Structural Requirements for Charged Lipid Molecules to Directly Increase or Suppress K^+ Channel Activity in Smooth Muscle Cells

Effects of Fatty Acids, Lysophosphatidate, Acyl Coenzyme A and Sphingosine

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ABSTRACT We determined the structural features necessary for fatty acids to exert their action on K^+ channels of gastric smooth muscle cells. Examination of the effects of a variety of synthetic and naturally occurring lipid compounds on K^+ channel activity in cell-attached and excised membrane patches revealed that negatively charged analogs of medium to long chain fatty acids (but not short chain analogs) as well as certain other negatively charged lipids activate the channels. In contrast, positively charged, medium to long chain analogs suppress activity, and neutral analogs are without effect. The key requirements for effective compounds seem to be a sufficiently hydrophobic domain and the presence of a charged group. Furthermore, those negatively charged compounds unable to "flip" across the bilayer are effective only when applied at the cytosolic surface of the membrane, suggesting that the site of fatty acid action is also located there. Finally, because some of the effective compounds, for example, the fatty acids themselves, lysophosphatidate, acyl Coenzyme A, and sphingosine, are naturally occurring substances and can be liberated by agonist-activated or metabolic enzymes, they may act as second messengers targeting ion channels.

INTRODUCTION

It is well recognized that certain lipid molecules, such as arachidonic acid and diacylglycerol, function as second messengers. We have previously demonstrated that

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a number of fatty acids, including arachidonic acid, directly activate K^+ channels in smooth muscle cells (Ordway, Walsh, and Singer, 1989; Ordway, Singer, and Walsh, 1991; Kirber, Ordway, Clapp, Walsh, and Singer, 1992), and others have shown similar direct regulation of different channel types by fatty acids (for example, Giaume, Randriamampita, and Trautmann, 1989; Kim and Clapham, 1989; Shimada and Somlyo, 1992; Huang, Xian, and Bacaner, 1992; Miller, Sarantis, Traynelis, and Atwell, 1992). In our earlier work we demonstrated that fatty acids with 14 carbons or more, provided they were sufficiently water soluble, directly open a 50 pS K^+ channel in membrane patches from smooth muscle cells isolated from the stomach of the toad, *Bufo marinus* (Ordway et al., 1989). Thus, the saturated fatty acid myristic acid (C14), the *cis*-monounsaturate, oleic acid (C18:1), the *cis*-polyunsaturate, arachidonic acid (C20:4) and the *trans*-polyunsaturate, linoelaidic (C18:2), all produced channel activation. In contrast, shorter chain fatty acids, such as the saturate, caprylic acid (C8), did not produce channel activation. Other than this information on chain length, nothing is known of the structural requirements necessary for amphiphiles like fatty acids to exert their action on ion channels. As a first step towards an understanding of these requirements, the actions of a variety of charged and uncharged amphiphiles were examined on the same 50 pS K^+ channel used in our earlier studies (Ordway et al., 1989). This channel's gating is only very weakly voltage sensitive (Ordway, 1990) but quite mechanosensitive (i.e., stretch activated [Ordway, Petrou, Kirber, Walsh, and Singer, 1992]). A brief account of some of this work has been reported elsewhere in abstract form (Petrou, Ordway, Singer, and Walsh, 1992).

METHODS

Recording Conditions

Smooth muscle cells were isolated from the stomach of the toad, *Bufo marinus* using procedures described elsewhere (Fay, Hoffman, Leclair, and Merriam, 1982; Lassingal, Singer, and Walsh, 1986), single channel currents were recorded using standard patch clamp techniques (Hammil, Marty, Neher, Sakmann, and Sigworth, 1981). For cell-attached and excised inside-out recordings the bathing solution usually contained (in millimolar): 130 K^+, 1 Mg^{2+}, 5 EGTA-KOH, 10 glucose, 114.5 Cl^- and 10 HEPES-HCl at pH 7.2 and patch pipettes usually contained: 127 Na^+, 3 K^+, 1 Mg^{2+}, 5 EGTA-NaOH, 114.5 Cl^- and 10 HEPES-HCl at pH 7.8. For excised outside-out recordings the solutions in the bath and patch pipette were reversed, with the exception that glucose was omitted from the patch pipette solution and added to the bathing solution. Recordings of K^+ channel activity were made with the membrane potential of the patches held at 0 mV.

Preparation and Application of Compounds

All the analogs used in this study were dissolved in bathing solution, with the exception of longer chain (> 10 C) fatty alcohols and myristic acid, which were first dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) and then diluted in bathing solution. Bath solution, or where appropriate, DMSO dissolved in bath solution (1:1,000 dilution), were used as controls and did not affect K^+ channel activity. Fatty acids and alcohols were obtained from Nu Chek Prep (Elysian, MN), primary alkyl amines and alkyl sulphonates were obtained from Aldrich Chemical Co. (Milwaukie, WI) (with the exception of tridecanesulphonate which was obtained from TCI America [Portland, OR]), lysophospholipids were obtained from Avanti

Polar Lipids (Birmingham, AL) and tetradecyltrimethylammonium, acyl Coenzyme A's and sphingosine were obtained from Sigma Chemical Co. All compounds were applied by pressure ejection (Picospritzer II; General Valve Corporation [Fairfield, NJ]) from a micropipette with a 1–2 μm tip (puffer pipette) placed 50–100 μm from the patch microelectrode.

Data Analysis and Display

All raw data were filtered at 5 kHz and then sampled onto video tape at 44 kHz using a Sony PCM device. Before analysis, data were filtered at 300 Hz then resampled at 1 kHz using the ERWIN suite of programs (kindly supplied by Dr. Michel Vivaudou, Lab de Biophysique Moleculaire et Cellulaire, CNRS, Grenoble, France). Idealized records were then produced from these resampled data files using the IPOCH suite of programs (Vivaudou, Singer, and Walsh, 1986) running on a PDP11/23 minicomputer. Mean open times ($\overline{To}$) and the average number of open channels (*NPo*; *N*, the number of channels in the patch; *Po*, the probability that a channel is open) were then determined from these idealized records. For the running *NPo* values shown in Figs. 1–4 *NPo* was calculated by determining average current and dividing by unitary current. All-points amplitude histograms and Gaussian curve fits were produced using the histogram module of the ERWIN suite of programs. Unless noted otherwise, data for displays were filtered at 100 Hz and then sampled at 300 Hz. For the purposes of display, electrical noise spikes have been removed from the figures.

RESULTS

Negatively Charged Lipid Compounds Mimic the Action of Fatty Acids

We first asked whether substitution of other negatively charged groups for the carboxyl group in the fatty acids could affect the ability to activate channels. Application of the negatively charged 14 carbon alkyl sulphonate, tetradecanesulphonate, to excised inside-out patches produced a large and reversible activation of the K^+ channels, thus mimicking the actions of myristic acid (Fig. 1, *A* and *B*). The same was true for the naturally occurring negatively charged 16 carbon palmitoyl lysophosphatidate (Fig. 1 *C*). Similar results were obtained in excised outside-out patches and in cell-attached patches (Table I). Thus, myristic acid, tetradecanesulphonate and palmitoyl lysophosphatidate which all bear negatively charged head groups activate K^+ channels even though the head group structures are quite different. Finally, as was the case with shorter chain fatty acids (Ordway et al., 1989), the shorter chain 8 carbon alkylsulphonate, octanesulphonate, did not activate the K^+ channels (Table I).

Neutral Lipid Compounds Neither Increase Nor Decrease K^+ Channel Activity

Because a variety of negatively charged compounds of diverse structure activate the K^+ channels, we questioned the necessity of the charge itself by employing neutral analogs of the effective fatty acids, for example myristyl alcohol and oleyl alcohol. These were without effect (Fig. 2 *A* and Table I) whereas myristic acid and oleic acid in the same patches increased channel activity. The same was true of the shorter chain alcohols, octanol, decanol and dodecanol (Table I). As a further test, we employed the lysolipid, palmitoyl lysophosphatidylcholine, a zwitterion which bears no net charge in the pH range used in this study. It too was without effect (Fig. 2 *B* and Table I) whereas the negatively charged palmitoyl lysophosphatidate tested on the same patches resulted in channel activation. The efficacy of negatively charged

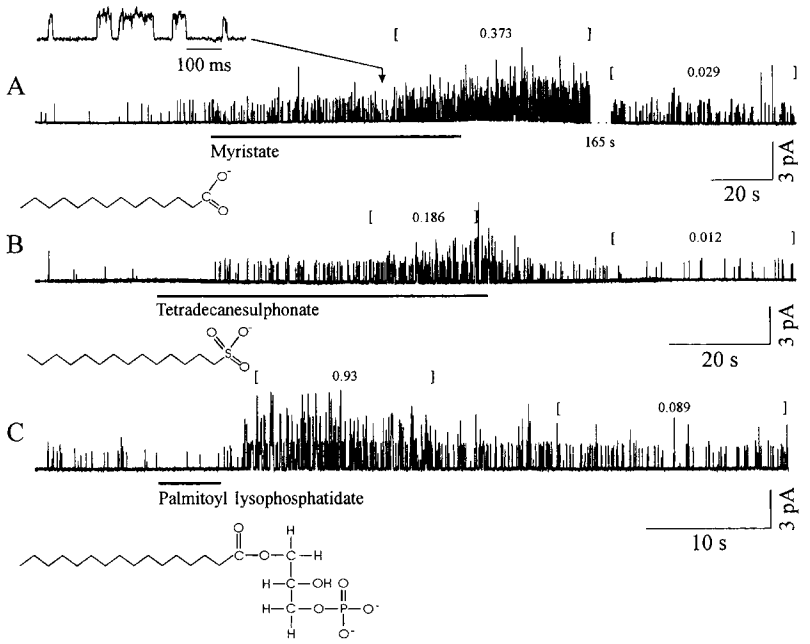

FIGURE 1. Negatively charged amphiphilic compounds mimic the actions of fatty acids by increasing K^+ channel activity. Myristate (50 μM) applied to a cell-attached patch produced a large and reversible increase in activity (*A;* note the break in the record) as did the negatively charged tetradecanesulphonate (50 μM) shown here applied to a cell-attached patch (*B*). For both compounds similar results were also observed in excised inside-out and excised outside-out patches (Table I). Palmitoyl lysophosphatidate (50 μM), shown here applied to an excised inside-out patch, also produced reversible increases in K^+ channel activity. Palmitoyl lysophosphatidate also increased channel activity in cell-attached and excised outside-out patches. (Because palmitoyl lysophosphatidate forms insoluble complexes with Mg^{2+}, Mg^{2+} was omitted from both the patch pipette and bathing solutions.) Single channel openings are illustrated by the inset which is an expanded view of the record shown in *A* at the time indicated by the arrow. In this case data were filtered at 300 Hz and then sampled at 1 kHz. *NPo* values were calculated (average current divided by unitary current) at various times for each channel record and are given above the trace in the region where the actual measurements were made. In each case, the solid bar indicates the period of application. V_m = 0 mV in this and all other figures. See Table I for complete summary of the compounds used, their actions in cell-attached, excised inside-out and excised outside-out patches and the number of patches in which each compound was tested. The differences in the time course of the responses are not necessarily indicative of the potency of the compounds as many factors, including puffer pipette tip size, the distance and geometry of the puffer in relation to the cell and the position of the membrane patch within the patch pipette, contribute to produce variation in the time required to develop a response. When the channel opens the resulting outward (positive) current is recorded as an upward transition. Records are shown on a compressed time scale such that the time courses of channel openings are not clearly resolved.

TABLE I

Negatively charged compounds	C-A*			I-O‡			O-O§		
	A[1]	S[2]	N[3]	A	S	N	A	S	N
Octanesulphonate (C8)			2			2			
Dodecanesulphonate (C12)	1		4	4		1			
Tridecanesulphonate (C13)							1		
Tetradecanesulphonate (C14)	12			16			10		
Myristic acid (C14) in DMSO	3			3					
Oleic acid (C18:1)	5			2					
Palmitoyl lysophosphatidate (C16)	9		1	14			4		
Palmitoyl lysophosphatidate (C16)+ 200 mM Na^+				3					
Neutral compounds‖	A	S	N	A	S	N	A	S	N
Octanol (C8) 500 μM			2			2			
Decanol (C10) in DMSO			3			4			
Decanol (C10) 150 μM in DMSO			1			1			
Dodecanol (C12) 20 μM			11			5			
Myristyl alcohol (C14) 20 μM in DMSO			1			1			
Oleyl alcohol (C18:1) 20 μM in DMSO			3			3			
Palmitoyl lysophosphatidylcholine (C16)			4			10			
Palmitoyl lysophosphatidylcholine (C16) 500 μM			1			1			
Positively charged compounds	A	S	N	A	S	N	A	S	N
Octylamine (C8)			3			1			
Decylamine (C10)		5	1		1	3			
Dodecylamine (C12)		4			2				
Tetradecylamine (C14)		7			3			2	
Oleylamine (C18:1)		3			3				
Sphingosine (C16:1)		6			15			1	
Tetradecyltrimethylammonium (C14)		5			16			2	
Negatively charged compounds that do not "flip"	A	S	N	A	S	N	A	S	N
Octanoyl Coenzyme A (C8)			3			3			1
Myristoyl Coenzyme A (C14)			6	3		2			2
Palmitoyl Coenzyme A (C16)			9	11					8

Table gives the number of patches used to test the activity of each compound. The number of carbons in the alkyl chains and their degree of saturation are given in parentheses. Unless noted, all compounds were applied at 50 μM concentration (in the puffer pipette) and were dissolved in bath solution. Because compounds are diluted with bathing solution as they exit the puffer pipette the final concentration of compound at the membrane surface is difficult to estimate. The concentration given is therefore the maximum possible concentration. DMSO: dimethylsulfoxide.

*Cell-attached configuration: compounds were applied to the external surface of the cell with the exception of the patch itself (i.e., the agents were not included in the patch pipette).

‡Excised inside-out configuration: compounds were applied solely to the intracellular surface of membrane patches.

§Excised outside-out configuration: Compounds were applied solely to the extracellular surface of membrane patches.

[1]Activate.

[2]Suppress.

[3]No effect.

Note that the magnitudes of these effects as expressed as NPo in Figs. 1–4 are typical for the results reported above.

‖In addition, the neutral methyl esters of the effective fatty acids were without effect on whole cell currents, but we do not include these results in Table I because we were uncertain that the methyl esters were sufficiently soluble to reach the cell membrane. The ineffectiveness of these fatty acid methyl esters on whole cell currents is indicated by the following summary (expressed as the ratio of number of negative results to the number of cells tested): myristoleic acid methyl ester (0/5), arachidonic acid methyl ester (0/1) and palmitoleic acid methyl ester (0/1).

compounds and the lack of effect of neutral compounds leads us to conclude that the presence of a charged group is a necessary condition for channel activation.

Positively Charged Amphiphilic Amino Compounds Suppress K^+ Channel Activity

To determine whether the charge required for channel activation must be negative, we employed a number of positively charged amphiphiles (Table I). Surprisingly, these compounds suppressed K^+ channel activity. Thus, the positively charged primary amines, the 14 carbon tetradecylamine (Fig. 3 *A*) and the 18 carbon oleylamine (Fig. 3 *B*), and the quaternary amine, the 14 carbon tetradecyltrimethylammonium, all suppressed K^+ channel activity. These are positively charged analogs

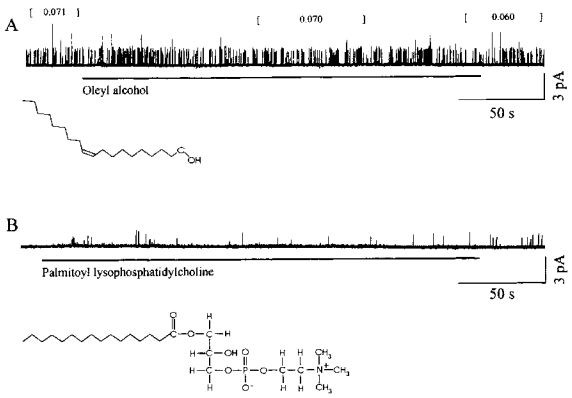

FIGURE 2. Long chain neutral compounds neither increase nor decrease K^+ channel activity. The neutral fatty alcohol, oleyl alcohol (50 μM), applied for a long period to this cell-attached patch did not affect K^+ channel activity (*A*). The neutral lysophospholipid, palmitoyl lysophosphatidylcholine (50 μM), applied here to an excised inside-out patch, was also ineffective (*B*). For the latter example Mg^{2+} was omitted from both the patch pipette and bathing solutions. *NPo* values were calculated (average current divided by unitary current) at various times for the channel record in *A* and are given above the trace in the region where the actual measurements were made. Similar results were also seen in excised inside-out patches (Table I) and in patches with varying levels of K^+ channel activity. The levels of channel activity needed to observe possible inhibitory responses were, in some cases, elicited by exploiting the mechanosensitive nature of these channels (Ordway et al., 1992). Long lasting increases in channel activity were elicited either by applying higher than usual levels of suction during seal formation, or by applying long pulses (2–10 min) of suction. The channel activity elicited in this way was not affected by application of control solutions. Note the differences in the head group structures of the two neutral, ineffective compounds (*A* and *B*).

of the fatty acids, myristic acid and oleic acid, which activated the channels. Similar to the negatively charged compounds, the shorter chain, less hydrophobic 8 carbon amine, octylamine, was ineffective (Table I). In addition, the naturally occurring amino alcohol, sphingosine, which also bears a positive charge at the pHs used in this study (Merrill, Nimkar, Mendalino, Hannun, Loomis, Bell, Tyagi, Lambeth, Stephens, Hunter, and Liotta, 1989) and has a 16 carbon hydrophobic region adjacent to the carbon carrying the amino groups, suppressed K^+ channel activity. After suppression of channel activity by positively charged compounds, activity only occasionally returned all the way to control levels. On those occasions where recovery did not occur, it was still possible to elicit increases in channel activity with fatty acids

or negatively charged compounds, indicating that the channels were still responsive. The failure of channel activity to recover completely does not appear to be a function of chain length as recovery was seen not only for the 10 carbon decylamine but also for the 14 carbon tetradecylamine.

Negatively Charged Compounds Which Do Not "Flip" across the Bilayer Are Effective Only When Applied to the Cytosolic Surface

Because both the positively and negatively charged compounds exert their effects in cell-attached patches, excised inside-out patches and excised outside-out patches it was unclear where the site of action might be located. To determine whether the site

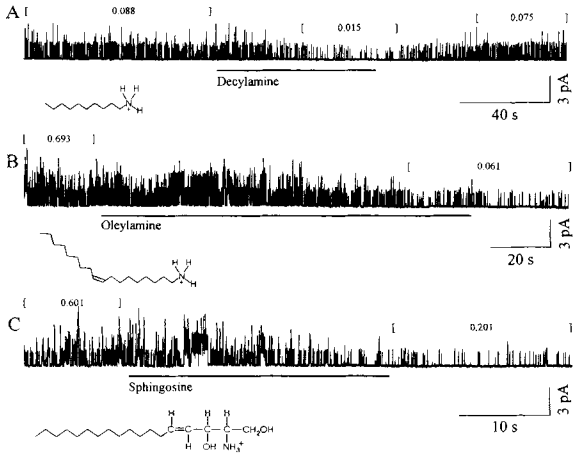

FIGURE 3. Positively charged compounds suppress K^+ channel activity. When applied to a cell-attached patch, the straight chain saturate, decylamine (50 μM), reversibly suppressed K^+ channel activity (*A*). The *cis*-monounsaturate, oleylamine (50 μM), suppressed K^+ channel activity (*B*) in a cell-attached patch. In this case the suppression was not reversible. Oleylamine was also effective when applied to excised inside-out patches. The positively charged amino alcohol, sphingosine (50 μM) suppressed K^+ channel activity in an excised inside-out patch (*C*). Sphingosine was also effective in cell-attached patches and in excised outside-out patches. *NPo* values were calculated (average current divided by unitary current) at various times for each channel record and are given above the trace in the region where the actual measurements were made. The levels of channel activity needed to observe these inhibitory responses was, in some cases, elicited by exploiting the mechanosensitive nature of these channels as discussed in the caption of Fig. 2. After exposure to positively charged inhibitory compounds channel activity did not usually recover to the levels seen before the exposure, although there were a few exceptions (for example, *A*). Again, note the structural diversity of these positively charged, inhibitory compounds.

of action of the fatty acids and similar lipids is limited to one side of the membrane bilayer, we examined the effects of a set of compounds which do not flip between membrane leaflets. We used a series of acyl Coenzyme A (CoA) molecules with negatively charged head groups which, according to recent NMR studies, are confined to the side of the membrane to which they are applied (Boylan and Hamilton, 1992). In contrast, fatty acids flip (Hamilton and Cistola, 1986) rapidly (Kamp and Hamilton, 1992) from one bilayer leaflet to the other. Both the 14 carbon acyl, myristoyl CoA and the 16 carbon acyl, palmitoyl CoA, increased channel activity when applied to excised inside-out patches but not when applied to cell-attached or

excised outside-out patches (Fig. 4 and Table I). As might be expected from the foregoing results, the shorter chain 8 carbon acyl, octanoyl CoA, had no effect (Table I). We conclude that the site of action for negatively charged, amphiphilic compounds that activate the K^+ channels is localized at the intracellular surface of the membrane.

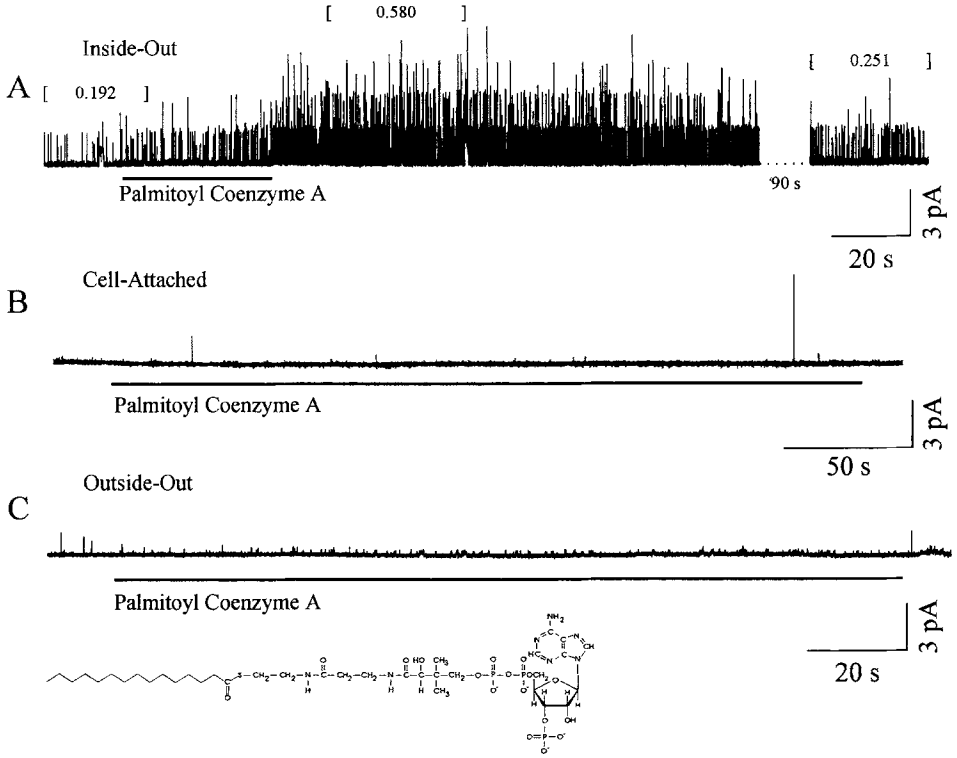

FIGURE 4. Compounds which do not flip across the lipid bilayer act only on the intracellular surface. Palmitoyl CoA (50 μM) applied to the intracellular surface of an excised inside-out membrane patch produced a reversible increase in K^+ channel activity (*A;* note the break in the record). When applied to the membrane surrounding a cell-attached patch (*B*) or to the extracellular surface of an excised outside-out patch (*C*), however, palmitoyl CoA did not increase K^+ channel activity. *NPo* values were calculated (average current divided by unitary current) at various times for the channel record in *A* and are given above the trace in the region where the actual measurements were made. Similar results were also obtained in patches with higher basal levels of K^+ channel activity.

In contrast to the CoA derivatives, the other amphiphiles are effective in cell-attached patches. Because the compounds were applied to the outside of the cell but not to the membrane area of the cell-attached patches themselves, these compounds had to traverse the membrane to exert their action. This is strong evidence that all but the CoA compounds are able to transit the bilayer to gain access to the site of fatty acid action.

The Negatively Charged Compound, Palmitoyl Lysophosphatidate Is Equally Effective in the Presence and Absence of High Ionic Strength Solutions

Charged lipid molecules that intercalate into lipid bilayers can alter membrane surface charge and thus increase the local concentration of counterions. As the activity of the K^+ channel under study is somewhat increased by application of low pH solutions applied to the intracellular surface (Dopico, A. M., M. Ugur, J. V. Walsh, Jr., and J. J. Singer, unpublished observations), we were interested in examining the possibility that an increase in the local intracellular concentration of H^+ or other counterion might be responsible for the channel activation. To test for this possibility we performed a series of experiments in which the negatively charged

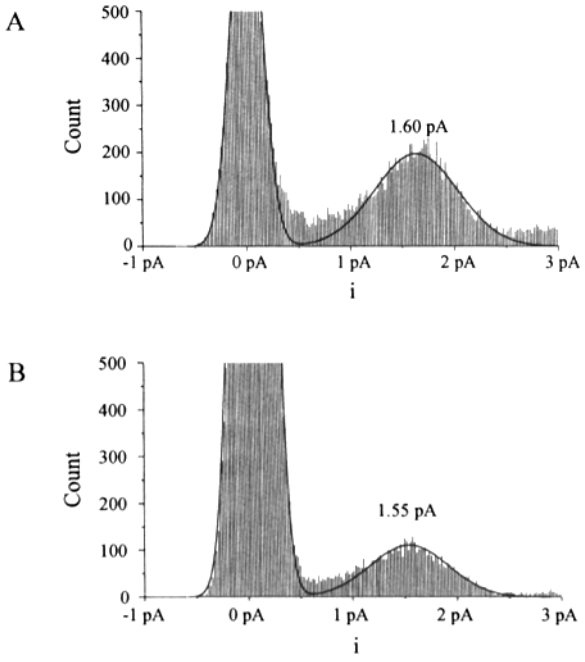

FIGURE 5. Sphingosine does not affect single channel conductance. Representative all-points amplitude histograms of channel data records made in the absence (*A*) and presence (*B*) of sphingosine (50 μM). Unitary conductance was not affected although *NPo*, determined at the same time, fell dramatically (from 0.614 to 0.062). The current values printed above each histogram indicate the difference between the peaks of each Gaussian curve fit. Similar results were obtained for decylamine, oleylamine, tetradecylamine, and tetradecyltrimethylammonium.

palmitoyl lysophosphatidate was applied to the intracellular surface of membrane patches in the presence of high ionic strength solution (200 mM NaCl). This should shield surface charge and thus reduce its possible effects on channel gating. We found that application of palmitoyl lysophosphatidate with 200 mM NaCl to excised inside-out patches (see Table I) produced an increase in channel activity qualitatively similar to that seen with palmitoyl lysophosphatidate alone. We conclude, that an alteration of surface charge is not sufficient to explain the actions of lysophosphatidate and, by extension, the actions of other charged compounds, on K^+ channel activity.

Positively Charged Compounds Are Not Open Channel Blockers

We examined whether positively charged compounds which suppress channel activity affect either single channel conductance or mean channel open times as would be

expected for open channel block. Amplitude histograms were constructed from digitized data records and mean open times were calculated from idealized data records in the periods before and during compound application. Sphingosine (Fig. 3 *C*) which produced a marked decrease in channel activity, had no effect on either the single channel conductance (Fig. 5) or the mean channel open time (Table II). The positively charged compounds oleylamine and tetradecyltrimethylammonium were also without effect on mean channel open time (Table II) and single channel conductance.

DISCUSSION

Endogenous Amphiphiles May Serve as Regulators of K^+ Channel Activity

The naturally occurring lipids utilized in the present study can be produced by metabolic and signal transduction pathways. Fatty acids (such as arachidonic acid) and lysophosphatidic acid can be liberated from membrane phospholipids, acyl CoA

TABLE II

Compound	Configuration	Control		Effect	
		NPo	$\overline{To}$	*NPo*	$\overline{To}$
Tetradecanesulphonate	C–A	0.023	16.4 ms	0.392	18.3 ms
Palmitoyl lysophosphatidate	I–O	0.064	8.4 ms	0.427	9.5 ms
Sphingosine	I–O	0.606	19.3 ms	0.060	18.1 ms
Oleylamine	C–A	0.387	14.2 ms	0.056	15.6 ms
Tetradecyltrimethylammonium	C–A	0.398	9.3 ms	0.061	7.1 ms

Comparison of the effects of various compounds on *NPo* and $\overline{To}$. Different patches were used for each compound but the same patch was used to determine *NPo* and $\overline{To}$ for control and effect (for each compound the data has been drawn from a single patch). In addition, visual inspection of single channel data from a number of patches (>3 for each compound) revealed no detectable change in To values, corroborating the results shown above. $\overline{To}$ and *NPo* were calculated from idealized records of multi-channel patches using procedures and software (IPOCH) described elsewhere (Vivaudou, Singer, and Walsh, 1986).

can be formed by the metabolic esterification of fatty acids to CoA, and sphingosine can be liberated from sphingomyelin by sphingomyelinase and sphingomyelin *N*-deacylase (Dennis, Rhee, Billah, and Hannun, 1991). Sphingosine is also thought to act as a second messenger (Hannun and Bell, 1989; Ballou, 1992) as is lysophosphatidate (Durieux and Lynch, 1993). The existence of such pathways raises the intriguing possibility that not only fatty acids (Ordway et al., 1989), but also lysophosphatidic acid and acyl CoA's may serve as endogenous activators of K^+ channels whereas sphingosine may serve as an endogenous inhibitor of K^+ channel activity.

Mechanisms of Amphiphile Action

The amphiphiles used in the present study might alter K^+ channel activity either by binding to target proteins or by affecting bilayer membranes (Ordway et al., 1989, 1991). Because amphiphiles readily partition into bilayer membranes, their actions are often ascribed to effects on the membrane itself; either an alteration of bulk membrane properties (Spector, 1986; Takenaka, Horie, and Hori, 1987; Takenaka,

Horie, Hori, and Kawakami, 1988; Bregestovski, Bolotina, and Serebryakov, 1989) or a change in membrane surface charge (Post, Ji, Leonards, and Langer, 1991; Ji, Weiss, and Langer, 1993). However, there is considerable evidence that amphiphiles can directly regulate the activity of a number of membrane bound (Merrill et al., 1989; Wallach and Pastan, 1976; Philipson, 1984; Philipson and Ward, 1985; Bottega and Epand, 1992) and soluble proteins (Braughler, Mittal, and Murad, 1979; Gerzer, Brash, and Hardman, 1986; Waldman and Murad, 1987; Touny, Khan, and Hannun, 1990). The results from the present study are inconsistent with a mechanism involving effects on the bilayer and they tend to support the idea that amphiphiles directly regulate K^+ channel activity for the following reasons.

The notion that "detergent effects" or alterations in "membrane fluidity" (Spector, 1986; Takenaka et al., 1987, 1988; Bregestovski et al., 1989) underlie the actions of fatty acids and polar lipids is open to question (Carruthers and Melchior, 1988; Cistola, Hamilton, Jackson and Small, 1988). The results from the present study not only fail to support such a mechanism but provide several lines of evidence to the contrary. First, positively charged, long chain compounds might be expected to have similar "detergent actions" and effects on "membrane fluidity" as negatively charged long chain lipids. Yet, the positively and negatively charged lipids had opposite effects on channel activity. Second, neutral lipids, such as lysophosphatidylcholine might also be expected to have similar effects on the lipid environment as fatty acids, yet they do not affect K^+ channel activity. Finally, as we have shown previously with fatty acids (Ordway et al., 1989) and have now demonstrated with alkylamines, saturated compounds and *cis*-monounsaturated compounds with like charge have similar effects on K^+ channel activity. If fatty acid action on K^+ channels involved effects on "membrane fluidity" one would expect to see some difference in the actions of lipids such as these with different chain conformations. Thus, it is unlikely that the mechanism of charged lipid action on ion channels demands perturbations of the bulk lipid environment.

We considered whether amphiphilic molecules, such as those used in the present study, might affect mechanosensitive ion channel activity via an extension of the bilayer couple hypothesis (Martinac, Adler, and Kung, 1990). According to this hypothesis, the preferential insertion of positive, negative or neutral amphiphiles into one lipid bilayer leaflet produces tension in the other leaflet and thus produces a change in ion channel activity. Because the K^+ channels examined in the present study are mechanosensitive (Ordway et al., 1992) it was possible that such a mechanism could be responsible for their activation by fatty acids. This model predicts that positively charged lipids should, if anything, increase K^+ channel activity and that palmitoyl CoA should act from either side of the membrane. But our results showed the opposite: suppression by positively charged lipids and a sidedness of palmitoyl CoA action. Another difference between our results and those attributed to the bilayer couple hypothesis is the time course of action. Fatty-acids and negatively charged lipids acted within seconds of application in our studies whereas the responses attributed to bilayer coupling (Martinac et al., 1990) take tens of minutes to develop.

Because charged lipids intercalate into the lipid bilayer and alter membrane surface charge, they can shift the voltage dependence of activation of voltage-gated

channels (Moczydlowski, Alvarez, Vergara, and Latorre, 1985; MacKinnon, Latorre, and Miller, 1989). We found no evidence, however, that fatty acids are modulating K^+ channels in this way. The K^+ channels examined in the present study are only weakly voltage dependent (93 mV for an e-fold increase in *Po* [Ordway, 1990]), in contrast to the more strongly voltage dependent Ca^{2+}-activated K^+ channels (9 mV for an e-fold increase in *Po* [Singer and Walsh, 1987]) which coexist in the same membrane patches. If fatty acids were regulating K^+ channels solely by shifting their voltage dependence, then activation of Ca^{2+}-activated K^+ channels is also to be expected. In fact, when we applied fatty acids, we saw a large increase in K^+ channel activity and no increase in Ca^{2+}-activated K^+ channel activity. Further evidence against a surface charge effect is provided by our observations on the sidedness of the palmitoyl CoA action. Palmitoyl CoA applied to the intracellular surface should increase the negative charge at the inner surface of the membrane and thus steepen the field within the membrane (equivalent to a membrane hyperpolarization) which should, if anything, decrease channel activity. But our results show quite the opposite; palmitoyl CoA applied to the intracellular surface of the membrane causes a marked increase in channel activity.

In addition to their effects on voltage-dependent channel gating, incorporation of charged lipids into membrane bilayers can also alter the ionic atmosphere around the channel (Moczydlowski et al., 1985; MacKinnon et al., 1989), which might increase the local concentration of counterions. If these counterions modulate channel gating, then it is plausible that charged lipids could also modulate channel gating via their effects on local concentrations of counterions. However, our results show that if we buffer local counterion concentrations by shielding surface charge with high ionic strength solutions we still see increases in K^+ channel activity, suggesting that charged amphiphiles do not act by an effect on local H^+ or other counterion concentration.

In summary, because there is little evidence that charged amphiphiles are acting on K^+ channels by affecting the lipid bilayer we favor the idea that these compounds are acting on the K^+ channel itself or some closely associated protein to cause channel activation.

The inhibitory actions of the positively charged compounds are intriguing and may point towards a common allosteric mechanism of action for both negatively and positively charged compounds. The inhibition of channel activity by positively charged amphiphiles can be readily explained by postulating an allosteric effect on the ion channel protein, although it is also possible that these compounds are acting as channel pore blockers. However, several lines of evidence argue against the latter explanation. First, although the positively charged compounds markedly suppressed channel activity they did not alter single channel conductance. Second, we occasionally observed that the K^+ channel under study entered a bursting mode of gating, where the channel moved rapidly from closed to open states for periods sometimes lasting a minute or more. On one such occasion sphingosine was applied and failed to affect the amplitude of the channel current or the channel bursting, although later application of sphingosine (to the same patch and after the bursting state was over) resulted in suppression of channel activity. This corroborates the absence of a

blocking mechanism and suggests that positively charged compounds can only suppress channel activity when they are not in a bursting mode. Third, even though sphingosine, oleylamine and tetradecyltrimethylammonium all produced marked decreases in channel activity, the mean open times were not altered (Table II), further corroborating the absence of a blocking mechanism. Thus, there is no evidence for either fast open channel block because single channel conductance is unchanged, or for slow open channel block because mean channel open time is unchanged. It is interesting to note that negatively charged excitatory compounds, which also have no effect on channel mean open times (Table II), are like the positively charged compounds in that they affect mean closed times, suggesting that both positively and negatively charged compounds act through a common allosteric mechanism.

An interesting precedent for these findings is the regulation of protein kinase C (PKC) activity (McPhail, Clayton, and Snyderman, 1984; Sekiguchi, Tsukuda, Ogita, Kikkawa, and Nishizuka, 1987; Seifert, Schächtele, Rosenthal, and Schultz, 1988; Morimoto, Nobori, Edashige, Yamamoto, Kobayashi, and Utsumi, 1988; Merrill et al., 1989; Bottega and Epand, 1992) and Na^+-Ca^{2+} exchange (Philipson, 1984; Philipson and Ward, 1985) by negatively and positively charged lipids. While PKC activity was increased by fatty acids (McPhail et al., 1984; Sekiguchi et al., 1987; Seifert et al., 1988; Morimoto et al., 1988; Touny et al., 1990), it was inhibited by a range of positively charged compounds with structural requirements for action similar to those found in the present study for the inhibition of K^+ channel activity (Merrill et al., 1989). (By this we do not mean to suggest that PKC-mediated phosphorylation is responsible for alterations in channel activity in the present study. This is highly unlikely in our experiments on excised patches since neither ATP nor any other energy source was present.) Similarly, the structural requirements for lipids that increase or decrease Na^+-Ca^{2+} exchanger activity were also like those found in the present study (Philipson, 1984). Given these precedents and the fact that data in the present study are inconsistent with mechanisms of fatty acid action on lipid membrane properties, membrane tension or surface charge, and our observation on the sidedness of palmitoyl CoA action, it is possible that fatty acids interact directly with ion channel proteins themselves as we have previously suggested (Ordway et al., 1989, 1991).

Further evidence for the idea that amphiphiles bind to ion channel proteins has been provided by a recent high resolution x-ray crystallographic study which revealed that the ion channel protein, porin, has a well defined amphiphile binding site (Weiss and Schulz, 1992). It remains to be seen whether fatty acids or other charged lipids can occupy this site and perhaps modulate porin activity. In addition to this structural demonstration of an ion channel protein-amphiphile interaction, our recent report (Petrou, Ordway, Singer, and Walsh, 1993) of a putative fatty acid-binding domain of the NMDA receptor, whose activity has been shown to be modulated by fatty acids (Miller et al., 1992), suggests that ion channel proteins not only bind amphiphiles but that binding modulates their activity.

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