Site of action of fatty acids and other charged lipids on BK$_{Ca}$ channels from arterial smooth muscle cells

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Clarke, Alison L., Steven Petrou, John V. Walsh, Jr., and Joshua J. Singer. Site of action of fatty acids and other charged lipids on BK$_{Ca}$ channels from arterial smooth muscle cells. Am J Physiol Cell Physiol 284: C607–C619, 2003. First published October 30, 2002; 10.1152/ajpcell.00364.2002.—Fatty acids and other negatively charged single-chain lipids increase large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channel activity, whereas sphingosine and other positively charged single-chain lipids suppress activity. Because these molecules are effective on both inside-out and outside-out patches and because they can flip across the bilayer, the location of their site of action is unclear. To identify the site of action of charged lipids on this channel, we used two compounds that are unlikely to flip across the lipid bilayer. Palmitoyl coenzyme A (PCoA) was used to identify the site of action of negatively charged lipids, and a positively charged myristoylated pentapeptide (myr-KPRPK) was used to investigate the site of action of positively charged lipids. The effect of these compounds on channel activity was studied in excised patches using patch-clamp techniques. In “normal” ionic strength solutions and in experiments where high-ionic strength solutions were used to shield membrane surface charge, PCoA increased channel activity only when applied to outside-out patches, suggesting that the site of action of negatively charged lipids is located on the outer surface of the membrane. A decrease in activity, similar to that of other positively charged lipids, was observed only when myr-KPRPK was applied to outside-out patches, suggesting that positively charged lipids suppress activity by also acting on the outer membrane surface. Some channel blockade effects of myr-KPRPK and KPRPK are also described. The sidedness of action suggests that modulation of channel activity by single-chain lipids can occur by their interaction with the channel protein.

Palmitoyl coenzyme A; myristoylated pentapeptide; channel blockade; calcium-activated potassium channel

Previously, we determined that fatty acids and other negatively charged lipids increase large-conductance, Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channel activity from rabbit pulmonary artery smooth muscle cells, whereas positively charged lipids decrease channel activity (3, 4). In addition, negatively charged lipids required a chain length of greater than eight carbons to be effective, and positively charged lipids with eight carbons were much less effective than their longer chain counterparts (3, 4). These studies also suggested that alterations in the properties of the lipid bilayer (e.g., detergent effects or changes in membrane fluidity, stiffness, and/or curvature) and changes in [Ca$^{2+}$], surface charge, and cytosolic and membrane-bound protein kinase or phosphatase activity are unlikely to be involved in the modulation of BK$_{Ca}$ channel activity by fatty acids and other charged lipids. Therefore, a reasonable hypothesis for the mechanism of action of charged lipids on BK$_{Ca}$ channel activity is that these molecules directly interact with the channel protein itself or, alternatively, a channel-associated protein or some other channel-associated component (3, 4).

If fatty acids and other charged lipid molecules affect BK$_{Ca}$ channel activity through some specific interaction with a binding site on the channel protein or some other channel-associated protein, it might be expected that they would be effective only when applied to one side of the membrane. However, because the fatty acids and other charged lipids used in the previous studies were capable of flipping across lipid bilayers (12) and were effective when applied to both excised inside-out and outside-out membrane patches, the location of their site(s) of action was unclear.

Thus, to obtain further evidence that fatty acids and other charged lipids interact with the channel protein itself and to determine the location of the site(s) of action of negatively and positively charged lipids, we studied the modulation of channel activity by two single-chain lipids, the negatively charged palmitoyl coenzyme A (PCoA) and a myristoylated positively charged pentapeptide, myr-Lys-Pro-Arg-Pro-Lys (myr-KPRPK). PCoA and myr-KPRPK, unlike most single-chain lipids and because of their multicharged head groups, should not be able to flip across the lipid bilayer (1, 12). Therefore, using PCoA and myr-KPRPK allowed us to determine whether negatively and positively charged single-chain lipids act from only one side.
of the membrane and, if so, whether they act at the internal or external membrane surface. Petrou et al. (24) previously used PCoA to determine the site of action of fatty acids and other negatively charged lipids on a small-conductance K⁺ channel from toad stomach smooth muscle. In that preparation, the site appeared to be located on the inner membrane surface.

A brief account of some of the present work has been reported elsewhere (5).

MATERIALS AND METHODS

The methods used for this study are similar to those presented by Clarke et al. (3) and are briefly described below.

Recording Conditions

Single-channel currents were recorded from excised inside-out (I-O) and excised outside-out (O-O) patches using standard patch-clamp techniques (11). Single-channel recordings were usually carried out in symmetrical solutions that were composed of (in mM) 130 K⁺, 1 Mg²⁺, 5 EGTA, 114.5 Cl⁻, and 10 HEPES-HCl at pH 7.4. Solutions containing 5 mM EGTA, zero Ca²⁺, and zero nucleotides were used to prevent changes in channel activity that could occur due to the involvement of Ca²⁺ and other second messengers. In experiments where we wished to shield membrane surface charge, recordings were made in symmetrical solutions composed of (in mM) 130 K⁺, 300 Na⁺, 5 EGTA, 10 HEPES-HCl, 1 Mg²⁺, and 415.5 Cl⁻ at pH 7.4; 330 K⁺, 5 EGTA, 10 HEPES-HCl, 1 Mg²⁺, and 314.5 Cl⁻ at pH 7.4; or 200 K⁺, 5 EGTA, 10 HEPES-HCl, 1 Mg²⁺, and 182.5 Cl⁻ at pH 7.4.

Preparation and Application of Compounds

Agents were applied to excised patches by pressure application from a glass pipette (“puffer pipette”) as described by Clarke et al. (3) while the bath was constantly perfused with recording solution. PCoA and octanoyl coenzyme A (OCoA) were purchased from Sigma Chemical. Concentrated stock solutions of these compounds were made up at a concentration of 1 mM in distilled water. Aliquots of this stock were diluted in the application (puffer) pipette solution to make up the desired concentration. PCoA and OCoA were usually applied to excised patches at concentrations of 10 and 50 μM, respectively. Myr-KPRPK and KPRPK were dissolved in water to make stock solutions of 50 and 75 mM, respectively. Myr-KPRPK and KPRPK were applied to excised patches at concentrations of 40 and 50 μM, respectively.

Oleic acid or tetradesanesulfonate were usually used to assess the responsiveness of patches to PCoA and myr-KPRPK. These compounds and tetracyclohexylmethylammonium bromide were prepared as described by Clarke et al. (3).

Synthesis of the Myristoylated and Nonmyristoylated Positively Charged Peptides

The myristoylated and nonmyristoylated positively charged peptides, KPRPK, were synthesized by Dr. Robert Carraway at the University of Massachusetts Medical School core peptide synthesis facility using 9-fluorenylmethoxycarbonyl (FMOC) technology and a Rainsin symphony peptide synthesizer (10). A prolyl residue was placed at the number 2 and number 4 positions to stabilize the peptide with regard to possible proteolytic degradation. Myristic acid was coupled to the α-amino group at the NH₂ terminus using HBTU [O-(benzotriazol-1-yl)-N,N,N',N'-tetramethylethyl]-hexafluorophosphate] activation. The peptides were cleaved from the solid phase by using trifluoroacetic acid, precipitated with ether, and lyophilized. After HPLC purification by reverse phase on μ-Bondapak C-18, the identity of each peptide was confirmed by amino acid analysis. The myristoylated peptide was considerably more hydrophobic during HPLC than the parent peptide.

Data Analysis and Display

Data acquisition and data analysis have been described previously (3). Briefly, qualitative changes in channel activity were determined by visual inspection of the current record. Some records were analyzed quantitatively using the analysis packages described below. For quantitative analysis, the change in activity was defined as a change in NP.o or the average number of open channels, where N is the number of channels in the patch (unknown) and P.o is the probability that an ion channel is in the open state. Patches chosen for analysis 1) showed low noise levels, 2) had very few or no other channel types evident in the patch that would have a major effect on the analysis, 3) could also show activation by a fatty acid control (when used), usually tetradesanesulfonate, and 4) were representative of each compound. If many patches could fit these criteria, patches used for analysis were chosen randomly from this group. The duration of the time period used for analysis before and during the application of a lipid was determined by visual inspection of the channel trace. Mean open times (T.o) were also determined over the same time periods as those used to determine NP.o.

Qualitative changes in NP.o are given in Table 1 and in the text, and more quantitative results are presented in Table 2. N.P.o, T.o, and i (unitary channel current) for Figs. 1 and 2 were calculated using pCLAMP6. For Figs. 3 and 4, the values of NP.o and i, given above the expanded traces for the periods before, during, and after the application of myr-KPRPK, were determined using the custom software package Erwin (see Ref. 3). The time period of the expanded traces shown, as well as a few seconds of data on either side of these expanded traces, was used to determine the values of NP.o and i shown (~5 s total). This was also the case for Figs. 8 and 9.

Table 1. Qualitative effects of palmitoyl coenzyme A and octanoyl coenzyme A on I-O and O-O patches

<table>
<thead>
<tr>
<th>Type of Compound</th>
<th>I-O</th>
<th>O-O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negatively Charged Compounds</td>
<td>A</td>
<td>S</td>
</tr>
<tr>
<td>Octanoyl coenzyme A (C8) in 130 mM K⁺</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Palmitoyl coenzyme A (C16) in 130 mM K⁺</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Palmitoyl coenzyme A (C16) in 200 mM K⁺</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Palmitoyl coenzyme A (C16) in 330 mM K⁺</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Palmitoyl coenzyme A (C16) in 130 mM K⁺, 300 mM Na⁺</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

A summary of qualitative effects of palmitoyl coenzyme A (PCoA) on I-O and O-O patches. The number of patches to which PCoA was applied and the qualitative effects are shown. The number of carbons in the carbon chain are given in parentheses. I-O refers to the excised inside-out patch configuration where compounds were applied to the intracellular surface of the membrane. O-O refers to the excised outside-out patch configuration where compounds were applied to the extracellular surface of the membrane. A, activation; S, suppression; N, no effect.
Expanded traces are taken from the compressed record in the same panel. The expanded channel trace representing a time period before the application of the test was taken sometime between 5 and 60 s before the compound was applied. The expanded channel trace representing a time period after the application of the compound was taken sometime between 5 and 120 s after the puffer was no longer ejecting the compound. The dotted lines in the expanded traces represent the single-channel current amplitude before the compound was applied.

Each patch was usually exposed to more than one agent, and an agent was usually applied more than once. In some cases there were residual effects on channel amplitude from previous exposures of the patch to myr-KPRPK and KPRPK. These residual effects on channel amplitude can sometimes be observed in the expanded traces (before and after application) and may be the consequence of the induction of channel substates or tethered channel blockade.

Data for Table 2 were analyzed using pCLAMP6. \( N_P \) and \( T_o \) were usually calculated for a period of time (10–120 s) before the application of the lipid and for 10–120 s during the time that the lipid exerted an effect. Because the activity of the channels in the patch was not always in a steady state (during application), the values of \( N_P \) and \( T_o \) represent an average over the time period used.

Channel activity varied greatly from patch to patch, so comparison of mean changes in \( N_P \) was not useful because consistent increases or decreases in channel activity could be masked by this natural variation. Therefore, to determine whether PCoA and myr-KPRPK alter BKCa channel \( N_P \) and \( T_o \), we compared these parameters (i.e., before and during the application of a lipid) with a paired t-test, where \( P < 0.05 \) was considered significant. Data are presented in Table 2 as mean fold changes in \( N_P \) and \( T_o \) because this most clearly illustrates the dramatic effects that these lipid compounds have on \( N_P \).

To determine whether PCoA and myr-KPRPK produced changes in \( N_P \) and \( T_o \) in charge screening solutions (330 mM K\(^+\), 5 mM EGTA or 130 mM K\(^+\), 300 mM Na\(^+\), 5 mM EGTA) similar to those seen in normal bathing solutions (130 mM K\(^+\), 5 mM EGTA), the fold changes in \( N_P \) in screening conditions were compared with the fold changes seen in normal bathing solutions with a t-test, where \( P < 0.05 \) was considered significant.

### RESULTS

#### Negatively Charged Lipids

To determine whether the site of fatty acid action and other negatively charged lipids is limited to one side of the membrane, we examined the effects of the negatively charged single-chain lipid PCoA on BKCa channel activity (see Ref. 24). PCoA is a single-chain lipid whose very large head group contains multiple negative charges. These features are most likely responsible for the inability of PCoA to flip across the lipid bilayer (12). PCoA, according to NMR studies, is confined to the side of the lipid bilayer to which it is applied (1).

PCoA has two different effects on channel activity but produces an increase in \( N_P \) only when applied to O-O patches. Application of PCoA to the outer membrane surface of O-O patches produced an increase in \( N_P \) (Fig. 1A, Table 1). In contrast, application of PCoA to the inner membrane surface of I-O patches produced a decrease in \( N_P \) (Fig. 1B, Table 1). Analysis of representative traces showed that both the increase in channel activity seen in O-O patches and the decrease in channel activity seen in I-O patches were significant (Table 2). Mean open time \( (T_o) \) was not significantly altered upon the application of PCoA to I-O and O-O patches in normal bathing solutions (Table 2), suggesting that PCoA alters channel behavior primarily through an alteration of the mean closed time of the channel (\( T_c \); see Ref. 3). There was no obvious alter-

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean Fold Change in ( N_P ) Before Application, ( \times 10^2 )</th>
<th>( N_P ) Significant Difference to Normal Solutions</th>
<th>Mean Fold Change in ( T_o ) Before Application, ( \times 10^2 )</th>
<th>Mean Fold Change in ( T_o ) During Application, ms</th>
<th>Mean Fold Change in ( T_o ) During Application, ms</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCoA O-O 130 mM K</td>
<td>0.25 ± 0.06</td>
<td>1.36 ± 0.60</td>
<td>9.5 ± 4.3</td>
<td>2.2 ± 1.4*</td>
<td>25.0 ± 19.0</td>
<td>41.3 ± 46.5</td>
</tr>
<tr>
<td>PCoA O-O 130 mM K</td>
<td>0.81 ± 0.18</td>
<td>0.92 ± 0.02</td>
<td>Yes</td>
<td>29.8 ± 7.5</td>
<td>22.0 ± 0.3</td>
<td>43.3 ± 17.6</td>
</tr>
<tr>
<td>PCoA O-O 300 mM Na</td>
<td>0.93 ± 0.32</td>
<td>1.04 ± 0.14</td>
<td>3.5 ± 1.9</td>
<td>3.2 ± 1.8</td>
<td>9.3 ± 0.2</td>
<td>9.8 ± 1.2</td>
</tr>
<tr>
<td>PCoA O-O 330 mM K</td>
<td>5.52 ± 5.2</td>
<td>1.70 ± 0.82</td>
<td>3.7 ± 2.5</td>
<td>17.0 ± 9.8*</td>
<td>11.1 ± 5.9</td>
<td>16.0 ± 4.6</td>
</tr>
<tr>
<td>PCoA O-O 330 mM K</td>
<td>3.20 ± 0.83</td>
<td>1.50 ± 0.11</td>
<td>No</td>
<td>6.8 ± 3.5</td>
<td>21.1 ± 10.6*</td>
<td>10.1 ± 1.4</td>
</tr>
<tr>
<td>PCoA O-O 130 mM K</td>
<td>4.64 ± 1.90</td>
<td>1.52 ± 0.22</td>
<td>No</td>
<td>13.7 ± 8.1</td>
<td>58.9 ± 31.1*</td>
<td>21.8 ± 10.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds</th>
<th>( N_P ) Significant Difference to Normal Solutions</th>
<th>Mean Fold Change in ( N_P ) Before Application, ( \times 10^2 )</th>
<th>Mean Fold Change in ( N_P ) During Application, ( \times 10^2 )</th>
<th>Mean Fold Change in ( T_o ) Before Application, ms</th>
<th>Mean Fold Change in ( T_o ) During Application, ms</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPRPK O-O 330 mM K</td>
<td>2.29 ± 0.73</td>
<td>1.25 ± 0.26</td>
<td>7.3 ± 2.1</td>
<td>16.8 ± 7.1*</td>
<td>15.0 ± 3.7</td>
<td>18.6 ± 6.1*</td>
</tr>
<tr>
<td>myr-KPRPK O-O 130 mM K</td>
<td>0.32 ± 0.10</td>
<td>0.91 ± 0.17</td>
<td>6.1 ± 1.0</td>
<td>1.8 ± 0.5*</td>
<td>9.9 ± 1.7</td>
<td>9.0 ± 2.4</td>
</tr>
<tr>
<td>myr-KPRPK O-O 330 mM K</td>
<td>0.42 ± 0.17</td>
<td>0.77 ± 0.38</td>
<td>No</td>
<td>17.9 ± 7.9</td>
<td>7.5 ± 4.4*</td>
<td>19.0 ± 5.5</td>
</tr>
</tbody>
</table>

Values are means ± SD for fold changes in channel activity (\( N_P \)) and mean open time (\( T_o \)) given for patches before and during the effect of PCoA and the myristoylated pentapeptide, myr-KPRPK. \( N_P \) and \( T_o \) were usually calculated for a period of time 10–120 s before the application of these compounds and for 10–120 s during the effect of these compounds. \( N_P \) and \( T_o \) (means ± SD) before and during the application of compounds are also shown. These values were calculated from patches selected using the criteria outlined in MATERIALS AND METHODS. The number of patches (\( n \)) used for these calculations is indicated. *Significantly different (using a paired t-test, where \( P < 0.05 \)). A yes (significant) or no (not significant) indicates which fold changes in \( N_P \) produced by the application of these lipids in screening conditions, are significantly or nonsignificantly different from the fold changes seen in normal bathing solutions (\( t \)-test where \( P < 0.05 \) was considered significant).

### Table 2. Effects of PCoA and myr-KPRPK on \( N_P \) and \( T_o \)

- PCoA is a single-chain lipid whose very large head group contains multiple negative charges.
- These features are most likely responsible for the inability of PCoA to flip across the lipid bilayer.
- PCoA, according to NMR studies, is confined to the side of the lipid bilayer to which it is applied.
- The site of fatty acid action and other negatively charged lipids is limited to one side of the membrane.
- To determine whether PCoA alters channel behavior primarily through an alteration of the mean closed time of the channel (\( T_c \)).
ation of the single-channel current amplitude \(i\) in either patch configuration. Thus application of PCoA to the outer surface of the membrane produces the same effect as fatty acids applied to either surface, an increase in \(N_{Po}\) (3, 16). However, the effect of PCoA at the inner membrane surface, a decrease in \(N_{Po}\), is not observed with fatty acids (3, 16).

Insertion of charged compounds into the lipid bilayer may alter membrane surface charge (3, 13). Because PCoA will only insert into the membrane leaflet to which it is applied (i.e., inner or outer) and has four negative charges, this compound is more likely to affect membrane surface charge than fatty acids, which bear only a single charge and may not preferentially insert into a membrane leaflet (3, 4). Therefore, we investigated whether one or both of the effects of PCoA resulted from an alteration in membrane surface charge. For example, incorporation of PCoA into the inner membrane leaflet could increase the negative charge associated with the inner membrane leaflet and cause a shift in the voltage dependence of channel activation to more negative potentials such that at any particular membrane potential, channel activity is decreased (3, 13). The opposite effect would be predicted if PCoA inserted into the outer membrane leaflet.

The effect of PCoA applied to the external surface of the membrane does not appear to be dependent on membrane surface charge. To determine whether the effects of PCoA on channel activity might be due to alterations in membrane surface charge, we applied PCoA to excised patches in the presence of high-ionic strength bathing and pipette solutions that are known to shield surface charge (18, 19). As the ionic strength of the solutions was increased, the fold decrease in \(N_{Po}\) was not as great and was seen less often when PCoA was applied to I-O patches (Fig. 2B, Tables 1 and 2). The fold change in \(N_{Po}\) in the high-ionic strength solution (130 mM K\(^+\)/11001, 300 mM Na\(^+\)/11001) was found to be significantly different from the fold change in \(N_{Po}\) seen in the normal ionic strength solution (130 mM K\(^+\)/11001; Table 2), suggesting that the effects on I-O patches may have been due to changes in membrane surface charge. However, in high-ionic strength solutions, the application of oleic acid, tetradecanesulfonate (not shown), and other negatively charged lipids to I-O patches could still produce a similar fold increase in \(N_{Po}\) (3, 4).

Under these same charge screening conditions, PCoA could also still activate BK Ca channels when applied to O-O patches (Fig. 2A, Table 1). The fold changes in \(N_{Po}\) produced by the application of PCoA to O-O patches in high-ionic strength solutions (i.e., 330 mM K\(^+\) vs. 130 mM K\(^+\), 300 mM Na\(^+\); Table 2) were not significantly different \((P > 0.1)\) from each other and from those seen under normal ionic strength conditions (i.e., 330 mM K\(^+\) or 130 mM K\(^+\), 300 mM Na\(^+\) vs. 130 mM K\(^+\); Table 2). Thus it is unlikely that the increase in \(N_{Po}\) produced by external application of PCoA was
due to alteration of membrane surface charge. \( T_o \) was altered by the application of PCoA to I-O and O-O patches in high-ionic strength solutions but could not account for the changes seen in \( N_{Po} \). Thus, like fatty acids and other negatively charged lipids (3), PCoA appears to alter \( N_{Po} \) primarily through changes in \( T_c \). The unitary current, \( i \), was unaltered.

OCoA, a short-chain derivative of PCoA, was ineffective at altering \( N_{Po} \) in O-O patches in normal bathing and pipette solutions (Tables 1 and 2). This result is consistent with our previous findings that negatively charged lipids, with eight carbons or less in their acyl or alkyl chains, are unable to alter BKCa channel \( N_{Po} \) (3, 4).

In summary, these results suggest that negatively charged lipids increase the \( N_{Po} \) of BKCa channels by acting on the extracellular (outer) membrane surface and that they do so through a mechanism that is most likely independent of changes in external membrane surface charge.

**Positively Charged Lipids**

To determine the site of action of positively charged lipids on the BKCa channel, we used a synthetic, positively charged myristoylated pentapeptide (myr-KPRPK). Myr-KPRPK has a 14-carbon chain with 5 amino acids (lysine, proline, arginine, proline, and lysine) attached to this chain. Lysine and arginine residues are positively charged at physiological pH, and thus myr-KPRPK carries three positive charges. The size and multiple charges of the head group make it unlikely that this compound would be able to flip across the lipid bilayer (14).

Myr-KPRPK decreased \( N_{Po} \) only when applied to the outer membrane surface and produced flickery channel openings when applied to the inner membrane surface. Myr-KPRPK produced a decrease in channel \( N_{Po} \) when applied to O-O patches (6 of 6 O-O patches; Fig. 3A), an effect consistent with other positively charged lipids (3, 4). Analysis of representative traces showed that this change was significant (Table 2). As with other positively charged lipids, this decrease in \( N_{Po} \) appears to result from an increase in \( T_c \) (Table 2), because \( T_o \) was not significantly altered and could not account for the changes seen in \( N_{Po} \) (see Ref. 3). In addition, application of myr-KPRPK to O-O patches appeared to produce a decrease in single-channel amplitude (Fig. 3B, during application).

Similar results were obtained in high-ionic strength solutions (330 mM K\(^+\); Fig. 4, Table 2), where a decrease in \( N_{Po} \) was seen in 8 of 8 O-O patches. Again, a small decrease in single-channel amplitude was observed, and upon analysis, the change in \( N_{Po} \) was shown to be significant in high-ionic strength solutions (Table 2). The average fold change in \( N_{Po} \) (Table 2) seen in high-ionic strength solutions (330 mM K\(^+\)) was not significantly different (\( P > 0.1 \)) from that seen under normal ionic strength conditions (130 mM K\(^+\)), making it unlikely that the change was due to an alteration of membrane surface charge.

Application of myr-KPRPK to I-O patches did not appear to produce a decrease in \( N_{Po} \). Instead, myr-KPRPK produced an effect that was not typically seen with the application of other positively charged lipids (e.g., sphingosine) on this channel: a decrease in single-channel amplitude accompanied by flickery channel
openings (9 of 9 I-O patches; Fig. 5, A and B). These flickery channel openings were also observed in the high-ionic strength solution of 330 mM K⁺/H₂O (3 of 3 I-O patches; Fig. 6). In some cases the flickery channel openings were accompanied by a delayed increase in channel activity that usually began and persisted after the application of myr-KPRPK ceased (see Fig. 5B for an example). Sometimes this increase in activity partially recovered, and sometimes it accompanied the loss of the patch. This increase in activity was more likely to occur if the patch had been previously exposed to the myr-KPRPK and was also observed in high-ionic strength solutions.

The changes in single-channel amplitude caused by myr-KPRPK and other agents (see below) in I-O and O-O patches were not studied in detail. The amplitude decrease was not uniform and did not seem to fully recover.

The results described so far suggest that the site of action of positively charged lipids, which decrease channel activity, is also (like negatively charged lipids) on the outer membrane surface. The decrease in channel activity by myr-KPRPK on O-O patches differed in one small way from those of other positively charged lipids: the response to the former was much more rapidly reversible upon cessation of application. This might be expected, because myr-KPRPK would not be likely to have the same effective binding affinity as the positively charged lipids used previously (Ref. 3, also see below).

**Application of Myr-KPRPK to I-O patches produced an effect similar to that of tetradecyltrimethylammonium bromide, a compound that acts like other K⁺ channel blockers.** The effect of the myr-KPRPK on channel openings, when applied to I-O patches, was reminiscent of other K⁺ channel blockers, such as tetraethylammonium (TEA) (2, 27). We applied tetradecyltrimethylammonium bromide (TDTMA-Br), a lipid compound that has a head group structure similar to that of TEA, to excised membrane patches.
TDTMA-Br is a 14-carbon lipid whose positively charged head group contains three methyl groups. Application of TDTMA-Br to I-O patches produced a similar response (5 patches) to the application of myr-KPRPK to O-O patches: a reduction of single-channel current amplitude and flickery channel openings (Fig. 7). In addition, channel activity appeared to increase after application ceased (Fig. 7). Thus, when applied to I-O patches, both TDTMA-Br and myr-KPRPK appear to act on channel openings in a manner similar to that of tetramethylammonium and TEA, known blockers of BKCa channels (2, 6, 28).

The nonmyristoylated peptide, KPRPK, did not decrease NPo when applied to the outer membrane surface. As a control we applied the nonmyristoylated peptide KPRPK. This peptide did not produce the decrease in NPo observed with the myr-KPRPK on O-O patches but, instead, produced an increase in NPo (2 of 2 O-O patches). This result is consistent with experiments showing that to effectively decrease NPo, positively charged lipids require a sufficiently long (C ≥ 8) carbon chain (3, 4). In addition, a small decrease in single-channel amplitude was observed in some patches, but this was not a consistent finding. These results were also obtained in high-ionic strength solutions (6 of 6 O-O patches), where KPRPK produced a significant change in NPo (Fig. 8; Table 2). The ability of KPRPK to increase channel activity when applied to O-O patches might, depending on the orientation of the molecule, counter the effect of the myr-KPRPK and therefore may also partially explain the rapid reversibility seen with the latter. KPRPK also produced a small but significant increase in To when applied to O-O patches in high-ionic strength solutions.

The nonmyristoylated peptide, KPRPK, produced an effect similar to that of the short-chain positively charged lipid, octylamine, when applied to the inner membrane surface. The peptide KPRPK did not produce the flickery channel openings found with myr-KPRPK but did produce a decrease in single-channel amplitude when applied to I-O patches (3 of 3 I-O patches; Fig. 9A). This decrease was also observed in high-ionic strength solutions (3 of 3 I-O patches). Interestingly, the effect of the nonmyristoylated peptide
on I-O patches was strikingly similar to that produced by the application of the short-chain positively charged lipid, octylamine (see Ref. 3), on O-O and I-O patches (Fig. 9B). Therefore, these results suggest that the longer carbon chain is required for myr-KPRPK to produce flickery channel openings when applied to the cytosolic surface of the channel.

**DISCUSSION**

It is well established that fatty acids modulate the activity of a large number of ion channels (17, 20–25). An understanding of the mechanism of modulation has been hampered, however, by the fact that fatty acids may be able to modulate ion channel activity through a large number of indirect mechanisms, particularly those involving alterations of the lipid bilayer, for example, by acting as detergents to perturb the lipid membrane or by altering membrane fluidity. In addition, it is possible that fatty acids affect channel behavior by altering membrane surface charge (see Ref. 3 and references therein for a discussion of these indirect mechanisms). In our previous study (3), we found no evidence that these indirect mechanisms are involved in the fatty acid modulation of BKCa channels from rabbit pulmonary artery smooth muscle cells.

Our findings here provide evidence that alterations in membrane curvature are also not involved in BKCa channel modulation by charged lipids. By using charged lipids that do not cross the bilayer, we limited their application to one side of the membrane. Therefore, we could determine whether insertion of the lipid into one leaflet (possibly causing bilayer curvature in one direction) would cause channel activation, whereas insertion into the other leaflet (causing curvature in the opposite direction) would cause channel inhibition, as predicted by the bilayer couple hypothesis where there is specific curvature dependence of channel activity (see Ref. 3 and references therein for a fuller discussion of this possibility). Under surface charge shielding conditions, channel activation was seen only when PCoA was applied to the extracellular side. There was a much smaller, nonsignificant effect when PCoA was applied to the intracellular surface.

The identification of fatty acid or lipid binding sites on these ion channel proteins is an important step toward demonstrating that ion channels could also be
direct targets of lipid signaling molecules. Evidence consistent with fatty acids modulating BK$_{Ca}$ channel activity by interacting with a protein site either on the ion channel itself or, alternatively, on some channel-associated protein is provided by this study, because it shows that charged lipids, which are unlikely to flip across the bilayer, produce their effects (3, 4) only when applied to one side of the membrane.

The Effects of PCoA Are Consistent With an External Site of Action for Negatively Charged Lipids

PCoA produced an increase in $N_p$ only when applied to O-O patches, suggesting that the site of action of negatively charged lipids is on the external membrane surface. The decrease in $N_p$ caused by the application of PCoA to the internal surface is most likely due to an alteration in membrane surface charge because charge screening conditions could almost eliminate this response, changing it from a large, significant fold decrease in activity to a small, nonsignificant fold decrease. Fatty acids and other negatively charged lipids did not produce this surface charge-induced decrease in channel activity. However, because PCoA has a large head group that contains multiple negative charges, it could be predicted that the very property that would prevent it from flipping across the bilayer (1) might also make it more likely to have an effect on membrane surface charge than fatty acids. These two features will most likely be a property of any natural compound appropriate for our purpose of identifying the sidedness of action. However, PCoA is indeed appropriate for this study because 1) its inability to flip across the membrane has been previously demonstrated (1), 2) like fatty acids, its ability to modulate channel activity is dependent on the length of the carbon chain, and 3) we were able to separate out the surface charge effects using high-ionic strength solutions. We have previously used PCoA to identify the site of action of fatty acids on a small-conductance K$^+$ channel from toad stomach smooth muscle. Similarly, Denson et al. (7) have successfully used arachidonoyl CoA to identify the site of action of fatty acids on a different type of BK channel in GH$_3$ cells.

OCoA, a compound with the same head group structure as PCoA but that has only an eight-carbon alky chain, did not affect BK$_{Ca}$ channel $N_p$ when applied to...
Fig. 7. Tetradecyltrimethylammonium bromide (TDTMA-Br) produces flickery channel openings when applied to I-O patches. As shown in this I-O patch held at +20 mV, application of TDTMA-Br causes a reduction in the single-channel current amplitude, and channel openings appear flickery (B–D). This recording was obtained in symmetrical 130 mM K⁺-containing solutions. Channel NP, is also increased after the application of TDTMA-Br (D). Expanded traces A–D show channel activity during these corresponding periods in the compressed, full-length channel record.

Fig. 8. The control, nonmyristoylated peptide (KPRPK) increased NP, when applied to the extracellular membrane surface. In an O-O patch held at +30 mV in high-ionic strength solutions (symmetrical 330 mM K⁺), the control, nonmyristoylated peptide KPRPK (40 μM) caused an increase in channel NP. Before the data shown were recorded, this patch had been exposed 4 times to myr-KPRPK and twice to KPRPK.
O-O patches. This result supports our previous finding that a chain length of more than eight carbons is required for BK<sub>Ca</sub> channel modulation (3, 4) and suggests that PCoA must either enter the membrane and/or interact with a hydrophobic domain of the ion channel or some other membrane-bound protein.

**The Effects of the Myristoylated Pentapeptide Are Consistent With an External Site of Action for Positively Charged Lipids**

When applied to the outside surface of the membrane, myr-KPRPK produced a response typical of the positively charged lipids, a decrease in NP<sub>o</sub>. In contrast, the nonmyristoylated KPRPK did not produce such a decrease when applied to O-O patches; instead, it produced an increase in activity. Thus, like charged lipids, which require a sufficiently long acyl or alkyl chain to effectively alter NP<sub>o</sub> (3), the decrease in activity produced by the myr-KPRPK required the presence of the 14-carbon chain.

Myr-KPRPK, when applied to the internal membrane surface, did not appear to affect channel activity in a way similar to that of other charged lipids (e.g., sphingosine). Instead, it produced a response similar to that of TEA and other alkyl-TEA derivatives: flickery channel openings that are consistent with channel blockade (2). Flickery openings were occasionally accompanied by a delayed and apparently irreversible increase in channel activity.

Interestingly, the flickery channel openings obtained by application of myr-KPRPK to I-O patches were also obtained by the application, to I-O patches, of the positively charged lipid TDTMA-Br, a compound whose head group structure is very similar to tetramethylammonium and TEA, both known blockers of BK<sub>Ca</sub> channels (2, 6, 28).

Flickery channel openings were not observed with the nonmyristoylated positively charged peptide KPRPK on I-O patches. Instead, KPRPK produced the same type of response as the short-chain positively charged lipid, octylamine: a decrease in single-channel amplitude suggestive of fast channel block (13). Therefore, the presence of the myristate group changes the effect that the positively charged peptide KPRPK has on this channel.

Thus the positively charged myristoylated pentapeptide has multiple effects on the BK<sub>Ca</sub> channel, with the characteristic positively charged lipid effect (i.e., a decrease in NP<sub>o</sub>) seen only when the peptide is applied to the outer membrane surface.

**Possible Sites of Interaction of Negatively and Positively Charged Lipids on the BK<sub>Ca</sub> Channel**

The ability of the positively charged myristoylated pentapeptide to act like TEA on I-O patches and as a positively charged single-chain lipid on O-O patches may help to explain the results of Choi et al. (2). They found that mutations in the P region of the Shaker K<sup>+</sup> channel, which effectively inhibited the blocking action of TEA, were much less effective at inhibiting channel block by the longer chain alkyl-TEA compounds. These experiments used alkyl-TEA compounds that had as many as 10 carbons in the alkyl chain. Because these experiments did not examine single-channel behavior but, instead, measured whole patch current, channel block was seen as a decrease in this current. Thus the decreased ability of these mutations to affect channel blockade by the longer chain alkyl-TEA compounds...
may be explained if the “longer” compounds could also decrease current by acting like sphingosine and other positively charged lipids to directly modulate channel activity. Such an interpretation would require that the site of action of positively charged lipids on the Shaker $K^+$ channel is on the inner membrane surface, and not the outer membrane surface that we found for the $BK_{Ca}$ channel.

Choi et al. (2) also found that mutations at amino acid position 469, a residue within $S6$, produced prominent effects on the affinities of the longer chain alkyl-TEA compounds (C8 and C10) but much smaller effects on the affinity of TEA itself. This result suggests that amino acid residue 469 is involved in the binding of the hydrophobic TEA blockers to the Shaker channel. Because we found that a chain length of eight carbons or greater is required for fatty acids and other charged lipids to effectively alter $BK_{Ca}$ channel activity (3, 4), a similar residue within $S6$ or another transmembrane-spanning region may also play a role in the binding of lipid compounds to the $BK_{Ca}$ channel.

The amino acid sites that presumably interact with the charge on the head group of the positively and negatively charged lipids to bring about changes in $BK_{Ca}$ channel activity are unknown. Although these sites are most likely found on the external membrane surface, it remains unresolved whether the positively and negatively charged lipids interact with the same or different amino acids. The fact that both positively and negatively charged lipids act from the external membrane surface is consistent with the previously proposed hypothesis (15) that these compounds may be interacting with the positively charged residues of the voltage sensor in $S4$.

In concurrence with our result, epoxyeicosatrienoic acid was shown to only increase the activity of $BK_{Ca}$ channels from airway smooth muscle cells when it was applied to the extracellular side of the channel in lipid bilayers (9). However, the sidedness of action of fatty acids on the $BK_{Ca}$ channel from the rabbit pulmonary artery is different from that identified for both the small-conductance $K^+$ channel of the toad stomach smooth muscle cells (24) and a $BK$ channel with different characteristics found in $GH_3$ cells (7). For the small-conductance $K^+$ channel, PCoA only increased $NP_{o}$ when applied to the cytosolic surface and had no effect when applied to O-O patches. Similarly in $GH_3$ cells, $BK$ channel activity was increased when arachidonoyl CoA was applied to the cytosolic surface. In this latter case, an intracellular site of action is in agreement with the mechanism of action of fatty acids identified for the channel: an increase in $Ca^{2+}$ sensitivity (8). These differences in the sidedness of action argue against fatty acids and charged lipids acting through a nonspecific mechanism to bring about channel modulation.

Evidence suggesting that fatty acids and other charged lipids, like sphingosine, act as endogenous messenger molecules within different cell types has accumulated over the past several years (20, 26). Thus the finding that the site of interaction between fatty acids and other charged lipids with the $BK_{Ca}$ channel is on the outer membrane surface is an interesting one. For the modulation of $BK_{Ca}$ channel activity by fatty acids and other charged lipids to be physiologically relevant, fatty acids released from membrane phospholipids would have to “flip” across the bilayer before acting on the channel. Alternatively, circulating fatty acids or fatty acids released from other surrounding cells may be the physiological source of fatty acids that act on $BK_{Ca}$ channels.

In conclusion, the data presented here suggest that the site of action of PCoA, when acting in a manner similar to fatty acids and other negatively charged lipids, is on the external side of the membrane. The site of action of myr-KPRLPK, when acting in a manner similar to other positively charged lipids like, for example, sphingosine, is also on the outer membrane surface.

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