Effect of Melittin on Electrophysiological Parameters in the Frog Cornea Epithelium

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GASPAR CARRASQUER,*,[1] SHEN YANG,* MANUEL SCHWARTZ,† AND MUMTAZ A. DINNO‡
Departments of Medicine (Nephrology) and Physics,† University of Louisville, Louisville, Kentucky 40292; and Department of Physics,‡ East Carolina University, Greenville, North Carolina 27858*

Abstract. **The effect of melittin on electrophysiological parameters of the bullfrog cornea was studied using an *in vitro* preparation. Epithelial cells of corneas were impaled with microelectrodes. Experiments were done under short-circuit current (I_{sc}) conditions. Melittin was added in concentrations of 10^{-5} *M* to the stromal or 10^{-6} *M* to the tear solution. The effects of melittin were as follows: (i) stromal side, a decrease in I_{sc}; an increase in the apical membrane fractional resistance, fR_o; no change in the transepithelial conductance, g_t; and a depolarization of the intracellular potential, V_o; (ii) tear side, an initial (first 10 min) increase and then a decrease in I_{sc}; a decrease in fR_o; an initial (first 10 min) increase with subsequent small decrease in g_t; and a depolarization of V_o. Changes in tear Na^+, but not in tear K^+, with melittin present in tear solution, induced changes in some electrical parameters. The effects on the tear side may be explained by opening of nonspecific channels in the apical membrane with some specificity for a Na^+ channel. The subsequent effects on the tear and the effects on the stromal side may be explained by an inhibition of the primary transport system, that is, of the Na^+/K^+-ATPase pump located in the basolateral membrane. In these experiments, there was no evidence of opening of channels in the basolateral membrane.** [P.S.E.B.M. 1996, Vol 211]

Melittin, a 26 amino acid polypeptide, contained in the honey bee venom (1), has been found to have an active interaction with membranes. It binds to negatively charged phospholipids (2) and has effects on the electrical properties of excitable tissues (3, 4). The venom forms channels in lipid bilayers (5) and also has been found to interact and inhibit the H^+/K^+-ATPase (6, 7), as well as the Na^+/K^+-ATPase (8). In one of the few studies using epithelial membranes, melittin was found (9) to increase the short-circuit current in the cornea, when placed on the stroma side, and in the skin of the toad (*Bufo marinus*). When placed on the tear side of the cornea, the short circuit current had a biphasic response, an early increase followed by a decrease.

We designed experiments to study the effects of melittin on the cornea using microelectrodes in order to obtain results not only on the short circuit current and conductance but also on the intracellular potential and apical membrane fractional resistance.

Materials and Methods

Bullfrog corneas (*Rana catesbeiana*) were mounted tear side up in a lucite chamber as previously described (10–12). The tissue was supported by a copper grid with a slightly less radius of curvature than that of the *in vivo* cornea. An opening of 0.4 cm^2 communicated the upper (epithelial) chamber (0.2 ml) with the lower (stromal) chamber (0.3 ml). Both chambers were continuously perfused at a rate of about 5 ml/min to insure complete exchange in 5–10 sec. A slight negative hydrostatic pressure was applied to the lower chamber to help secure the cornea to the copper grid. Control solutions contained (in m*M*) (stroma): Na^+, 102; K^+, 4.2; Ca^{2+}, 1; Mg^{2+}, 0.8; Cl^-, 106.2; SO_4^{2-}, 0.8; phosphate 1; and glucose 10; (tear): Na^+, 100;

[1] To whom requests for reprints should be addressed at Department of Medicine, Division of Nephrology, University of Louisville, Louisville, KY 40292.

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K^+, 4; Ca^{2+}, 1; Cl^-, 97; HCO^{3-}, 5; phosphate, 1; and glucose, 10. Choline was used to substitute for Na^+ for low Na^+ solutions. Melittin was added to the stromal solution to a final concentration of 10^{-5} *M* (initially 10^{-6} *M* with no effects) or to the tear solution to a final concentration of 10^{-6} *M*. The pH was 7.3–7.4 for the stromal solutions or 8.5–8.6 for the tear solutions, and remained stable during the experiment. Candia (13) showed that high pH in the tear solution was favorable for high I_{sc} and Cl^- fluxes. Two pairs of macroelectrodes and one microelectrode were used. One pair was used to measure the transepithelial potential difference (calomel electrodes connected via KCl bridges to within 0.5 mm of tissue surfaces); the other pair (AgCl-coated Ag wire loop electrodes, 4 mm from the tissue on either side) was used to send current. The intracellular potential, V_o, was recorded with 3 *M* KCl–filled microelectrodes, which had an input resistance of 50–70 Mohm. Corneas were short-circuited using an automatic clamp device (Biomed. Inst., Germering, Germany) except for brief perturbations that lasted about 200 ms, during which the transepithelial potential was clamped at +10 mV (stroma side positive). These perturbations were repeated every 1–2 sec and were used for measurement of the transepithelial conductance ($g_t = \Delta I_t/\Delta V_t$). Also the apical membrane fractional resistance ($fR_o = R_o/(R_o + R_i) = \Delta V_o/\Delta V_t$) could be obtained. V_t and I_t are the transepithelial voltage and current, and R_o and R_i are the resistances across the apical and basolateral membranes, respectively. The values of short-circuit current (I_{sc}), g_t, fR_o, and V_o were recorded together with the microelectrode resistance on a multichannel strip chart recorder (Linseis, TYP 2065). I_{sc} is defined as positive when the direction of current is from tear to stroma via the tissue. Hyperpolarization of V_o is defined as an increase in the negative intracellular potential. Depolarization is used as the opposite of hyperpolarization. Student's *t* test with paired observations was performed to determine the level of significance when applicable. For experiments with "several (two) planned comparisons" with one control, we have used the "Adjusting the Level Downward" (ALD) method similar to the Bonferroni adjustment (14) for which $\alpha = 0.025$ (0.05/2) when two comparisons are used with the same control.

Results

Effect of Adding Melittin to a Concentration of 10^{-5} *M* in the Cornea Stromal Solution. Figure 1 shows the effects of melittin when added to the stromal solution. The curves present the mean values, from 20 experiments, of the short circuit current, I_{sc}, the apical membrane fractional resistance, fR_o, the transepithelial conductance, g_t, and the intracellular potential, V_o, plotted versus time, with zero being the time of addition of melittin.

Table I presents the mean control values and the mean changes of the parameters at 10 and 30 min after addition of 10^{-5} *M* melittin. In 10 and 30 min, respectively, I_{sc} decreased by 0.9 and 3.3 from a control of 4.7 $\mu A/cm^2$; g_t did not change; fR_o increased by 0.14 and 0.35 from 0.44 control; and V_o depolarized by 12.0 and 36.1 from −37.9 mV control. All these changes were significant by the Student's *t* test ($P < 0.01$) and by the ALD method ($\alpha = 0.025$).

Effect of Adding Melittin to a Concentration of 10^{-6} *M* in the Cornea Tear Solution. Figure 2 shows the effects of melittin when added to the tear solution. The curves present the mean values from nine experiments and the parameters are the same as in Figure 1.

Table II presents the mean control values and the mean changes of the parameters at 10 and 30 min after addition of melittin. In 10 and 30 min, respectively, I_{sc} increased by 5.3 and 1.1 from a control of 5.3 $\mu A/cm^2$; g_t increased by 0.54 and 0.43 from a control of 0.14 mS/cm^2; fR_o decreased by 0.36 and 0.43 from 0.48 control; and V_o depolarized by 41.5 and 46.6 from −54.3 mV control. All the changes, except $I_{sc} = 1.1$ $\mu A/cm^2$, were significant by the Student's *t* test ($P < 0.01$) and by the ALD method ($\alpha = 0.025$).

Effect of Changing Tear Na^+ from 100 to 10 and back to 100 m*M* without and with 10^{-6} *M* Melittin in the Tear Solution. Figure 3 shows the effects of

Table I. Effects of Adding 10^{-5} *M* Melittin to Stromal Solution

	Control ($n = 20$)	Change in parameter	
		10 min	30 min
I_{sc} ($\mu A/cm^2$)	4.74 ± 0.34	−0.89 ± 0.22[a]	−3.26 ± 0.26[a]
fR_o (mS/cm^2)	0.44 ± 0.03	0.14 ± 0.03[a]	0.35 ± 0.04[a]
g_t (unitless)	0.12 ± 0.01	0.00 ± 0.00[ns]	0.02 ± 0.01[ns]
V_o (mV)	−37.9 ± 2.4	12.01 ± 2.54[a]	36.1 ± 2.8[a]

Note. Values are means + SEM. *n*, number of experiments; Control, values obtained before the addition of melittin. The other values are the changes obtained, 10 and 30 min after the addition of melittin.
[a] $P < 0.01$.
[b] $P < 0.05$.
[ns] $P > 0.05$.

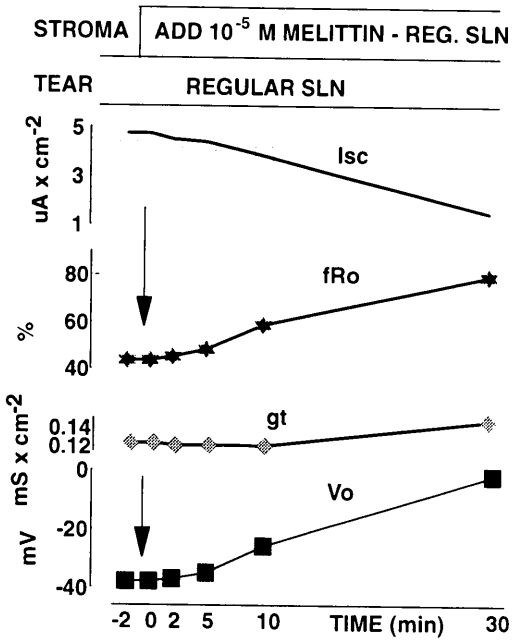

Figure 1. Effect of 10^{-5} *M* melittin in the stromal solution. Short-circuit current in μA/cm², I_{sc}; apical membrane fractional resistance, fR_o; transepithelial conductance in mS/cm², g_t; intracellular potential in mV, V_o, plotted versus time. Zero time when melittin was added.

changing the tear Na^+ concentration before and after melittin. The curves present the mean values from one representative experiment and the parameters are the same as in Figure 1. We have not presented the plot of the means in the present case since there is some variation in the time course of each experiment.

In six experiments, there were no significant

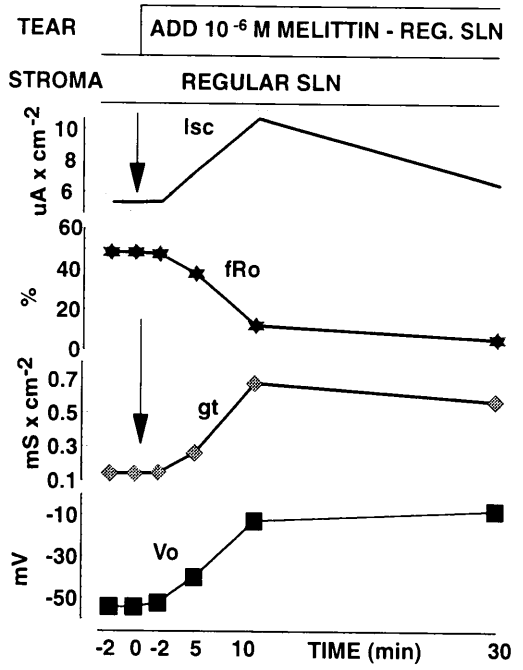

Figure 2. Effect 10^{-5} *M* melittin on the tear solution. Symbols as in Figure 1.

Table II. Effects of Adding 10^{-6} *M* Melittin to Tear Solution

	Control (n = 9)	Change in parameter	
		10 min	30 min
I_{sc}	5.32 ± 0.6	5.34 ± 0.83[a]	1.11 ± 0.78[ns]
fR_o	0.48 ± 0.04	−0.36 ± 0.05[a]	−0.43 ± 0.04[a]
g_t	0.14 ± 0.01	0.54 ± 0.07[a]	0.43 ± 0.06[a]
V_o	−54.3 ± 2.6	41.50 ± 3.87[a]	46.61 ± 3.27[a]

Note. Values and symbols as in Table I.

changes in the parameters for changes in tear Na^+ from 100 to 10 m*M* and back to 100 m*M* without melittin.

Table III presents the mean control values and the mean changes of the parameters at 10 min after changing the Na^+ concentration in the presence of melittin. In changing the tear Na^+ concentration from 100 to 10 m*M*, I_{sc} decreased by 4.3 from a control of 6.4 μA/cm²; g_t decreased by 0.14 from a control of 0.79 mS/cm²; fR_o did not change; and V_o hyperpolarized by 1.6 from −4.4 mV control. All the changes were significant ($P < 0.01$). In returning from 10 to 100 m*M* tear Na^+, I_{sc} increased by 4.2 from a control of 2.2 μA/cm² ($P < 0.01$); g_t did not change; fR_o decreased by 0.02 from 0.02 control ($P < 0.05$); and V_o did not change.

We note that changes of K^+ from 4 to 79 m*M* and back to 4 m*M* in the tear solution without and with melittin gave no significant changes in the electrical parameters.

Discussion

When melittin was placed on the tear side (10^{-6} *M*), the results on the short circuit current obtained by us in the bullfrog cornea were similar to those obtained in the toad (9). An initial increase in I_{sc} (Fig. 2), was followed by a decrease, although it remained above the control level at 30 min. The increase in I_{sc} was accompanied by a depolarization of the intracellular potential, V_o, an increase in the transepithelial conductance, g_t, and a decrease in the apical membrane fractional resistance, fR_o. These findings are similar to those observed by an increase in the apical membrane Cl^- conductance, as it occurs with epinephrine (10, 15). Therefore, melittin could have increased the Cl^- conductance. Effects of epinephrine on Cl^- transport in the cornea were reported earlier by Chalfie *et al.* (16).

Perhaps melittin creates nonselective channels in the apical membrane like amphotericin B (17, 18). With amphotericin B in the tear solution, changes in Na^+ concentration in the tear solution result in bioelectrical parameters effects compatible with the presence of a Na^+ channel in the apical membrane (19). In the present experiments, changes in Na^+ concentration from 100 to 10 m*M* in the tear solution gave, with

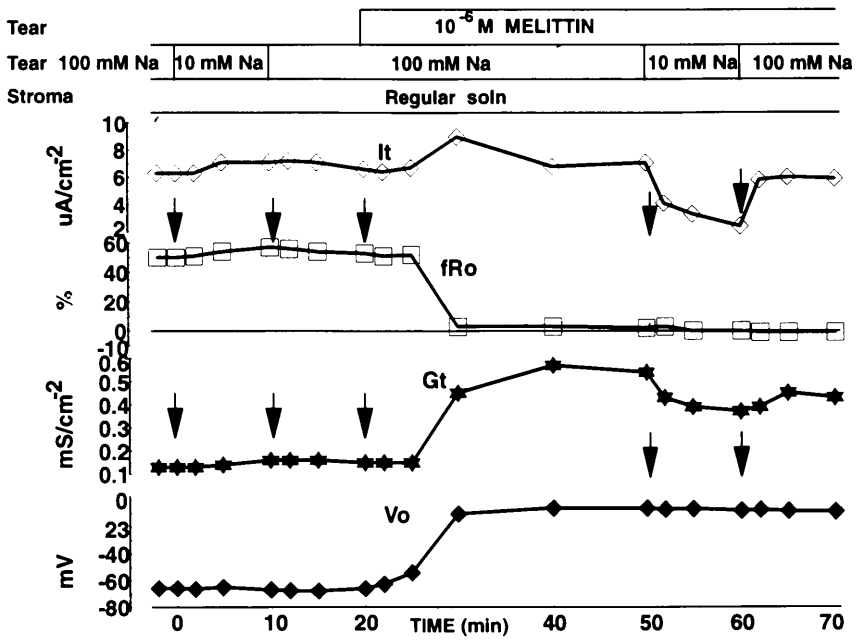

Figure 3. Effect of changing the concentration of Na^+ in the tear solution concentration in one experiment. On the left is before melittin and on the right is after addition of 10^{-6} *M* melittin tear solution. Symbols as in Figure 1.

melittin in the tear solution, a decrease in I_{sc} and g_t, but no change in fR_o. In going from 10 to 100 m*M* Na^+, there was an increase in I_{sc}, no change in g_t and a small decrease in fR_o. These results suggest a limited specificity of melittin for a Na^+ channel. This effect of melittin on Na^+ is much smaller than that produced by amphotericin B (19). An opening of a Na^+ channel is supported by McGahan *et al.* (9), who found an increase in the Na^+ fluxes by melittin in the toad cornea.

Since the electrochemical potential of K^+ is higher in the cell (20) than in the tear solution, opening of a K^+ channel in the apical membrane should result in a hyperpolarization of V_o and a decrease in short circuit current, I_{sc}, contrary to observation. Furthermore, we have changed the concentration of K^+ in the tear solution with no effects on the bioelectrical parameters without and with melittin. This is contrary to our observations with amphotericin B (19). In the latter case, changes in K^+ concentration in the tear solution gave significant changes in I_{sc}, g_t, fR_o, and V_o compatible with a K^+ channel.

While the initial increase in I_{sc} following addition of melittin to the tear solution may be due to the creation of Na^+ channels or nonspecific channels, the subsequent decrease in I_{sc} is probably due to an effect of melittin on the Na^+/K^+-ATPase pump as observed by Cuppoletti and Abbot in other tissues (8).

The results on short-circuit current with 10^{-5} *M* melittin in the stromal solution in our micropuncture preparation of the bullfrog cornea are quite different from those obtained in the toad cornea with 10^{-6} *M* using a different type of chamber (9). In the toad they found an increase in short-circuit current, which was inhibited by bumetanide. This implies that melittin stimulated the bumetanide-sensitive (15) cotransporter located in the basolateral membrane. In our preparation, we did not find an effect with 10^{-6} *M* melittin, and the effect with 10^{-5} *M* was of an inhibitory nature: decrease in I_{sc}, without a change in g_t, which remained below 0.15 mS/cm² after 30 min of exposure to melittin.

In general,

$$V_o = E_b - I_{sc}R_b = E_b \times fR_o$$

where E_b is the EMF of the pump located in the basolateral membrane and R_b is the basolateral mem-

Table III. Effects of Changing Tear Na^+ Concentration with 10^{-6} *M* Melittin in the Tear Solution (Six Experiments)

	Change Na^+ 100 to 10 m*M*		Change Na^+ 10 to 100 m*M*	
	Control	Δ = 10 min	Control	Δ = 10 min
I_{sc}	6.4 ± 0.6	−4.3 ± 0.3[a]	2.2 ± 0.4	4.2 ± 0.4[a]
fR_o	0.03 ± 0.01	−0.01 ± 0.004[ns]	0.02 ± 0.007	−0.02 ± 0.007[b]
g_t	0.79 ± 0.12	−0.14 ± 0.04[a]	0.66 ± 0.12	0.00 ± 0.03[ns]
V_o	−4.4 ± 5.6	−1.6 ± 0.1[a]	−6.0 ± 5.5	0.1 ± 0.9[ns]

Note. Control, values obtained before the change in Na^+ concentration. The other values are the changes obtained 10 min after the change in Na^+ concentration. Symbols and units as in Table I.

brane resistance. In these experiments, V_o decreased and fR_o increased. Hence, E_b must have decreased. Since the primary transport mechanism is the Na^+/K^+-ATPase, it follows that melittin inhibited the pump when placed in the stromal side as it is suggested above with the late effects of melittin in tear solution. The increase of fR_o, without a change in g_t, may suggest that there may have been some minor simultaneous decrease in the apical and increase in the basolateral conductances.

Although our results on the bullfrog cornea are somewhat different from those obtained by McGahan *et al.* (9) on the toad cornea, we find, as they did, that the effect of melittin in the stromal solution is quite different from the effect of melittin in the tear solution. Some of the differences between our results and theirs, when melittin is placed on the stromal solution, may be due to differences in the experimental chambers. For our micropuncture preparation, the basolateral membrane of the corneal epithelium is separated from the bathing medium not only by the stroma and the endothelium but also by a copper grid to keep the cornea fixed during the experiment. The cornea is kept immobile by a negative pressure, while in non-micropuncture chambers a positive pressure of 2 or more cm of water is applied to the stromal side. This could be the reason for the higher concentration needed to elicit any effects. Also, the toad may differ in some respects from the bullfrog cornea.

In summary, the effects of adding melittin (10^{-6} *M*) to the tear solution may be explained by opening of nonspecific channels in the apical membrane with some specificity for a Na^+ channel. There is no evidence at present for the presence of a K^+ channel. The late effect of melittin on the electrophysiological parameters on the tear side may be due to an inhibition of the Na^+/K^+-ATPase located in the basolateral membrane. The effect of higher (10^{-5} *M*) concentrations in the stromal solution suggest an inhibition of the Na^+/K^+-ATPase, with minor changes in the conductances of the apical and basolateral membranes.

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