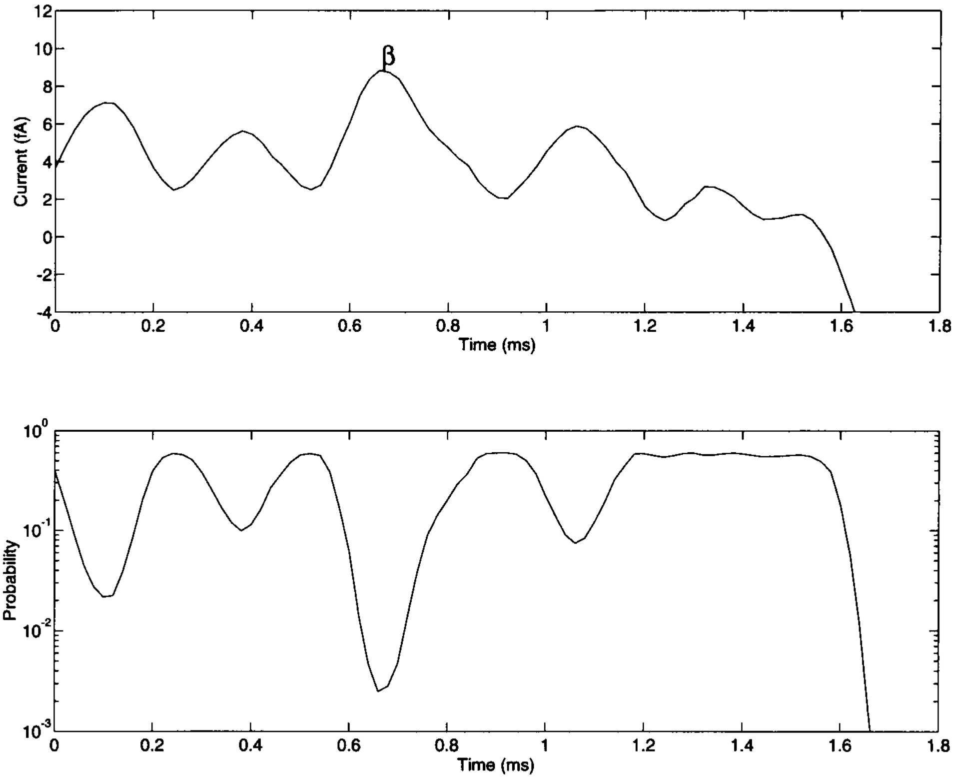


FIGURE 6 The probability that the pre-open current level is the same as the noise level. The probability was calculated using analysis of variance with a null hypothesis that the two means are equal. The probability for each time point in the pre-open signal was calculated against each time point of baseline current. The mean results, for each time point of the pre-open signal, are plotted in the bottom plate. The top plate show the pre-open signal of Fig. 1 for reference. The probability that the β peak have the same mean as the noise is on the average 0.0022 (the maximum probability is 0.008). At the two sides of the β peak the probability raises steeply to values in the order of 0.1, indicating that the β peak is an abrupt change and not a mean level of a distribution. Other peaks in the pre-open current are difficult to ascertain at the given signal-to-noise ratio. The low level of 0.01 at the beginning of the analyzed time, corresponding to the α peak, is close to channel closures and may thus result from the filter overshot in response to channel closure.

traces. Using this assumption, current traces that were suspected of containing fast opening were removed from the traces before averaging. The criterion for removal was based on the fact that the noise is normally distributed (Sigworth, 1985) and that ionic current is unidirectional and therefore will cause asymmetry between the two sides of the pre-open noise amplitude histogram, which has a Gaussian distribution. The process was iterative; traces which had peaks that deviated from a selected range around the mean current were removed from the averaging process. A new amplitude histogram was constructed and the symmetry was checked again. The process continued until no symmetry deviation could be detected. Removing traces with suspected fast openings from the averaged data markedly enhanced the amplitude of the γ peak (Fig. 7), suggesting that it is a "true" gating current transient. The effect on the β peak was minor, indicating that unresolved openings do not significantly influence its behavior.

DISCUSSION

The results of the presented work may have significant implications to a wide spectrum of scientific areas. The most general is related to physical chemistry. Our understanding of the nature of chemical processes is usually based on observations and measurements made on a macroscopic scale, i.e., on an average, or some other statistical property of a population of molecules. Therefore, the characteristics of a process, such as a conformational or a dipole moment change, occurring in an individual specific molecule cannot be monitored by these means. The uniqueness of this study is in that it provides us with the amplitude and timing of a series of charge displacements occurring during conformational changes taking place in a single specific macromolecule. The apparent time locking between the β charge distribution change and channel opening time may be interpreted in terms of two apparently different kinetic mechanisms. According to one, the results may indicate that some molecular processes or state transitions, for example, conformational changes, are deterministic in nature. In such cases, the locking in time must not necessarily be absolute; however, the spread in timing of the events is expected to be Gaussian. This is in contrast to the general belief that such molecular processes are stochastic, having an exponential time distribution. A second possible interpretation of the results is that the unusual timing is due to an average of many stochastic transitions. However, this case is equivalent to a continuous process which mimics the behavior of a process of deterministic nature.

As to the specific molecule studied, the implications are to the gating process in general and to the specifics of the charge displacements occurring during gating. As estimated in this study, the largest equivalent amount of charge displaced through the membrane in a single transition between two states, is on the order of 4–5 electrical charges. If we assume that the whole gating process consists of a number of such voltage-dependent transitions, the net charge displacement would be of the same order as the net charge estimated from macroscopic gating currents, i.e., about 12 charges (Schoppa et al., 1992; Stühmer et al., 1991). The

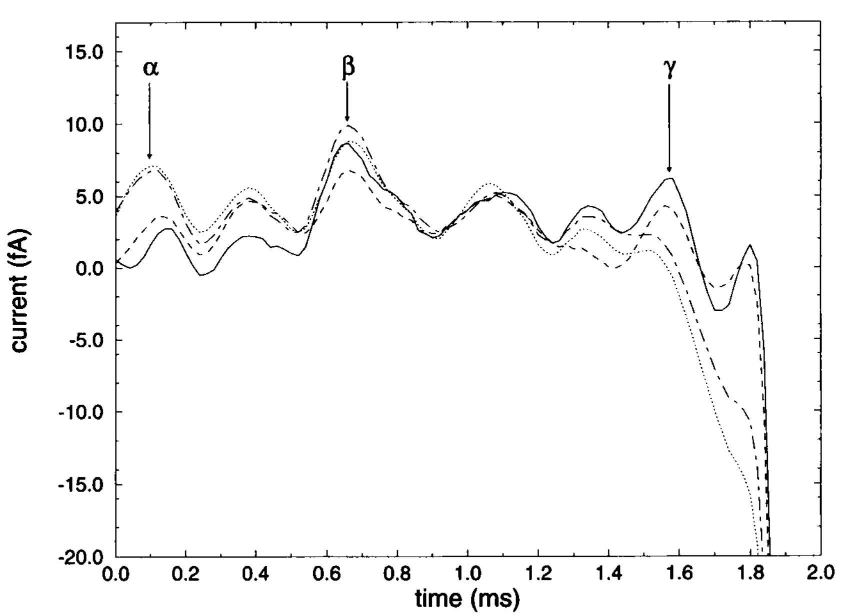


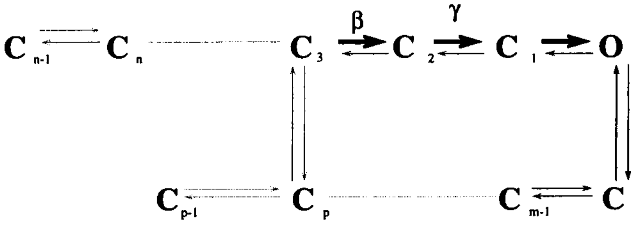
FIGURE 7 Aligned and averaged current traces obtained at different stages of the "unresolved openings" (very short openings) elimination procedure. The procedure makes use of the fact that closed channels current noise amplitude is normally distributed. Therefore, any unidirectional current flowing during a brief opening will result in an asymmetrical current amplitude histogram. Channel openings were selected to be included in the averaging procedure only when the pre-open noise level did not exceed a defined threshold; in this case, the initial value was set at 440 fA. An amplitude histogram was constructed from the selected pre-open signals and its asymmetry was assessed. The threshold level was then reduced in steps until no deviation from symmetry could be detected (240 fA in this case). Obviously, the number of traces that passed the selection criterion decreased with each step. (*Dotted line*) Average of 7692 pre-open traces, using a 440-fA threshold. (*Dashed/dotted line*) Average of 6923 pre-open traces, using a 400-fA threshold. (*Dashed line*) Average of 4754 pre-open traces, using a 280-fA threshold. (*Solid line*) Average of 2962 pre-open traces, using a 240-fA threshold. The current traces are markedly changed by the said process only during the first 400 μs before the opening (including the γ peak). Note the enhancement of the γ peak when the "unresolved openings" are eliminated. The β peak does not change while the early part of the trace changes slightly in a consistent way; its amplitude is reduced as the threshold is lowered.

charge displacement during a single transition, as estimated by single channel gating currents, is 2–3 times higher than that indirectly estimated from macroscopic gating currents (Schoppa et al., 1992; Bezanilla et al., 1991; Stühmer et al., 1991; Conti and Stühmer, 1989).

The following significant difference between the measurements described here and the reported macroscopic gating current measurements must be emphasized. The macroscopic gating currents are always linked to induced membrane potential steps. In contrast, we average pre-open currents that occur along a steady potential 250 ms in duration. Only 5% of the averaged segments preceded the first opening, while >80% were at times >10 ms after the voltage step, i.e., when the macroscopic gating current is completely undetectable. Thus the results reflect mostly the behavior of the channel molecule in a steady state. This difference will effect any comparison between the single channel results reported here and macroscopic gating currents which occur immediately following the voltage step and are attenuated exponentially. The above characteristics of the described method mean that it can be utilized to measure gating currents generated by conformational change in a ligand-dependent, stretch-dependent, as well as other voltage independent ion channels, as long as the conformational changes are accompanied by some charge displacement.

The most significant potential interpretation regarding ion channel gating process is that it may consists of a combination of: 1) "classical" state transitions of stochastic nature, and 2) deterministic, or at least, apparent deterministic transitions, which are time-locked to the channel opening. In both cases we are dealing with voltage-dependent transitions.

Within this framework we wish to outline a general structure of a partial channel model. The model relates to the behavior of the channel molecule in steady state and includes only the states adjacent to the channel open state, as the pre-open gating currents reflect the transitions preceding the channel opening. The suggested partial scheme of gating transitions is depicted below. The states connected with dotted lines represent states not supported by the gating current results.



Within the framework of a multi-state gating process that includes both stochastic and apparent deterministic processes, the time locking of the β and possibly the γ transients to the opening suggests that either no stochastic transitions intervene between them and the opening transition, or that the time constants of such putative stochastic transitions are fast (relative to the system's response time). Furthermore, since the close time between openings does not contain a detectable deterministic component, some conformational changes of stochastic nature must precede these deterministic transients. These facts are reflected in the upper pathway of the suggested partial scheme.

The relatively steady gating current component is not compatible with the above combined stochastic-deterministic transition pathway to the open state. Therefore, it may indicate the existence of another pathway (lower pathway), of regular stochastic nature, leading to the open state. If we assume that, except for the deterministic processes, all the conformational changes that produce gating currents follow a Markovian stochastic behavior, then the averaging process generates a kind of weighted histogram of the single gating events. Thus, in the averaged signal, the individual electric events are "smeared" to various degrees and have a multiexponential time course. We suggest that the measured relatively steady current results from numerous fast charge displacements occurring in the lower pathway characterized by state transitions of stochastic nature.

Within the above model in steady state, at the resting potential or during hyperpolarization, the channel molecule fluctuates randomly between various closed states or conformations. These could be states C_{n-1}–C_n and C_{p-1}–to C_{m-1} in the given scheme. It is possible that there are transitions between these two groups of states. Transitions to states C_3 or C_m are rare and, as a consequence, so are channel openings. However when the membrane is depolarized, the probability of the channel to be in the right hand states, C_1 and C_m, increases. Under these conditions, the channel may reach the open state, O. The transition to O can be achieved by two different types of mechanisms and pathways. From state C_m the transition to O (lower pathway) is the regular stochastic, energy-barrier-dependent, conformational change (thin arrows). This process occurs with a specific probability determined by the membrane potential. In contrast, the transition from state C_3 to O (upper pathway) is, at least apparently, deterministic in nature. These transition, denoted by the thick arrows, represent a different type of state transitions. It may represent a continues movement of a charge or a practically infinite series of small charge displacements or transitions. The measured β and γ peaks, time-locked to the channel opening, most probably reflect such transitions (from C_3 to C_2 and from C_2 to C_1, respectively). Once in the O state, the channel may close again by a stochastic transition to C_m, C_{m-1}, etc. It is possible that it may also go to state C_1, etc., by stochastic processes (thin reverse arrows). It is also possible, but unlikely, that a reverse deterministic reaction occurs at the same potential in the steady state. In the case that there is no reverse reaction in the upper pathway, transition must be possible between the two pathways. Note that when the membrane is at a depolarized potential and the channel reaches steady state it resides in C_3–C_1, C_p–C_m, and O groups of states.

A possible interpretation of the results in terms of molecular behavior may be that the channel undergoes stochastic conformational changes which, at random times, removes barriers that prevent the movement of the charges that produce the β transient, and subsequently initiate the opening of the channel. Alternatively, some other mechanism may trigger the said movement. However, the above charge displacement can not be the direct or immediate cause of the channel opening, since the last occurs ~1 ms after the termination of the former. Therefore, we suggest that the charge displacement triggers or enables yet another deterministic process, which takes ~1 ms to complete and eventually leads to the channel opening. This process could, for example, be driven by mechanical "spring-like" molecular forces. The channel opening may change the prevailing electromechanical forces so as to enable resetting of the system.

In summary, new information provided by our data includes: 1) the measurement of single channel charge displacement, previously estimated only on the basis of indirect measurements; 2) the determination of some temporal relationships and kinetics of the channel state transitions by a direct, practically model independent, evaluations; 3) a breakdown of the integrated macroscopic gating current into some of its components; 4) evidence that some of the pre-open molecular events are time-locked to the transition to the open state, i.e., they can be possibly interpreted as, at least partially, deterministic or apparently deterministic in nature; 5) the presentation of a new partial scheme for the gating process that consists of two pathways, one in which deterministic transitions intervene between the stochastic transitions and the open transition, and another of purely stochastic nature.

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