Absence of Effects of Low-Frequency, Low-Amplitude Magnetic Fields on the Properties of Gramicidin A Channels

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ABSTRACT The effects of static and low-frequency magnetic fields on gramicidin A channels have been investigated using bilayer patch clamp recording and a bridge technique capable of detecting 0.3% changes in the conductance of glyceryl monooleate membranes containing many channels. In the bridge technique the conductance was assessed using 10-ms voltage pulses applied at 10 Hz. Measurements were made for LiCl, KCl, and CsCl using magnetic fields of 50, 100, 500, and 5000 μT with the frequency scanned from 10–200 Hz. The combinations of static and low-frequency fields employed include the "cyclotron resonance" conditions at which effects had been predicted to occur. In no case was there any detectable change in conductance when the magnetic fields were applied or changed. Potassium currents through single gramicidin channels have been recorded for patches in which several channels may be open at once. Fields were applied for 2 min periods interleaved with 2 min controls. Methods have been developed to analyze the multichannel records to reveal the amplitude and duration of the channels together with the frequency, depth, and apparent period of flickers. No significant differences were observed between the control and field-exposed recording periods. The peak of the distribution of opening and closing transitions always coincided for fields on and off within the resolution, 0.4%, of the recordings. There are at least two types of flicker, one with typical period less than 0.1 ms, the other with typical period from 0.3–0.8 ms. Most of the latter were not complete closures with the conductance during a flicker 15–20% above the level for a full closure.

INTRODUCTION

After the initial report (Wertheimer and Leeper, 1979) several, but not all, epidemiological surveys have suggested that a small increase in the incidence of certain cancers may be associated with long-term exposure to low-frequency magnetic fields (for references and discussion see Hamnerius, 1992; European Bioelectromagnetics Association, 1992; National Radiological Protection Board, 1992; U.S. Department of Energy, 1992). If low-intensity, low-frequency magnetic fields do affect cell function in the longterm, then there must be some process in the cells that can be affected acutely, at least to a small extent. Because magnetic fields act on moving charges, ion transport across membranes has been considered as a possibility for such a process (Liboff et al., 1987; Liboff, 1992). A particularly influential study proposed that combinations of parallel low-frequency and static magnetic fields satisfying a "cyclotron resonance" condition were able to alter the rate of calcium transport across the external membrane of diatoms (Smith et al., 1987).

There has been considerable reluctance (e.g., see Adair, 1991) to accept that low-frequency (<200 Hz), low-amplitude (~50 μT) magnetic fields can affect the movement of ions across cell membranes. Skepticism arises at least partly because simple direct interaction between the fields and an ion is certain to be too weak. Thus, theories that have been put forward all incorporate features to increase the sensitivity of the responding system, e.g., by invoking cooperative transitions (Adey, 1975, 1981, 1992; Blackman, 1992) or by proposing some form of resonance that allows the response to develop over a period of time (Liboff et al., 1987; Liboff and McLeod, 1988; Liboff, 1992; Blackman, 1992; Chiabrera et al., 1992). In the cyclotron resonance hypothesis as originally proposed, the resonance was held to occur between the fields and individual ions as they are transferred across the membrane. The resonance condition relates the frequency of the varying field, f, the magnetic flux density of the static field. B, and the ratio of charge to mass for the transported ion, q/m.

$$f = \frac{1}{2\pi}\frac{q}{m}B$$

Smith et al. (1987) proposed an effect of the magnetic fields on ion transport to explain the observation that the fields altered the dependence of cell motility on the calcium concentration in the medium. A strong argument for the importance of the magnetic fields per se and the absence of nonfield-related artifact was that merely changing the relative orientation of the low-frequency and static fields to perpendicular completely abolished the effect. Blackman et al. (1985, 1990) agree that combinations of static and low-frequency magnetic fields can affect calcium movements in cells, but in their experiments with chick hemispheres in vitro only perpendicular combinations were effective. Not surprisingly results like these have provoked further work including attempts at replication of the diatom experiments (Reese et al., 1991; Saalman et al., 1992; Parkinson and Sulik, 1992; Davies, et al., 1992); theoretical studies (McLeod and Liboff, 1987; Liboff and McLeod, 1988; Halle,

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1988; Durney, 1992, Galt et al., 1993a), at least one assertion that any such effect is impossible for magnetic field strengths below 50 μT (Adair, 1991, 1992; but see Kirschvink, 1992), and projects like this one aimed at demonstrating the presence or otherwise of a resolvable effect of the fields on transmembrane ion transport in simpler, better defined conditions.

Gramicidin A is a linear pentadecapeptide that forms pores that are selective for monovalent cations. The structure and function of these channels are understood in greater detail than for any other type of channel (Hladky and Haydon, 1972; Hladky, 1988, Woolley and Wallace, 1992). Despite their small size these pores manage to mimic a number of kinetic features that have been observed with more complicated channels formed by large proteins, e.g., concentration-dependent permeability ratios, the anomalous mole fraction effect, and flux ratio exponents greater than 1 (see Hladky and Haydon, 1984; Hladky, 1988), gramicidin was chosen for this study because the process by which an ion passes through the pore is likely to be similar to that in much more complicated systems, but it has the advantages that it is more easily manipulated and has very reproducible properties. A lipid bilayer membrane containing gramicidin A is probably the simplest system that might display effects of magnetic fields on ion transport through pores.

The bridge experiments described in this paper for membranes containing many gramicidin channels are capable of detecting any change in average conductance greater than 0.3% that can develop within 10 ms or that can develop over 2 min while no net current is flowing. No effects were seen. This technique would fail to detect canceling effects on single-channel conductance and the probability of finding a channel open. We also report experiments for a restricted range of field combinations in which the magnetic fields are allowed to act upon the transport process for 2 min at a time. We have measured single-channel amplitude, the frequency of channel formation, channel durations, and the periods and frequency of flickers. Parts of this work have been reported in a brief communication (Wang and Hladky, 1992) or presented as a poster (Wang and Hladky, 1993).

MATERIALS AND METHODS

Materials

Salts (AR grade) were roasted at 500°C to remove organic impurities. Water was distilled in a commercial apparatus (Fisons, Loughborough, U.K.) modified by replacing all tubing containing plasticizers with polytetrafluoroethylene (PTFE) tubing. Solid components that came into contact with the aqueous or lipid solutions were cleaned with a dichromate-sulphuric acid mixture. Lipid bilayers were formed from 20 mM solutions of glyceryl monooleate (Sigma Chemical Co., Poole, U.K.) in either *n*-hexadecane (Puriss, Fluka, Buchs, Switzerland) or *n*-decane (Koch-Light, Haverhill, U.K.). The *n*-alkanes were passed through an alumina column before use to remove polar impurities. The gramicidin A used in the single-channel experiments was a gift of Dr. J. Sandblom. We have observed no differences between this sample and the 25-year-old sample (purified by counter-current distribution, gift of Dr. E. Gross, National Institutes of Health, Bethesda, MD) in normal use in our laboratory, which was used for the multichannel study.

Many-channel recording: the bridge technique

The experimental cell is shown in Fig. 1. The Ag:AgCl electrodes were made from silver wire or silver tube (>99% pure, Goodfellows Metals Ltd., Cambridge, U.K.). Lengths of PTFE tubing (Jencons Scientific, Leighton Buzzard, 0.8 mm inside diameter and 1.5 mm outside diameter) were pushed over the ends of a 1 cm length of the silver tube, and the other end of the short PTFE tube was pushed through a hole in the floor of the PTFE cup. The aqueous solution of the experiment was added by pouring a small quantity into the cup and withdrawing the syringe plunger (250 μl gastight, Hamilton Company, Reno, NV) until the column of solution extended through the silver tube. Black lipid membranes were formed by placing a drop of lipid solution across the end of the PTFE tube and removing the excess lipid solution either by bulging the membrane over the outer edges of the tubing or by wiping with an air bubble on the end of a pipette.

The experimental cell was mounted in a pivoted plastic frame to allow orientation of the membrane for visualization in reflected light. For magnetic field production three pairs of mutually perpendicular Helmholtz coils were secured to the mounting frame (see Fig. 2). Each coil contained 200 turns of 0.5 mm enameled copper wire (RS Components, Corby, U.K.) and was covered with copper foil tape wound in a spiral. The foil was required to reduce the electrostatic coupling between the coils and the rest of the experimental system. The currents driven by the electromotive force produced in the recording loop by the applied low-frequency magnetic fields were not detectable in the bridge measurements. (These currents do, however, preclude the use of this setup for the measurement of single-channel currents).

Each pair of coils could be driven by an arbitrary linear combination of a constant current and a current at the frequency of the experiment using a locally constructed controlled current source indicated schematically in Fig. 3. The current through the coils was very nearly the command potential divided by 1 Ω. (For frequencies above 1 kHz the impedance of the wire-wound resistor increased as a result of its inductance. Below 200 Hz this error was less than 1%.) Each circuit could produce $\pm$4 A at 25 V.

The magnetic fields produced by the coils were calibrated using a three-axis fluxgate magnetometer with output voltage of 10 V for 100 μT (Bartington Instruments, Oxford, U.K.). The output for each axis could be monitored on an oscilloscope or on a digital meter constructed to read separately the average of the output and the rms amplitude of the low-frequency signal (7.07 V for 100 μT mean to peak, 3% attenuation at 10 Hz). All values of varying fields are stated as mean to the peak. With no currents in the coils the ambient constant-field at the site of the membrane was ~45 μT and the ambient field at 50 Hz was <0.05 μT. Each axis of the magnetometer head was repeatedly centered at the position occupied by the membrane during an experiment, and the steady currents through the coils were adjusted until the components of the static field in all three directions were zero. Repeated determinations revealed an uncertainty in the dial settings for the nulled state corresponding to 5 μT. All applied fields were relative to this nulled state. The fields were proportional to the driving currents, and for frequencies up to 200 Hz they were also proportional to the dial settings of the potentiometers used to vary the size of the command voltages. A small test coil was used to confirm that the fields increased linearly (within 1%) with the applied current up to 5 mT for frequencies from 10 to 1000 Hz. Low-frequency fields were applied to one or occasionally two pairs of coils as indicated in Table 1.

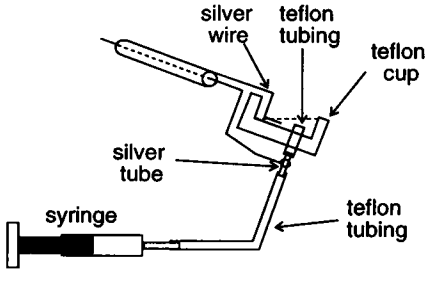

FIGURE 1 Bilayer cell used in the bridge experiments.

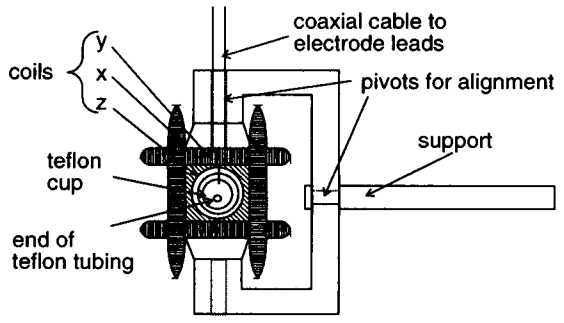

FIGURE 2 Coils and cell mounting. The three pairs of coils have internal diameters of 2.54, 4.1 and 6.2 cm, respectively. A disposable syringe barrel sheathes the coaxial cable leading to the electrode leads and serves as one of the pivots.

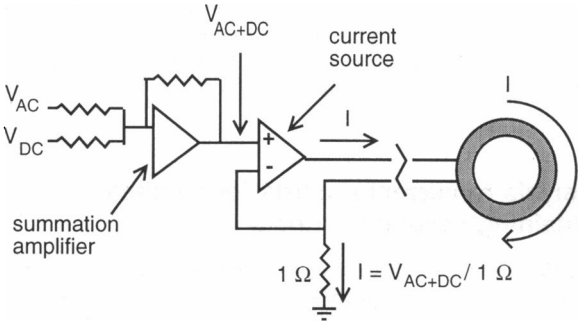

FIGURE 3 Schematic diagram of the current source used to drive on a pair of coils. A common power supply is used to drive three such circuits. The AC signal is obtained from a Wayne-Kerr Audio Frequency oscillator. The DC signal is set using potentiometers driven from the power supply.

Sufficient gramicidin A was added to the front aqueous solution to bring the membrane conductance into the range from 10^{-7} to 10^{-6} S (roughly 10^4 pores). The current across the membrane was measured using a bridge circuit (Hainsworth and Hladky, 1987) (see Fig. 4) with a standard virtual earth circuit as the null detector. (It was not necessary to use an integrator as in the earlier work because for permeation concentrations of 0.1 M and higher, the ionic currents can be chosen to be much greater than the capacitative currents but still much less than the limits imposed by diffusion polarization.) In the "unknown" arm of the bridge applied pulses are attenuated 10-fold and applied to one side of the membrane. The other side of the membrane is connected to the virtual earth. In the "known" arm of the circuit the potential pulse is inverted and applied to a 10-turn adjustable potentiometer. The variable potential from this potentiometer is used to force a current through a known resistor into the virtual earth. Most of the time the potential applied to this circuit was 0. Ten times per s, 75 mV, 10 ms pulses were applied to the bridge (see Fig. 5) to assess the membrane conductance.

Each experiment started with the magnetic fields nulled. The "known" current was adjusted until it was equal and opposite to that through the membrane, giving an output such as that in the third trace of Fig. 5. This setting established the baseline. The magnetic fields were then applied and any change in the output of the detector noted. Under the conditions of the survey, the resolution was typically 0.2%. The fields were then turned off and the output noted to see if there had been any drift in the conductance. In practice it was found that the drift was sufficiently slow, except immediately after an addition of gramicidin, that there was not perceptible change in conductance on return to the reference conditions. The frequency of the varying magnetic field was scanned from 10 to 200 Hz over about 2 min.

Control experiments with no gramicidin present demonstrated that the leak or background currents were always at least 1000 times smaller than those recorded in these experiments. The currents were also always at least 100 times smaller than those observed in the absence of a membrane, and there was no observable decrease or droop in the current during the 10-ms pulses.

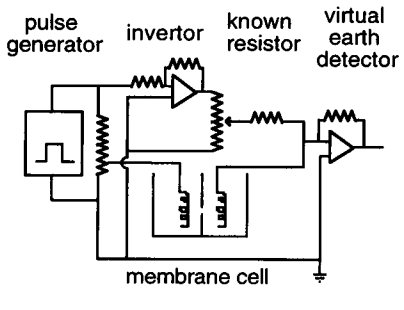

FIGURE 4 The bridge circuit used to measure the current across the membrane. The membrane current is compared with an adjustable reference current. The virtual earth circuit holds at 0 the potential at its input, which is connected to one electrode and one end of the known resistor. The output of the detector is proportional to the difference between the experimental and reference currents.

Single-channel recording

Bath and electrode solutions were unbuffered 1 M potassium chloride (pH ~5.6). Currents were recorded using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). The apparatus (see Fig. 6) and methods were similar to those introduced by Anderson (1983) and developed by E. Neher (Sigworth et al., 1987; Sigworth and Shenkel, 1988). A length of Teflon tubing was attached to a syringe and clamped in position with the opening of the free end pointing slightly upward just above the floor of a plastic culture dish. The dish and the tubing were filled with an aqueous solution, and a lipid bilayer is formed across the end of the Teflon tube and viewed using a TMS phase contrast inverted microscope (Nikon Corporation, Tokyo, Japan) with a 10× phase contrast objective. The amplifier headstage, the pipette holder with side arm (HL-1–12, Axon Instruments), and the pipette were mounted on a mechanical micromanipulator (MX-1, Narishige Scientific Instrument Laboratory, Tokyo, Japan) tilted to 45°. The microscope and the manipulator were firmly attached to the base plate of an AVT 700 air table (Wentworth Laboratories Ltd., Bedford, U.K.).

Patch pipettes were pulled from 1.2 mm outside diameter filamented borosilicate glass capillaries (GC120F-10; Clark Electromedical Instruments, Pangbourne Reading, U.K.) using a PP-83 puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and heat polished by bringing the

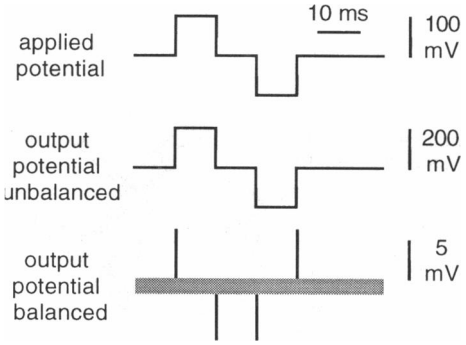

FIGURE 5 Diagram of the applied potential used to measure the membrane current and the output traces from the null detector before and after balancing the reference arm of the bridge. As indicated a membrane current that produces 200 mV output when unbalanced can be balanced so that changes of less than 0.4 mV can be detected repeatedly.

pipette tip close to a ~0.4 mm diameter glass bead on a glowing 0.1 mm platinum wire. The pipette shanks were bent to an angle of about 45° by heating in the flame of a methanol burner so that in the final position the axis of the pipette tip was nearly vertical. After fabrication the pipettes were silanized. The pipettes rested tip upward in holes drilled through an aluminum block raised on feet so that vapor could enter the base of the pipettes. The block was placed in a covered crystalizing dish on a hot plate (Corning PC-35) and the plate turned on full for 5 min to drive off any water vapor. The dish was then removed and allowed to cool until it could be touched. Several drops of 10% (v/v) dimethyldichlorosilane (Sigma Chemical Co.) in chloroform (AR grade) were spotted around the base of the dish, and the dish with the cover replaced was put on the hot plate, which was then turned back on for 3–5 min. The dish was then removed and allowed to cool with the cover shifted to allow vapor to escape. Use of more concentrated solutions of dimethyldichlorosilane frequently produced clogged electrodes. Insufficiently silanized electrodes usually broke the large bilayer.

Pipettes were filled with 1 M KCl, which was filtered through a 0.22-μm filter (Millex GS, Millipore SA, Molsheim, France). Pipette resistance was about 4 MΩ. A silver wire was used to make electrical contact with the solution in the pipette; the reference electrode was a partially sheathed Ag/AgCl pellet. Single-channel currents were observed on an oscilloscope and recorded onto digital audio tapes using a modified Sony DTC-1000ES DAT recorder (Fentronics, Haddenham, U.K. CB6 3PR). Subsequently the records were played back via an eight-pole Bessel filter (902LPF2, Frequency Devices, Haverhill, MA) set at 5 kHz, sampled at twice this frequency, and stored directly on hard disk using a CED 1401 interface (Cambridge Electronic Design, Cambridge, U.K.) controlled using the Continuous Sampling module of the CED Patch 5.5 software. One trace was filtered at 2 kHz and sampled at 5 kHz to allow registration of a larger number of channels.

The lipid bilayer could be positioned easily by adjusting the syringe. Either the pipette or the bilayer was advanced until the pipette tip crossed the bilayer, pressure was applied to clear the pipette tip, and the pipette or the membrane was withdrawn until the tip with a patch attached was back in the bath.

As shown in Fig. 6, three mutually perpendicular coils were positioned in slots cut into the plastic stage of the microscope so that the coil axes intersected on the axis of the objective just above the bottom of a plastic culture dish. The maximum size of the coils was dictated by the size of the stage; the orientation was chosen to avoid hitting the objective turret. Single coils were acceptable because it was only necessary to control the magnetic fields over a small region, less than 1 mm on a side. Each coil contained 200 turns of 0.5 mm enameled copper wire (RS Components, Corby, U.K.), and was covered with a layer of copper foil tape, a layer of insulating tape, and a second layer of the copper tape. The artifact produced by electrostatic coupling between a coil and the recording circuit was assessed by applying a 25-V pp sinusoidal voltage relative to earth to both leads of the coil. The electrostatic artifact was made undetectable by earthing the inner foil to the current source and the outer to the headstage of the recording circuit. Electromagnetic coupling produced by the component of the varying magnetic field perpendicular to the recording loop was minimized by careful arrangement of the lead to the reference electrode. With this care the magnetic coupling artifact was also too small to see in the experimental traces.

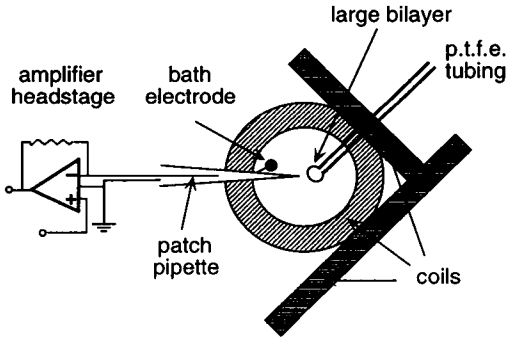

FIGURE 6 Top view of the arrangement of the large bilayer, the patch pipette, the coils, and the reference electrode. Each coil was driven from an independent current source.

The magnetic fields at the position to be occupied by the cell (with the objective in position) were measured using a probe constructed from a Hall effect integrated circuit (304–267, Radiospares, Corby, U.K.). To obtain the null condition the center of the chip was located at the position to be occupied by the pipette tip during recording. The currents in the coils were then adjusted until inverting the chip produced no change in the output signal. The current settings required to produce 100 μT fields (AC mean to peak and DC) imposed on top of this null condition were also measured with the Hall probe, which was calibrated using the Helmholtz coils of the bridge apparatus. Fields of different strengths were produced by proportional changes in the current through the coils. These indirect procedures were used because the head of the fluxgate magnetometer was too large for its sensitive area to be positioned correctly above the objective.

With no currents in the coils, the constant field at the site of the membrane was about 45 μT, whereas the ambient 50 Hz field was undetectable with the Hall probe. The field at 50 Hz measured nearby with the fluxgate magnetometer was less than 0.05 μT. Repeated determinations revealed a maximum uncertainty in the current settings for the nulled state of the constant field corresponding to 5 μT. All amplitudes of low-frequency fields are stated as mean to peak.

Analysis of current records for patches containing several channels

Examples of current vs. time records for channels in membranes made from glyceryl monooleate with *n*-hexadecane or *n*-decane are shown in Figs. 7 and 8. The brief decreases in conductance that are visible in the records are called flickers.

Accurate comparison of single-channel properties with fields on and off required registration of data for the largest possible number of channels (to achieve the best statistical accuracy) in the shortest possible time (to avoid drift in properties with time), which could only be reconciled by developing procedures for the analysis of multichannel records. The records were analyzed offline in stages. The CED Patch 5.5 software was used to construct a Cambridge File System results file, which describes an idealized trace. By inspection of the data a baseline and six equally spaced thresholds were set defining the closed state and six open levels of increasing conductance. Transitions between levels were recognized as threshold crossings. The period of a result is the time between two consecutive crossings, and its amplitude is the average of the current measurements during the period. The CED software allows visual comparison of the results file with the data and manual editing of the results (e.g., to delete the switching transient from our drying oven). The results file can also be edited using programs written in Pascal or C.

Distributions of channel conductances for gramicidin (e.g., Figs. 9 and 10) clearly show a main peak but there are also openings of nonstandard size (Hladky and Haydon, 1972; Busath and Szabo, 1981). A nonstandard opening is prominent in Fig. 7, and two nonstandard levels that occurred as partial closures or long-lived flickers are marked *a* and *c* in Fig. 8. When a nonstandard conductance falls on top of a preset threshold, the CED system produced a long string of small repeated transitions between levels. These were edited out of the record by a program that merges three successive results whenever the first and third are at the same level, the amplitude of the second differs from the first by less than some tolerance value (here set equal to the threshold), and the period of the second is less than some assigned value (here set to 10 ms). The new merged result can be the first result for the next merge. A merged result in the "dejittered" output file has a period that extends from the start of the first to the end of the last result included in a chain of merges and an amplitude set equal to the average of all the data points during the merged period. (Although the editing procedure is effective at removing the "jitters," one consequence remains. The CED software imposes a limit of 32,000 results in a single file. At 10 kHz, "jittering" can fill a results file in only 3 s. Fortunately in practice the limit was usually longer than 4 min.) The procedure was evaluated by superimposition

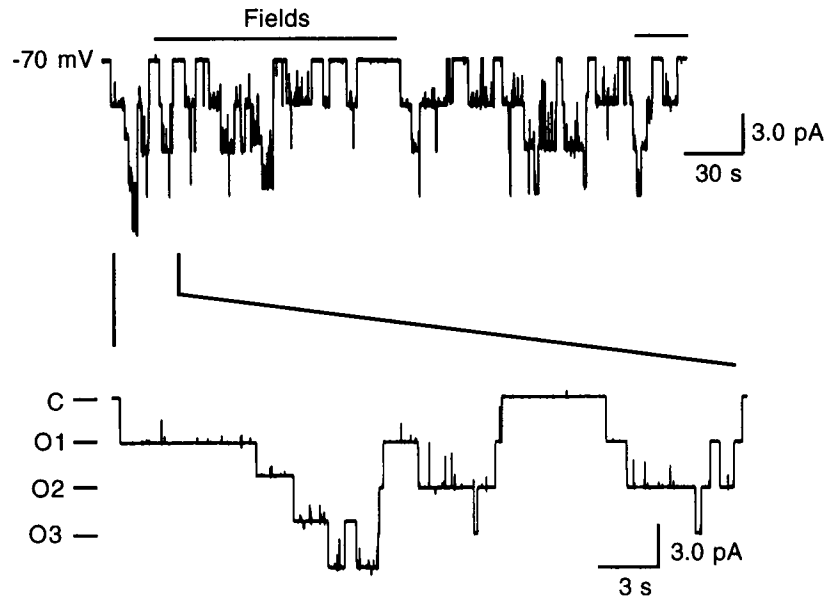

FIGURE 7 Current vs. time record for gramicidin channels in patches containing *n*-hexadecane. Trace obtained by playback of the taped trace through a Gould 2200-S pen recorder (effective bandwidth ~100 Hz). Note the occurrence of brief interruptions. In the lower trace note the nonstandard opening, second from the left, from level 1 to level 2 that pairs with the second closure from level 4 to level 3, which in turn is immediately followed by a closure from level 3 to level 2.

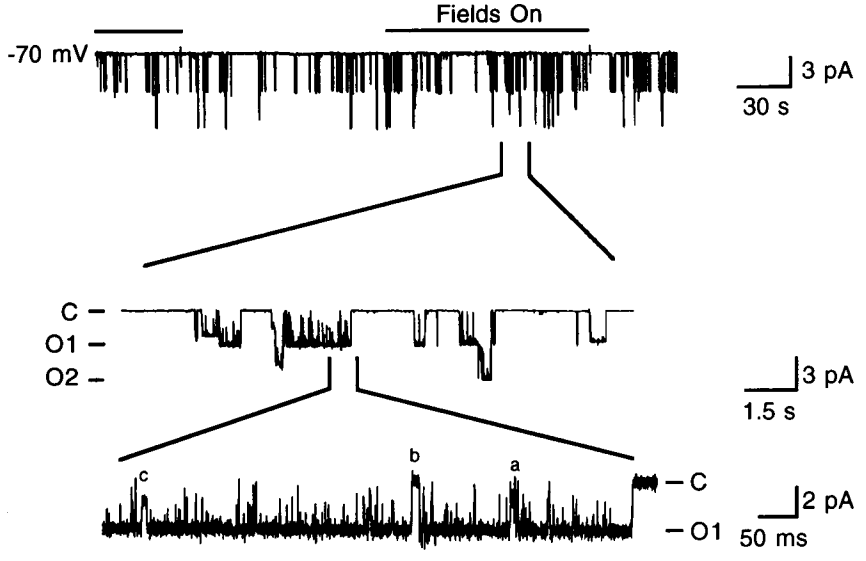

FIGURE 8 Current vs. time trace for gramicidin in a patch containing *n*-decane. The top traces were produced using the Gould recorder (see Fig. 7); the bottom trace is the plotter output of the digitized trace produced by the CED software. The "noise width" of the trace is the envelope of the signal. The width of this trace is typical. *a* and *c* mark relatively long-lived partial closures of a channel, *b* probably a full, temporary closure of a channel (see text). In this figure time advances from right to left.

of the new results file and the original data. For gramicidin channels and our recording conditions, the edited file appears to be a faithful description with three exceptions. The half-height threshold misses openings of very small conductance channels (Busath and Szabo, 1981), many flickers that persist for less than about 100 μs are missed because they do not cross a threshold, and occasional shoulders on openings and closures (Sigworth et al., 1987; Sigworth and Shenkel, 1988) are taken to be part of one of the neighboring levels. Openings of less than half full size occurred in our traces, but rarely; typically only one or two in a period during which more than 100 openings occur.

Some criterion must be adopted to distinguish channel closing from the onset of a flicker. Preliminary inspection of closed time histograms revealed that transitions from level 1 to level 0 fall into two easily distinguished populations. The first are followed by long-lived closed times that depend on the number of channels. These correspond to the breakup of a channel. The amount of gramicidin added was adjusted so that these times were usually much longer than 100 ms. The second population was followed by much shorter closed times, usually <1 ms. Because the gaps between channels were so much longer than the flickers, the latter could be recognized simply as a decrease in conductance level followed by a return to the original level within 10 ms.

Auxiliary programs were used to process the results file to calculate the average amplitude of the full-conductance state, the number of channel openings, the channel duration (equivalent to the burst duration for permanently formed channels), and the periods and frequency of the flickers. The average full-conductance amplitude between successive opening or closing events was found by merging the levels across the flickers such that the period of the merged level runs from the start of the first to the end of the last and the amplitude is the time-weighted average excluding the period during the flickers. (When several channels are open simultaneously, there is a possibility of overlapping flickers. These were rare and could be eliminated by multiple passes of the program for merging levels.) The merged levels correspond within the width of the idealized trace line on the screen to the levels that would be drawn by eye. The full conductance of a channel is then determined as half the separation of the opening and closing peaks on a histogram of the amplitudes of the opening and closing events, i.e., the changes in level that are not the onset or end of flickers (see Figs. 9 and 10).

The average number of channels open, n, is calculated as

$$n = (\text{level} \times \text{level period})/T$$

where T is the total duration of the record. The average channel duration or

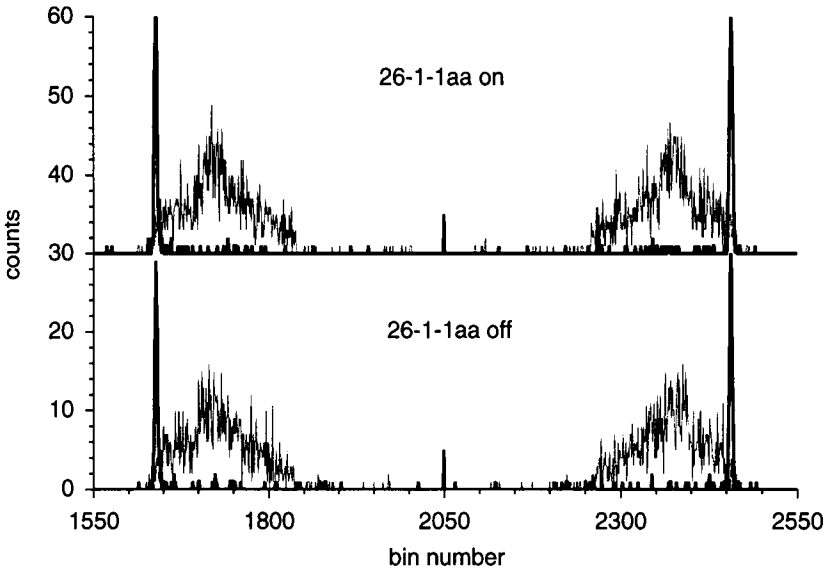

FIGURE 9 Comparison of transition amplitude histograms with fields on and off. The bin number indicates the size of the transition between two levels, (2049 = 0 pA, 1550 = −3.852 pA, 2550 = 3.868 pA). The heavy trace is for channel openings and closings; the light trace is for flickers onsets and ends. Increases in conductance, i.e., openings and the ends of flickers, are shown to the right of center and decreases to the left. Note the sharpness of the peaks for opening and closing transitions. The position of these peaks is the same for fields on or off. Similarly no difference is apparent in the distribution of flicker amplitudes. (Decane-containing membrane, −70 mV, 254 μT static and 254 μT, 50 Hz both vertical).

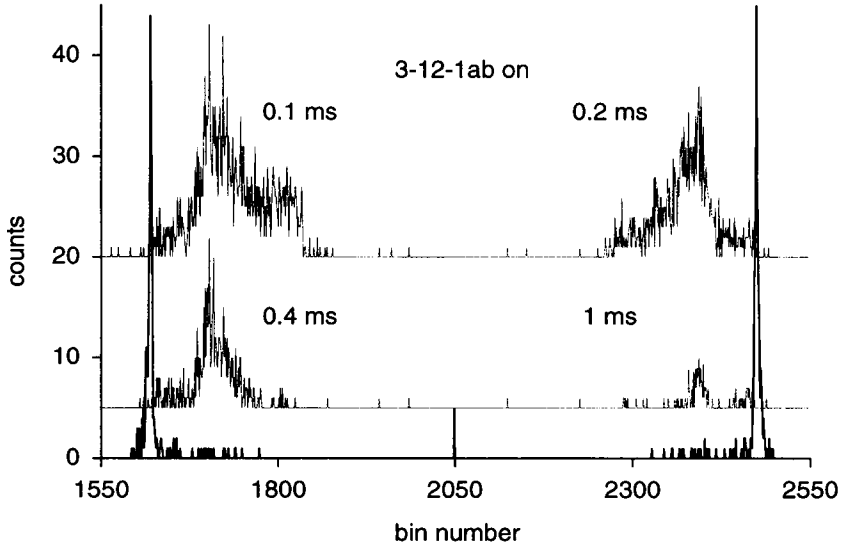

FIGURE 10 Distribution of flickers with periods between the minimum shown and 10 ms. The sharp peaks and lowest baseline display the statistics for openings and closings (cf. Fig. 4). Only half of each flicker trace is shown, closures and flicker starts for 0.1 and 0.4 ms, openings and flicker ends for 0.2 and 1 ms. The baselines are shifted upward by five counts and 20 counts. The prominent hump toward 0 pA in the 0.1 ms distribution reflects flickers that had not reached full size at the time of the sample. The position of the peak is the same for all flickers and those that are longer-lived, and thus this position is not affected by the filtering. In this figure the mode of the flicker depth distribution is 81% of a full closure. (2049 = 0 pA, 1550 = −3.852 pA, 2550 = 3.868 pA. 70 mV applied potential, 127 μT static and 127 μT, 50 Hz, both vertical).

lifetime, τ, is then calculated using the relation

$$N \times \tau = n \times T$$

The flicker frequency reported in Table 1 is calculated as the number of flickers observed with periods less than 10 ms divided by the average number of channels open and the duration of the data sample. (The number of flickers calculated from curves fitted to flicker-period histograms as in Fig. 11 was usually about 70% of the number obtained by direct counting. (see Table 2) Direct counting must be below the correct value, as many rapid flickers have been missed.) A separate tabulation verified (data not shown) that the flicker rate when two channels are open is double that when only one is open.

RESULTS

Many-channel conductances

A variety of steady and varying fields were tried for cesium, lithium, and potassium as the permeant ions. As indicated in Table 1 we used field strengths of 50, 100, 500, and 5000 μT applied perpendicular or parallel to the membrane and perpendicular or parallel to each other. For each of the specified conditions responses were sought by slowly varying the frequency from 10 to 200 Hz. In no case was there any detectable deviation of the output of the null detector when the magnetic fields were applied or changed.

Single-channel properties

Examples of experimental records are given in Figs. 7 and 8 and histograms of the amplitudes of channel openings and closings, and the sizes of the onset and end of flickers are shown in Figs. 9 and 10. The channel amplitude, the average channel duration, and the frequency, depth, and apparent period of the flickers are listed in Tables 3 and 4.

Channel properties in the absence or presence of applied fields

For gramicidin the conducting unit is almost certainly a dimer (for reviews see Hladky and Haydon, 1984; Woolley and Wallace, 1992). Channel formation coincides with

TABLE 1 List of field combinations and salt concentrations

The following combinations were used for CsCl at 0.1 M and 1 M, LiCl at 0.1 M, 1 M, and 3 M, and for KCl at 1 M and 3 M.

A	B	A	B
C_z at 50, 100, or 500	none	L_z at 50, 100, or 500	C_y at 50, 100, or 500
L_z at 50, 100, or 500	none	L_z at 50, 100, or 500	L_y at 50, 100, or 500
C_z and L_z at 50, 100, or 500	none	L_z at 50, 100, or 500	C_y and L_y at 50, 100, or 500
C_z at 50	L_z at 100 or 500	L_y at 50, 100, or 500	none
C_z and L_z at 50, 100, or 500	L_y at 50, 100, or 500	C_y and L_y at 50, 100, or 500	none
C_y at 50	L_y at 100, 250, or 500	C_x and L_y at 50, 100, or 500	none
C_x at 50	L_y at 100, 250, 500		

The following combinations were used for CsCl at 0.1 M and 1 M, LiCl at 3 M, and KCl at 3 M.

A	B	A	B
C_z at 1000 or 5000	none	C_z at 100	L_z at 5000
L_z at 1000 or 5000	none	C_y at 1000 or 5000	none
C_z and L_z at 1000 or 5000	none	L_y at 1000 or 5000	none
L_z at 100, 500, 1000, or 5000	C_y at 100, 500, 1000, or 5000	C_y and L_y at 1000,or 5000	none
C_y at 100	L_y at 5000	C_y at 500 or 5000	L_y at 100
C_z at 500 or 5000	L_z at 100	L_y at 100, 500, 1000, or 5000	C_x at 100, 500, 1000, or 5000
L_y at 100, 500, 1000, or 5000	C_z at 100, 500, 1000, or 5000		

Each experiment combines one selection of field from column A with one selection from the adjacent column B. All fields not stated were nulled. All possible combinations indicated have been investigated. The type and orientation of the fields are indicated by the following symbols.
C = constant field in μT; L = low-frequency field, mean to peak in μT; "C and L" means that C and L each took on the same stated value; x = field parallel to the membrane and perpendicular to the recording circuit loop; y = field parallel to the membrane and to the recording circuit loop; z = field perpendicular to the membrane and parallel to the recording circuit loop.

dimerization and channel closure with the dimer dissociation. In all cases the conductance change when a channel opens or closes was sharply defined (see Figs. 9 and 10). With membranes made with *n*-hexadecane as solvent, channel durations were between 3 and 7, and flicker frequencies ranged between 1.5 and 6.5 (flickers crossing a threshold and lasting for less than 10 ms). With *n*-decane the channel duration was shorter (200 ms to 5 s) and the flicker frequency higher (2–60 s^{-1} $channel^{-1}$). There was a pronounced variation in properties between patches.[1]

In both types of lipid bilayer the recorded flickers were not complete closures (see Figs. 9 and 10); the conductance during a flicker remained 15–20% above the level for a full closure as reported previously by Sigworth and colleagues (Sigworth et al., 1987; Sigworth and Shenkel, 1988). As shown by the two examples in Fig. 11, histograms of flicker periods could not be fitted accurately with a single exponential. In all cases there were substantially more brief flickers (even though the brief flickers were more likely to fail to reach a threshold). There was also an excess of longer events that were not full closures (such as those flagged *a* and *c* in Fig. 10). However, because data sampled at 10 kHz are not adequate to characterize the faster events, the best-fit (see legend to Fig. 11) single exponential time constant, the "apparent flicker period," has been used to compare the observations with fields on and off. The observed range, 0.3–0.8 ms, and the depth of the flickers are both consistent with the properties of the slower component of flickers observed for gramicidin in diphytanoyl phosphatidylcholine + *n*-decane membranes by Sigworth and Shenkel (1988). The flickers we observed are also similar to those reported by Ring (1986) for gramicidin in glyceryl monooleate membranes. Fig. 8 displays an example of an apparently full closure with a duration of ~10 ms. These full closures were rare.

Lack of observed effect of the applied magnetic fields

Channel properties were compared in the presence and absence of fields by collecting data continuously with the fields off (i.e., nulled) or on for alternating 2 min periods. The dejittered results file was split into corresponding segments for determination of the channel properties.

Figs. 9 and 10 show examples of histograms of the amplitudes of the transitions between levels with fields on (upper traces) and off (lower traces). In all cases the peak positions for fields on or off were either in the same bin or in adjacent bins of the histograms (see Table 3), corresponding to a maximum difference of 0.4% in an individual experiment. The relative difference in the "apparent flicker period," ((on-off)/off) × 100, was 1.8% ± 4.3% (mean ± SEM) for membranes formed with *n*-decane, and 1.4% ± 3.6%

[1] The variability in channel formation rate, channel duration, and flicker frequency may be related to changes in the solvent content of the patches (cf. Ring, 1986; Sigworth et al., 1987). Planar membranes made from glyceryl monooleate with *n*-decane are known to be thicker and to contain more solvent than those made with *n*-hexadecane (Andrews et al., 1970). In the former the channel duration is shorter (Hladky and Haydon, 1972; Elliott et al., 1983), whereas the flicker frequency is higher (Ring, 1986). When a membrane patch is formed (cf. Sigworth et al., 1987; Sigworth and Shenkel, 1988) solvent may be able to escape onto the surface of the silanized electrode, and once the pipette tip is withdrawn into the bath solution the solvent can no longer be replenished from the large reservoir of the lipid-hydrocarbon solution. The thickness of the membrane patch will then depend on the amount of solvent remaining, which may be poorly reproducible. The data displayed in Figs. 8 and 10 report data for the *n*-decane-containing patch with the shortest channel duration (~200 ms) and presumably the largest amount of solvent.

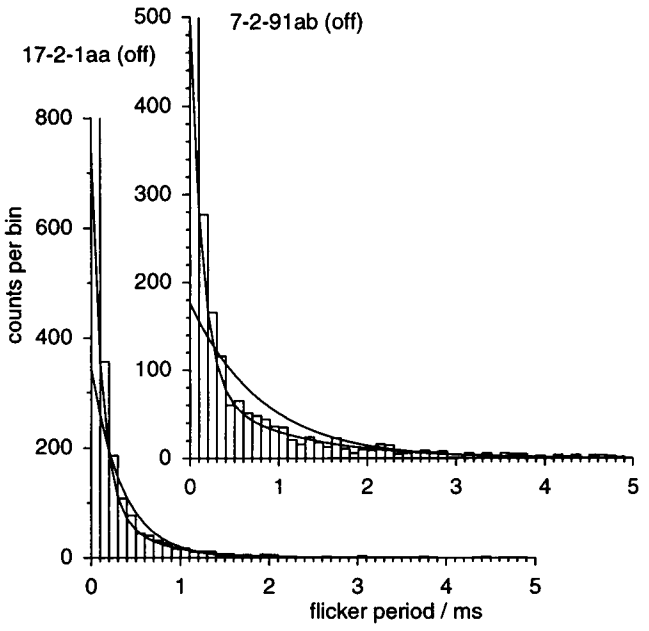

FIGURE 11 Histograms of flicker periods (experiments 17-2-1aa, hexadecane; 7-2-91ab, decane). A results file was created in which all decreases in level next followed by an increase to the original level within 10 ms (100 samples) are called flickers. The curves are least-sum-of-squares fits to all flickers persisting for at least two samples. The data clearly display at least two exponential components. The initial bin ($0 < t \leq 0.1$ ms) contains 1250 counts in 17-2-1aa and 770 counts for 7-2-91ab. The fitted parameters used for the curves are shown in Table 2.

TABLE 2 Fitted parameters used for curves in Fig. 11

		First component		Second component	
Dataset	Experiment no.	Maximum counts	Time constant (ms)	Maximum counts	Time constant (ms)
17–2-laa	1	344	0.35		
	2	624	0.12	109	0.52
7–2–9lab	1	176	0.80		
	2	422	0.14	78	1.04

for those formed with *n*-hexadecane. For the flicker frequency the comparable figures are $-1.4\% \pm 1.5\%$ and $0.59\% \pm 1.8\%$. For the channel duration the figures are $3.1\% \pm 5.5\%$ and $8.5 \pm 6.9\%$. None of these values are significantly different from 0.

DISCUSSION

In the "cyclotron resonance" model that was used to interpret the diatom results (Smith et al., 1987) it was envisaged that magnetic fields act directly on the permeating ions to alter their trajectories, presumably by imposing some tendency toward helical motion. Gramicidin was suggested as a suitable experimental system to test the model partly because the atoms that coordinate an ion during its passage through the pore are arranged in a helical fashion. Furthermore, based on theoretical simulations (Skerra and Brickman, 1987) it has been proposed that ion trajectories in the channel are helical. However, direct interaction of the fields and the permeating ions, as envisaged in the resonance hypothesis, is unlikely. The ion moves in a viscous medium that tends to destroy the coherent motion required for resonance. Furthermore the time that any one ion remains within a pore, less than 1 μs, is much smaller than the tens of milliseconds required for the development of a resonance effect (Liboff and McLeod, 1988; Halle, 1988; Durney et al., 1988; Galt et al., 1993b). Partly for this reason, both our study and the independent investigations by Galt and colleagues (Galt, 1990; Galt et al. (1993b) were designed to look for effects whether they occur as a result of a change in the current through open channels or the probability of finding the channels open.

Galt and coworkers (Galt, 1990; Galt et al., 1993b) compared the conductance through multichannel membranes measured with two out of the three of the static field amplitude, the low-frequency field amplitude, and the frequency held constant, and the third varied over a range of values. The swept parameter was increased 10-fold over a 10 s period, each time starting from the value predicted by the "cyclotron resonance" model. Two hundred such repetitions were averaged to see if there were any changes in the conductance. They excluded any variations greater than about 4% for 0.1 M KCl.

In the many channel recordings we have used a bridge circuit (Hainsworth and Hladky, 1987) to allow rapid, accurate measurements. The accuracy with which changes in the current across a membrane containing many gramicidin channels can be measured is ultimately limited by the drift in the conductance that occurs whether or not magnetic fields are applied. The effects of this drift can be reduced either by taking repetitive measurements in such an order that the effect of drift can be distinguished from that of the fields (Galt, 1990; Galt et al., 1993b) or by comparing the current in the presence of the fields with that in their absence within a sufficiently short period of time that the drift is negligible.

The many channel results in this paper show that for frequencies between 10 and 200 Hz and for magnetic field strengths up to 5 mT, the wide range of combinations of low-frequency and static magnetic fields indicated in Table 1 have no observable effect on the product of the number of channels open and the single channel conductance as assessed by the membrane conductance measured at 100 μs intervals. Furthermore, there is no change in this product during the 10 ms pulses while the magnetic fields and a 75 mV electrical potential are applied simultaneously. This time is sufficient to allow more than 10^5 ions to pass through each pore. A systematic change of about 0.3% would have been seen.

Galt and colleagues (Galt, 1990; Galt et al., 1993b) also investigated the effect of "cyclotron resonance" fields on single-channel properties of gramicidin in glyceryl monooleate membranes. They reported relative differences between properties in the presence and absence of fields (K^+

TABLE 3 Measured channel properties for patches formed from membranes containing *n*-hexadecane; 70 mV applied potential, 1 M KCl, 50 Hz AC field; filtered at 5 kHz, sampled at 10 kHz

		Channel properties			Flickers		Flicker distribution (single exponential)	
Experiment	Fields	Transition amplitude (pA)	Channel duration (s)	Opening rate (s^{-1})	Flicker frequency (s^{-1})	Flicker depth (pA)	Initial counts	τ (ms)
2311-laa	127 μT DC and AC Vert.	2.84	4.64	0.101	2.16	2.28	305	0.34
	nulled	2.84	3.74	0.101	2.05	2.28	319	0.34
3–12-lab	127 μT DC and AC Vert.	3.29	2.64	0.394	1.77	2.67	248	0.34
	nulled	3.28	2.59	0.371	1.65	2.61	245	0.39
2–12-laa	127 μT DC and AC Vert.	3.29	2.81	0.419	2.11	2.68	120	0.40
	nulled	3.29	3.02	0.443	2.16	2.64	183	0.36
4–12-laa	127 μT DC and AC Vert.	3.50	5.61	0.269	1.23	2.91	140	0.57
	nulled	3.51	5.54	0.261	1.3	2.92	148	0.61
17–2-laa	127 μT DC Hor., AC Vert.	3.33	6.99	0.181	5.63	2.67	427	0.37
	nulled	3.33	7.93	0.247	5.43	2.71	344	0.35
17–2-lab	127 μT DC Hor., AC Vert.	3.28	9.53	0.319	6.1	2.59	400	0.38
	nulled	3.3	6.81	0.324	6.24	2.3	290	0.33
17–2-lac	127 μT DC Hor., AC Vert.	3.23	6.75	0.376	6.16	2.63	842	0.41
	nulled	3.23	6.06	0.392	6.3	2.66	782	0.41

TABLE 4 Measured channel properties for patches formed from membranes containing *n*-decane; 70 mV applied potential, 1 M KCl, 50 Hz AC field; filtered at 5 kHz, sampled at 10 kHz

		Channel properties			Flickers		Flicker distribution (single exponential)	
Experiment	Fields	Transition amplitude (pA)	Channel duration (s)	Opening rate (s^{-1})	Flicker frequency /s^{-1}	Flicker depth /pA	Initial counts	τ (ms)
26–1-laa	254 μT, DC and AC Vert.	3.16	2.48	0.260	5.23	2.54	456	0.34
	nulled	3.16	2.58	0.218	5.43	2.56	438	0.33
26–1-lac*	359 μT, DC and AC Vert.	3.20	3.31	0.219	1.96	2.58	408	0.34
	nulled	3.20	3.56	0.208	2.02	2.62	413	0.33
26–1-lad	508 μT, DC and AC Vert.	3.11	3.30	0.301	11.1	2.51	803	0.33
	nulled	3.11	2.49	0.348	10.6	2.48	817	0.31
27–1-laa	254 μT, DC and AC Vert.	3.09	5.71	0.159	1.67	2.49	257	0.44
	nulled	3.08	5.48	0.155	1.61	2.48	233	0.47
27–1-lab	508 μT, DC and AC Vert.	3.06	5.13	0.176	1.59	2.47	201	0.4
	nulled	3.06	4.69	0.175	1.65	2.48	189	0.41
27–1-lac	508 μT, DC and AC Vert.	3.20	3.31	0.219	1.96	2.58	113	0.49
	nulled	3.20	3.56	0.208	2.02	2.62	173	0.4
7–2–9lab	254 μT DC and AC Hor.	3.11	0.179	1.18	58.3	2.29	147	0.69
	nulled	3.12	0.191	1.47	61.2	2.12	176	0.8
7–2–9lba‡	254 μT DC and AC Hor.	3.12	0.224	1.05				
	nulled	3.12	0.254	1.16				

* Experiment at 90 mV.

‡ Filtered at 2 kHz and sampled at 5 kHz. 7–2–9lab is the first 4 min of this trace.

ions, 50 μT, 20 Hz fields) as follows: single-channel conductance ((on-off))/off, mean ± SEM), 0.5 ± 0.7%; channel duration, 0.7 ± 5.0%; and formation rate, 1.7 ± 6.5%. None of these are significantly different from 0. The much larger scatters seen for the duration and the formation rate reflected a marked drift in properties with time. Our bilayer patch clamp experiments confirm and extend their results.

Low-frequency magnetic fields including those chosen to satisfy the proposed cyclotron resonance condition for the bare potassium ion (127 μT at 50 Hz) had no detectable effects on the rate of K^+ ion transport through individual gramicidin channels. As the observed differences in individual experiments never exceeded the resolution of the equipment (0.4%), the difference is yet smaller. Thus the magnetic fields had no detectable effect on the ion flow through a channel within the 2 min periods during which the fields were applied. This result at the single-channel level and the absence of effect on the averaged current through many channels during 10 ms voltage pulses (maximum effect <0.3%) observed in the bridge experiments establish that these fields also had no detectable effect (i.e., <0.3%) on the equilibrium between conducting and nonconducting forms of gramicidin in the membrane at 0 applied voltage. The one remaining possibility (for gramicidin A, K^+, and glyceryl monooleate membranes) is that magnetic fields interact with the consequences of an applied voltage or the ion flow and

by this interaction alter channel gating in some manner that takes substantially longer than 10 ms to develop. The marked variability between patches, which is probably related to the drift observed previously (see footnote 1, Galt, 1990; Galt et al., 1993b), makes it difficult to draw a final conclusion, but the data do not support the existence of an effect. In two independent studies no effect has been found on channel duration. Furthermore, if Sigworth and Shenkel (1988) are correct that flickers with periods in the 0.1–1 ms range represent brief visits to an intermediate state in the opening and closing process, then any effect of magnetic fields on gating would be expected to alter the properties of the flickers. Again no effects have been seen.

In light of the results reported here and by Galt and colleagues (Galt, 1990; Galt et al., 1993b) it is very unlikely that any effect of low-frequency, low-amplitude magnetic fields will be observed with gramicidin. That in turn supports the view that an effect of these fields directly on the permeating ions is unlikely. However, no study on a model system can finally rule out the possibility that magnetic fields act on specific properties of some other channel. Höjevik et al. (1990) failed to find an effect of magnetic fields satisfying the resonance condition for calcium on whole-cell calcium currents in RINm5F-cloned insulin-producing β-cells. We report elsewhere (Wang and Hladky, 1994) that fields satisfying the resonance condition for potassium are without observable effect on ATP-sensitive potassium channels in excised patches of membranes from the CRI-GI insulin-secreting cell line.

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