corresponds to a concentration of labeled glutamine within the vesicles of about 20–30 μM (i.e., an approximate 100-fold enrichment). We have found no evidence for either membrane potential or ion gradient requirements to account for this concentrative uptake. External ions are not needed, and the usual preincubation buffer containing potassium can be replaced with no significant change in uptake kinetics. The concentrative effect we are seeing is probably due to an exchange reaction between the external labeled glutamine and unlabeled glutamine that has been retained within the vesicle preparation. Indeed, amino acid analysis of several vesicle preparations indicated that even after extensive washing L-glutamine is present at about 50 μM levels. This is not surprising given the high endogenous levels of glutamine in the CNS (3–5 mM) (Kvamme, 1983), and this level is sufficient to account for the observed concentration of the labeled glutamine by the system.

References

Balcar, V. J., & Johnston, G. A. R. (1975) *J. Neurochem. 24*, 875–879.

Balcar, V. J., & Hauser, K. L. (1978) *Proc. Eur. Soc. Neurochem. 1*, 498.

Baldessarini, R. J., & Yorke, C. (1974) *J. Neurochem. 23*, 839–848.

Benjamin, A. M., Verjee, Z. H., & Quastel, J. H. (1980) *J. Neurochem. 35*, 67–77.

Cohen, S. R., & Lajtha, A. (1972) in *Handbook of Neurochemistry* (Lajtha, A., Ed.) Vol. 7, pp 543–572, Plenum Press, New York.

Cotman, C. W., & Taylor, D. (1972) *J. Cell Biol. 55*, 696–711.

Johansen, L., Roberg, B., & Kvamme, E. (1987) *Neurochem. Res. 12*, 135–140.

Kanner, B. I. (1978) *Biochemistry 17*, 1207–1211.

Kanner, B. I., & Sharon, I. (1978) *Biochemistry 17*, 3949–3953.

Keynan, S., & Kanner, B. (1988) *Biochemistry 27*, 12–17.

Kvamme, E. (1983) in *Handbook of Neurochemistry* (Lajtha, A., Ed.) Vol. 3, 2nd ed., pp 405–422, Plenum Press, New York.

Kvamme, E., Torgner, I. Å., & Svenneby, G. (1985) in *Methods in Enzymology* (Meister, A., Ed.) Vol. 113, pp 241–256, Academic Press, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Minn, A., & Besagni, D. (1983) *Life Sci. 33*, 225–232.

Roberts, P. J. (1976) *Adv. Exp. Med. Biol. 69*, 165–178.

Roberts, P. J., & Keen, P. (1974) *Brain Res. 67*, 352–357.

Robinson, M. B., Crooks, S. L., Johnson, R. L., & Koerner, J. F. (1985) *Biochemistry 24*, 2401–2405.

Schousboe, A., Hertz, L., Svenneby, G., & Kvamme, E. (1979) *J. Neurochem. 32*, 943–950.

Shank, R. P., & Campbell, G. L. (1982) *Neurochem. Res. 7*, 601–616.

Walum, E., & Weiler, C. (1978) *Proc. Eur. Soc. Neurochem. 1*, 499.

Weiler, C. T., Nystrom, B., & Hamberger, A. (1979) *J. Neurochem. 32*, 559–565.

Yu, A. C. H., & Hertz, L. (1982) *J. Neurosci. Res. 7*, 23–35.

Functional Modification of a Ca^{2+}-Activated K^+ Channel by Trimethyloxonium†

Roderick MacKinnon‡ and Christopher Miller*

Howard Hughes Medical Institute and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received April 7, 1989; Revised Manuscript Received June 12, 1989

ABSTRACT: Single Ca^{2+}-activated K^+ channels from rat skeletal muscle plasma membranes were studied in neutral phospholipid bilayers. Channels were chemically modified by briefly exposing the external side to the carboxyl group modifying reagent trimethyloxonium (TMO). TMO modification, in a "multi-hit" fashion, reduces the single-channel conductance without affecting ion selectivity. Modification also shifts the voltage activation curve toward more depolarized voltages and reduces the affinity of the channel blocker charybdotoxin (CTX). CTX, bound to the channel during the TMO exposure, prevents the TMO-induced reduction of the single-channel conductance. These data suggest that the high-conductance Ca^{2+}-activated K^+ channel has carboxyl groups on its external surface. These groups influence ion conduction, gating, and the binding of CTX.

Ion channels are integral membrane proteins that serve as the molecular basis for cellular electrical excitability. They perform the seemingly simple task of allowing ions to diffuse passively down their electrochemical gradients. However, to do this in a physiologically useful way, ion channels must exhibit several sophisticated properties. In response to external signals, such as transmembrane voltage changes or binding of specific ligands, they must be able to switch rapidly between nonconducting and conducting states. The open channel must be able to discriminate among various ions, allowing only one or a few types to permeate. Moreover, many channels are specifically altered in function by natural toxins or synthetic drugs which bind to receptor sites on the channel protein.

† Supported by NIH Research Grant GM-31768 and by NIH Training Grant NS-07292.

‡ Present address: Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.

0006-2960/89/0428-8087$01.50/0

These three characteristics of ion channels—voltage- and ligand-dependent gating, specific ion conduction, and toxin block—are all subject to experimental attack at high resolution through the use of single-channel recording methods.

The high-conductance Ca^{2+}-activated K^+ channel from mammalian skeletal muscle has been under close study for several years. It displays a wealth of mechanistically interesting properties, including high conductance and strong ionic selectivity (Blatz & Magleby, 1984; Yellen, 1984; Moczydlowski et al., 1985; Eisenman et al., 1986), voltage-dependent gating which is sensitive to internal Ca^{2+} (Barrett et al., 1982; Magleby & Pallotta, 1983; Moczydlowski & Latorre, 1983), and high-affinity block by charybdotoxin (CTX), a peptide inhibitor from scorpion venom (Miller et al., 1985; Anderson et al., 1988; MacKinnon & Miller, 1988). In each of these functions, negatively charged protein residues are thought to be involved (Latorre, 1986; Anderson et al., 1988). In this study, we investigate this possibility experimentally by chemically modifying the Ca^{2+}-activated K^+ channel using trimethyloxonium (TMO), a cationic methyl group donor. Trialkyloxonium reagents are known to react specifically with carboxylate residues in several enzymes (Nakayama et al., 1970; Parsons et al., 1969), and TMO in particular has been shown to affect profoundly the properties of an ion channel, the excitable membrane Na^+ channel (Shrager & Profera, 1973; Sigworth & Spalding, 1980; Spalding, 1980; Worley et al., 1986).

Our results show that TMO modification alters the function of the high-conductance Ca^{2+}-activated K^+ channel in three separate ways. Modification of the channel from the external solution lowers the single-channel conductance, shifts the voltage activation curve to more depolarized potentials, and greatly reduces the affinity of CTX block. The effects of TMO modification become increasingly apparent as ionic strength is lowered. Our results are consistent with the view that this channel has several carboxyl groups close to its externally facing mouth and that these negatively charged groups affect the channel conductance, the CTX affinity, and the transmembrane electric field sensed by the gating mechanism. In the following paper (MacKinnon et al., 1989), we show that these TMO-induced changes in channel function are all natural consequences of removal of negative surface charges from the external face of the channel protein.

Materials and Methods

Biochemical Reagents. Lipids were 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and the corresponding phosphatidylethanolamine (POPE), obtained from Avanti Polar Lipids, Inc., Birmingham, AL. TMO was obtained as the tetrafluoroborate salt from Fluka Chemical Corp., Ronkonkoma, NY. Transverse tubule membranes were prepared from rat skeletal muscle as previously described (Eisenman et al., 1986). CTX was purified from the lyophilized venom of *Leiurus quinquestriatus* (Latoxan, Rosans, France) by a previously described method (Anderson et al., 1988).

Fusion of Channels with Planar Bilayers and Chemical Modification. Planar lipid bilayers were formed by applying a drop of lipid solution (14 mM POPE + 6 mM POPC in *n*-decane) to a 250-μm-diameter hole in a plastic partition separating two chambers filled with an aqueous solution. Single Ca^{2+}-activated K^+ channels were inserted into the bilayer, using rat skeletal muscle plasma membrane vesicles as the source of channels, under conditions promoting vesicle fusion with the bilayer (Latorre et al., 1982). Vesicles (1–5 μg/mL) were added to the internal solution, which was 145 mM KCl, 10 mM MOPS, 0.01 mM $CaCl_2$, and 6 mM KOH, pH 7.4; the external solution was either 4 mM MOPS/2.5 mM NaOH, pH 7.4, or 10 mM MOPS/6 mM NaOH, pH 7.4. Under these conditions, the channels always incorporated with unambiguous physiological orientation, i.e., with the Ca^{2+} activation site facing the internal solution. After incorporation of a single channel, further insertion was suppressed by adding the desired concentration of KCl to the external solution; in some cases, vesicles were removed by extensive perfusion of the internal chamber.

Ca^{2+}-activated K^+ channels were modified by TMO after incorporation into planar bilayers. Following the insertion of a single channel as described above, a control current record was taken, and the 60 mM MOPS/48 mM KOH, pH 7.8, was added to the external solution. Solid TMO, to give a concentration of 50 mM, was added while stirring vigorously, followed by 35 mM KOH added over 10 s after introducing the TMO. Vigorous stirring was necessary to allow TMO to diffuse up to the bilayer membrane before being hydrolyzed; under these conditions the TMO hydrolysis rate constant is 0.14 s^{-1}, as determined by the rate of proton liberation. Thirty seconds after addition of TMO to the external solution, the chamber was perfused extensively with at least 10 volumes of fresh solution containing the initial ionic composition. The single-channel current was again measured; these conditions resulted in functional modification (a reduction in the current) in almost every attempt.

Data Collection and Analysis. The voltage clamp circuit for measuring single-channel currents was as previously described (Hanke & Miller, 1983). A desired voltage was applied across the bilayer, and the transmembrane current was measured with a low-noise current-to-voltage converter circuit. The current signal was filtered at 0.5–1 kH using an eight-pole Bessel filter and recorded on videotape. The voltage command was applied by a laboratory computer, which was also used for data analysis. The electrophysiological voltage convention is used, with the external side of the channel defined as zero voltage.

In this paper, we analyze three separate properties of Ca^{2+}-activated K^+ channels: the open-channel current amplitude, the equilibrium between the open (conducting) and closed (nonconducting) states of the channel, and channel block by CTX. The association and dissociation rates of CTX were determined from the statistical distributions of the blocked and unblocked dwell times measured directly from the single-channel record. Blocking events were defined as nonconducting intervals longer than 500 ms, and the usual corrections for missed events were employed (Miller et al., 1987).

Results

TMO Modification Reduces the Single-Channel Current. Figure 1 shows single-channel events before and after modification by TMO from the "external" side of the membrane. After TMO exposure, the chamber was extensively perfused with fresh solution so that channels were always observed under identical ionic conditions. In the experiment shown here, the current was reduced by both the first and second exposures to TMO, but additional exposures had no further effects. In some experiments, all of the current reduction occurred after a single exposure to TMO, but under these conditions, a partial reduction in current was the most common response to the first TMO treatment. Since a single site modification should produce an all-or-none effect, we interpret the stepwise response to mean that TMO affects the current by modifying the channel at more than one site.

TMO reduces the channel current by modifying the channel protein, not the membrane lipids. In Figure 2A (upper trace),

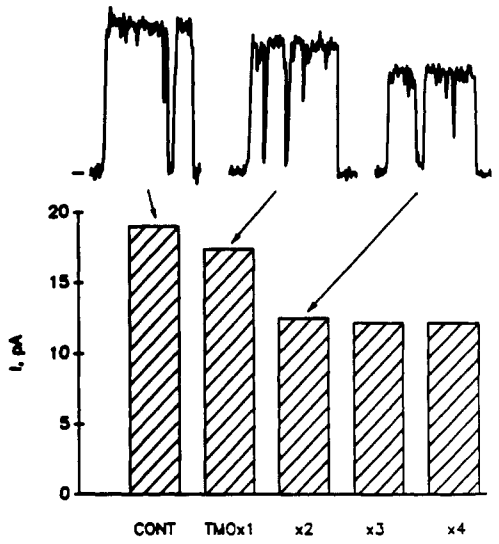

FIGURE 1: TMO treatment reduces the open-channel current. Current through a single channel was measured at 20 mV. The internal solution contained 150 mM KCl, and the external solution was 4.0 mM MOPS/NaOH, pH 7.4 (control). Then 50 mM TMO was added to the external solution as described under Materials and Methods. After 30 s, the external chamber was perfused back to 4.0 mM MOPS/NaOH, and the open channel current was again measured. This sequence was repeated 4 times. The top of the figure shows the open channel before and after the first and second TMO exposures.

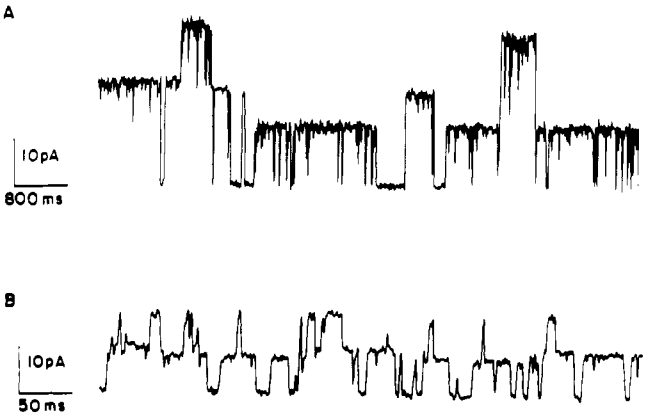

FIGURE 2: TMO reduction of single-channel current does not reflect lipid modification. A single channel was fused into a bilayer and was modified as in Figure 1. After perfusion of all excess TMO from the external chamber, a second channel was fused into the bilayer. The internal solution contained 150 mM KCl. The external solution was 4 mM MOPS/NaOH, pH 7.4 (A), and then 150 mM NaCl was added to the outside (B). Both the modified and unmodified channels can be seen fluctuating in the same membrane.

a single channel was modified by TMO, and then, after replacing the chamber with fresh solution, a second channel was incorporated into the same membrane. The single-channel currents are clearly different. The larger channel, incorporated after TMO modification, displays a current characteristic of a normal, unmodified channel. Since the two channels share the same membrane, we conclude that the effect of TMO arises from modification of the channel protein itself, and not of the membrane lipids.

The effect of TMO modification depends on ionic strength. Figure 2B shows the same two channels as in Figure 2A, but at higher external salt concentration (150 mM NaCl). Single-channel current is reduced 40% by TMO at low salt (Figure 2A), but only 21% at high salt (Figure 2B). This masking of the TMO effect by raising the salt concentration is a clue that an electrostatic mechanism might contribute to the overall current, as we confirm quantitatively in the following paper (MacKinnon et al., 1989).

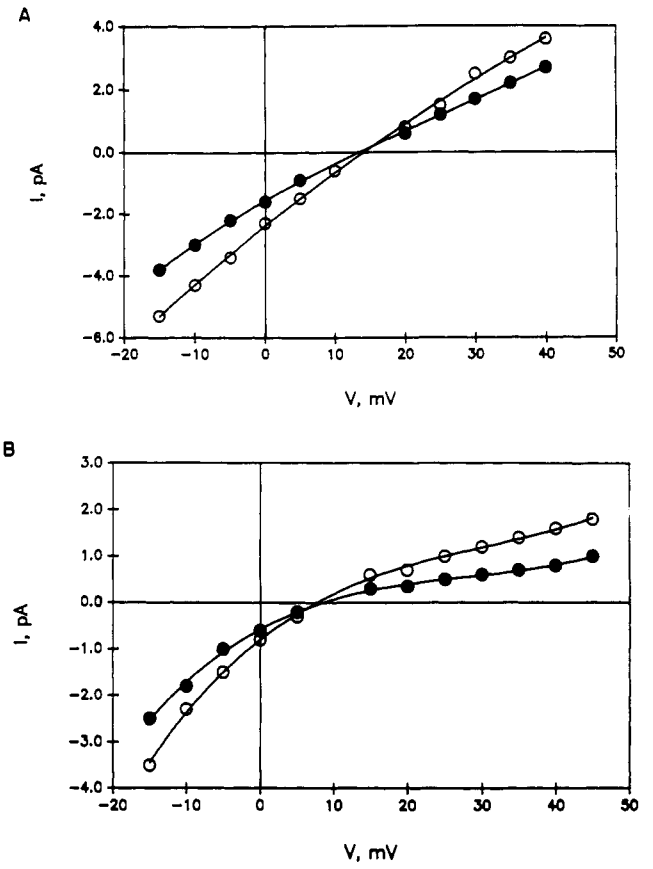

FIGURE 3: TMO modification does not alter ion selectivity. The open-channel current–voltage relation was measured for a control channel (open circles) and a TMO-modified channel (closed circles). In all cases, the external solution contained 100 mM K^+. The internal solution contained 50 mM K^+ and 50 mM Na^+ (A), and 100 mM Rb^+ (B).

Ionic Selectivity. One of the characteristic features of the high-conductance Ca^{2+}-activated K^+ channel is its strong selectivity for K^+ over Na^+. By any measure of permeability, Na^+ appears to be completely impermeant (Yellen, 1984; Blatz & Magleby, 1984; Neyton & Miller, 1988). In Figure 3, we assess the effect of TMO modification on the channel's ionic selectivity. Figure 3A shows the current–voltage relationship in a control and modified channel under asymmetric ionic conditions. The current is reduced at all voltages in the modified channel, but it reverses at 14 mV, a value very close to the K^+ equilibrium potential for this experiment; thus, Na^+ is still impermeant after TMO treatment. The weak selectivity for K^+ over Rb^+ is also preserved after modification, as shown in Figure 3B. Here, with K^+ on one side of the membrane and Rb^+ on the other, the TMO-modified channel displays lower current at all voltages, but the reversal potential is the same as in the control channel. We conclude that TMO reduces the conductance of the channel but does not alter the selectivity among monovalent cations.

Gating. Gating of the Ca^{2+}-activated K^+ channel, i.e., the conformational change leading to channel opening, is sensitive to both voltage and internal $[Ca^{2+}]$. It is known that channel opening is favored by micromolar concentrations of internal Ca^{2+}. Under our conditions, Ca^{2+} activates the channel with a Hill coefficient of about 2 (Moczydlowski & Latorre, 1983; Golowasch et al., 1986; Oberhauser et al., 1988). This result is interpreted to mean that at least two Ca^{2+} binding sites are involved in the activation process. Figure 4 displays Hill plots for Ca^{2+} activation in a control and a TMO-modified channel.

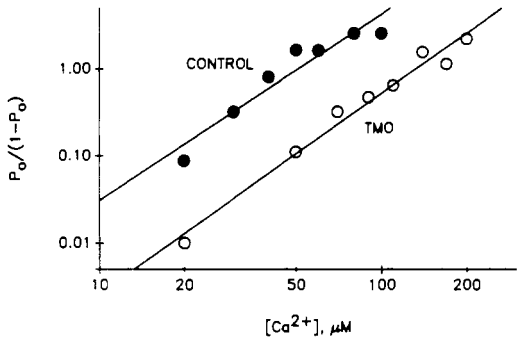

FIGURE 4: Hill coefficient for Ca^{2+} activation is not affected by modification. The single-channel open probability, P_o, was measured as a function of internal Ca^{2+} concentration in a channel that was modified by external TMO (open circles) and in a control channel (closed circles). Solid lines are drawn with Hill coefficients of 2. Membrane voltage was 40 mV.

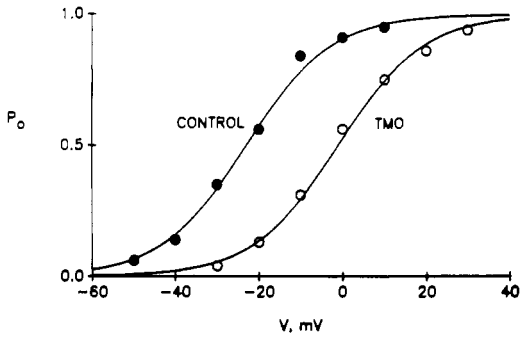

FIGURE 5: Modification shifts voltage activation to more depolarized voltages. In a single channel, the open probability, P_o, was measured as a function of membrane voltage, V, before (closed circles) and after the same channel was modified by TMO (open circles). The internal solution contained 150 mM KCl and 10 μMCa^{2+} and the outside 10 mM MOPS/KOH, pH 7.4. The points are fit by eye to the equation $P_o = \{1 + \exp[zF(V - V_0)/RT]\}^{-1}$ where the "gating charge" $z = -2.4$ and $V_0 = -23$ mV (control) and −1.0 mV (TMO).

The important result here is that the slope is the same, about 2, for both channels; TMO modification does not alter the apparent molecularity of Ca^{2+} activation.

Figure 5 shows the open probability as a function of voltage in the same channel before and after TMO modification. The steepness of the relationship is not altered by modification, but the activation curve is shifted toward more depolarized voltages. Thus, after TMO treatment, the channel responds to voltage as usual, but 25-mV stronger depolarization is needed to attain an equivalent level of activation as in the unmodified channel. In these experiments, the external salt concentration was kept low (5 mM ionic strength); in the following paper, we show that this TMO-induced voltage shift, like the current reduction, is ionic strength dependent (MacKinnon et al., 1989).

Charybdotoxin Block. The high-conductance Ca^{2+}-activated K^+ channel is blocked in a bimolecular fashion by nanomolar concentrations of externally applied CTX (Miller et al., 1985; Anderson et al., 1988). The blocked state results from a single CTX molecule physically occluding the channel's external ion entryway, or "mouth" (MacKinnon & Miller, 1988; Miller, 1988). Figure 6A shows raw records of a control and modified channel in the presence of CTX. The quiet periods that separate bursts of channel gating correspond to block durations, when a single toxin molecule is blocking the channel. For the simple bimolecular reaction:

$$\mathrm{CTX} + \mathrm{C} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \mathrm{C{\cdot}CTX}$$

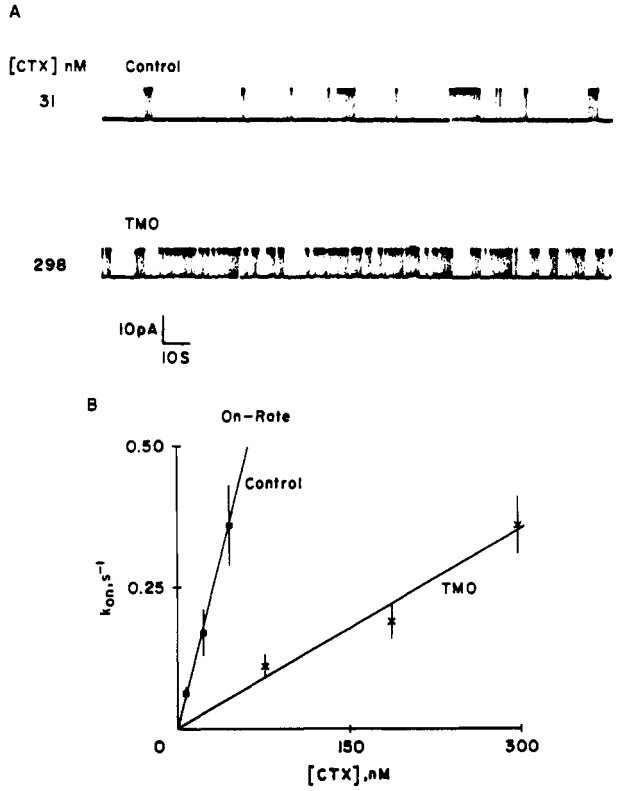

FIGURE 6: TMO modification diminishes sensitivity to CTX block. (A) A single unmodified channel (upper trace) was observed in the presence of 31 nM CTX, with symmetrical 150 mM KCl, at 40 mV. The recording is displayed at low chart speed, so individual openings and closings are not resolved. The long quiet periods correspond to block durations. The bottom trace shows a TMO-modified channel in the presence of 298 nM CTX, under the same conditions. (B) CTX on-rates were measured in control (squares) and modified (crosses) channels, as in (A). The slope, α, of the solid line is 7.8×10^6 M^{-1} s^{-1} for control and 1.1×10^6 M^{-1} s^{-1} for the modified channel.

The mean block time, τ_b, and the mean unblock time, τ_{burst}, are related to the kinetic rate constants α and β by

$$\tau_b = 1/\beta$$

$$\tau_{burst} = 1/\alpha[\mathrm{CTX}]$$

TMO modification shortens the mean CTX block time, i.e., it increases the off-rate, β, about 5-fold in the example of Figure 6A. It is also apparent that the CTX association rate α is lower for the modified channel since a much higher CTX concentration is required to achieve similar burst durations. Both block and unblock times are singly exponentially distributed (data not shown). In Figure 6B, a plot of the pseudo-first-order association rate constant, k_{on} ($=\tau_{burst}^{-1}$), against CTX concentration yields a second-order association rate constant which is 7-fold slower for the modified channel. Thus, the affinity of CTX for the modified channel is weaker both because the association rate is slower and because the dissociation rate faster.

Earlier we observed that modification can result in a partial effect on single-channel current, as though more than one site can be modified. Just as a single exposure to TMO has a variable effect on single-channel current, there is also a variable effect on CTX block. This is illustrated in Table I which lists the on- and off-rate constants and the dissociation constant K_D for nine different channels after modification. The large variability (nearly 2 orders of magnitude) in K_D is in contrast with the K_D for CTX block of unmodified channels, which is tightly distributed around 10 nM (Anderson et al., 1988).

Table I: CTX Block Kinetics of Modified Channels[a]

channel	β (s^{-1})	$\alpha \times 10^{-6}$ (M^{-1} s^{-1})	K_D (nM)
1	1.1	1.7	650
2	0.38	3.8	100
3	0.19	0.50	380
4	0.36	1.8	200
5	0.43	3.8	110
6	0.36	0.05	7200
7	0.49	1.1	450
8	2.4	0.71	3400
9	1.0	0.66	1500

[a] Single TMO-modified channels were observed in the presence of external CTX. The CTX dissociation rate, β, and second-order association rate, α, were determined from 14–75 blocked and unblocked intervals. The invernal and external solutions were 150 mM KCl, and the membrane voltage was 40 mV.

CTX Protects the Channel from TMO Modification. CTX is a highly basic protein, having a net charge of about +5 at neutral pH (Smith et al., 1986; Gimenez-Gallego et al., 1988). Since electrostatic forces are known to contribute strongly to CTX binding, carboxyl groups have been postulated to be crucially involved in the toxin–channel interaction (Anderson et al., 1988). Our demonstration here that TMO treatment reduces the CTX affinity is fully in harmony with this idea. Do the carboxyl groups involved in CTX binding have anything to do with the modification sites that influence the single-channel conductance? We addressed this question with a series of experiments showing that CTX protects the channel from TMO modification of conductance. We treated channels with TMO in the presence or absence of a high concentration (100 nM) of CTX. Under conditions which, in the absence of toxin, resulted in modification of the single-channel current in every attempt (16 trials), CTX prevented modification in 6 out of 13 trials. In these experiments, we do not expect 100% protection, because of the relative rates of CTX dissociation, 0.03 s^{-1}, and TMO hydrolysis, 0.14 s^{-1}. The probability that the channel will be CTX-blocked when TMO is added is close to unity, since a high concentration of CTX is present. The channel becomes susceptible to modification when that CTX molecule dissociates. Because of the relative rates of TMO hydrolysis and CTX dissociation, there is a 50% chance that a CTX molecule, originally present at the moment of TMO addition, will dissociate before most of the TMO has been hydrolyzed. Thus, we observe protection only some of the time.

Discussion

Specificity of the TMO Reaction. In this study, we demonstrate that TMO modifies the high-conductance Ca^{2+}-activated K^+ channel. TMO is generally considered to be a carboxyl group modifying reagent. Does this highly reactive methyl donor modify groups other than carboxyls? Several studies address this question. The closely related reagent triethyloxonium (TEO) rapidly esterified carboxyl groups of free amino acids and dipeptides in aqueous solution, including glutamate and aspartate side chains (Yonemitsu et al., 1969). However, TEO also alkylated the sulfur of methionine and the imidazole nitrogen of histidine. The reagent failed to modify hydroxyl, amino, or guanidinium groups.

In another study, TEO exclusively esterified carboxyl groups on lysozyme (Parsons et al., 1969). Other amino acids, including methionine and histidine, were unmodified. Likewise, TEO also modified only carboxyl groups on trypsin (Nakayama et al., 1970). Interestingly, when TEO modification of trypsin was compared to the more standard method of carboxyl group modification using a carbodiimide and glycine ethyl ester, TEO preferentially esterified carboxyl groups near the active site. This result suggested that TEO, being cationic, was directed toward trypsin's anionic cleft which is designed to bind cationic amino acid residues. This picture may apply to the K^+ channel here, since negatively charged groups might be expected to reside at the "active site" or pore entrance to a cation channel (Apell et al., 1979; Dani, 1986; Jordan, 1987). Like TEO, TMO is a small cation that would be electrostatically directed to such a region of negative potential. In the absence of amino acid composition analysis, we cannot firmly conclude that TMO methylates only carboxyl groups on the Ca^{2+}-activated K^+ channel. However, the biochemical precedent reviewed above makes it likely that this is the case.

Analogy to the Na^+ Channel. TMO modification of the high-conductance Ca^{2+}-activated K^+ channel is similar to TMO modification of the excitable membrane Na^+ channel. TMO, on the outside of the Na^+ channel, has been shown to reduce the conductance without affecting ionic selectivity, and the specific inhibitor tetrodotoxin (TTX) protects the channel from modification (Shrager & Profera, 1973; Baker & Rubinson, 1975; Spalding, 1980; Sigworth & Spalding, 1980; Worley et al., 1986). Furthermore, the channel's TTX sensitivity is markedly diminished by TMO. Thus, the Na^+ channel and the Ca^{2+}-activated K^+ channel both appear to have carboxyl groups that influence ionic conduction and participate in toxin block.

There is one fundamental difference between our results and the results of Na^+ channel modification that deserves attention. Modification of single Na^+ channels has been reported to be all-or-none, an effect which led Worley and colleagues (Worley et al., 1986) to propose that a single carboxyl group is involved. In contrast, the progressive reduction of single-channel current upon repeated TMO treatment (Figures 1) argues that more than one site is modified on the Ca^{2+}-activated K^+ channel. The CTX block kinetics (Table I) provide independent evidence that multiple sites are involved. Not only do we see a large variation in the K_D for CTX block of modified channels but also there is little correlation between the degree to which the on-rate is decreased and the off-rate increased by modification in a given channel. Carboxyl groups that are close to, but not part of, the CTX binding site might be expected to affect the on-rate, while carboxyl groups that interact intimately with bound CTX might be expected to change both rates. It is difficult to rationalize these results by proposing fewer than three modifiable sites, which would allow seven distinguishable modified states.

Conclusion. This paper offers a phenomenological description of the influence of TMO modification on the high-conductance Ca^{2+}-activated K^+ channel. We propose that the external face of the channel carries ionized carboxyl groups which influence ionic conduction, voltage-dependent gating, and CTX block. There are several obvious questions that remain unanswered. What is the charge density on the outside of the channel? How do charged groups located on the outside of the channel influence outwardly directed K^+ current? Is the channel also charged on its internal face? Most importantly, what is the physical mechanism by which these charged groups influence the various functions of the channel? In the following paper (MacKinnon et al., 1989), we develop a mechanistic description of the role of local electrostatic potentials on the function of the high-conductance Ca^{2+}-activated K^+ channel.

References

Anderson, C., MacKinnon, R., Smith, C., & Miller, C. (1988) *J. Gen. Physiol. 91*, 317–333.

Apell, H. J., Bamberg, E., & Lauger, P. (1979) *Biochim. Biophys. Acta 552*, 369–378.

Baker, P. F., & Rubinson, K. A. (1975) *Nature (London) 257*, 412-414.
Barrett, J. N., Magleby, K. L., & Pallotta, B. S. (1982) *J. Physiol. 331*, 211-230.
Blatz, A., & Magleby, K. (1984) *J. Gen. Physiol. 84*, 1-23.
Dani, J. A. (1986) *Biophys. J. 49*, 607-618.
Eisenman, G., Latorre, R., & Miller, C. (1986) *Biophys. J. 50*, 1025-1034.
Gimenez-Gallego, G., Navia, M. A., Reuben, J. P., Katz, G. M., Kaczorowski, G. J., & Garcia, M. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3329-3333.
Golowasch, J., Kirkwood, A., & Miller, C. (1986) *J. Exp. Biol. 124*, 5-13.
Hanke, W., & Miller, C. (1983) *J. Gen. Physiol. 82*, 25-45.
Hille, B. (1984) *Ionic Channels of Excitable Membranes*, Sinauer Associates, Sunderland, MA.
Jordan, P. C. (1987) *Biophys. J. 51*, 297-311.
Latorre, R. (1986) in *Ion Channel Reconstitution* (Miller, C., Ed.) pp 431-468, Plenum Press, New York.
Latorre, R., Vergara, C., & Hidalgo, C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 805-809.
MacKinnon, R., & Miller, C. (1988) *J. Gen. Physiol. 91*, 335-349.
MacKinnon, R., Latorre, R., & Miller, C. (1989) *Biochemistry* (following paper in this issue).
Magleby, K. L., & Pallotta, B. S. (1983) *J. Physiol. 334*, 585-604.
Miller, C. (1988) *Neuron 1*, 1003-1006.
Miller, C., Moczydlowski, E., Latorre, R., & Phillips, M. (1985) *Nature (London) 313*, 316-318.
Miller, C., Latorre, R., & Reisin, I. (1987) *J. Gen. Physiol. 90*, 427-449.
Moczydlowski, E., & Latorre, R. (1983) *J. Gen. Physiol. 82*, 511-542.
Moczydlowski, E., Alvarez, O., Vergara, C., & Latorre, R. (1985) *J. Membr. Biol. 83*, 273-282.
Nakayama, H., Tanizawa, K., & Kanaoka, Y. (1970) *Biochem. Biophys. Res. Commun. 40*, 537-541.
Neyton, J., & Miller, C. (1988) *J. Gen. Physiol. 92*, 569-586.
Oberhauser, A., Alvarez, O., & Latorre, R. (1988) *J. Gen. Physiol. 92*, 67-86.
Parsons, S. M., Jao, L., Dahlquist, F. W., Borders, C. L., Groff, T., Racs, J., & Raftery, M. A. (1969) *Biochemistry 8*, 700-712.
Shrager, P., & Profera, C. (1973) *Biochim. Biophys. Acta 318*, 141-146.
Sigworth, F. J., & Spalding, B. C. (1988) *Nature (London) 283*, 293-295.
Smith, C., Phillips, M., & Miller, C. (1986) *J. Biol. Chem. 261*, 14607-14613.
Spalding, B. C. (1980) *J. Gen. Physiol. 305*, 485-500.
Worley, J. F., French, R. J., & Krueger, B. K. (1986) *J. Gen. Physiol. 87*, 327-349.
Yellen, G. (1984) *J. Gen. Physiol. 84*, 157-186.
Yonemitsu, O., Hamada, T., & Kanqoka, Y. (1969) *Tetrahedron Lett. 23*, 1819-1820.

Role of Surface Electrostatics in the Operation of a High-Conductance Ca^{2+}-Activated K^+ Channel†

Roderick MacKinnon,‡ Ramon Latorre,§ and Christopher Miller*
Howard Hughes Medical Institute and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received April 7, 1989; Revised Manuscript Received June 12, 1989

ABSTRACT: This paper demonstrates that local electric fields originating from negatively charged groups on a K^+-specific ion channel modify its behavior. Single high-conductance, Ca^{2+}-activated K^+ channels were studied in neutral phospholipid bilayers. The channel protein surface charges were manipulated experimentally by carboxyl group esterification using trimethyloxonium (TMO) or by electrolyte screening. Three channel properties—ion conduction, ion blockade, and voltage-dependent gating—are affected by surface electrostatics. Negative charges increase the affinity of cationic pore blockers by establishing a local negative potential at the pore entrance; these charges modify channel gating by establishing a potential gradient across the ion channel; finally, both effects influence ion permeation through the pore.

Electrostatic forces originating from charged groups on protein surfaces influence a variety of macromolecular functions. The distribution of charges on the surface of superoxide dismutase is important for electrostatic guidance of substrate into the active site (Getzoff et al., 1983; Sharp et al., 1987), and a similar electrostatic steering mechanism may act in the encounter of cytochrome *c* with cytochrome *c* peroxidase (Northrup et al., 1988). Likewise, charged groups are known to affect the binding of protons in subtilisin (Russell & Fersht, 1987) and of Ca ions in subtilisin (Pantoliano et al., 1988) and intestinal calbindin (Linse et al., 1988). In these well-studied cases, for which high-resolution structures are available, it is clear that the charged groups influence ion binding at distant sites through electrostatic forces operating over distances up to 2 nm. The effects are as expected intuitively: a negative charge near to, but not right at, a binding site for a positively charged ligand enhances the binding of that ligand.

† Supported by NIH Research Grant GM-31768 and Training Grant NS-07292 and by Grant 0451-1988 of the Fondo Nacional de Investigaciones de Chile.
‡ Present address: Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.
§ Permanent address: Centro de Estudios Cientificos de Santiago, Santiago, Chile.

0006-2960/89/0428-8092$01.50/0