

Effects of Tetraethylammonium on Potassium Currents in a Molluscan Neuron

A. HERMANN and A. L. F. GORMAN

From the Department of Physiology, Boston University School of Medicine, Boston, Massachusetts 02118. A. Hermann's present address is Department of Biology, University of Konstanz, D-7750 Konstanz, Federal Republic of Germany.

ABSTRACT The effects of tetraethylammonium (TEA) on the delayed K^+ current and on the Ca^{2+} -activated K^+ current of the *Aplysia* pacemaker neurons R-15 and L-6 were studied. The delayed outward K^+ current was measured in Ca^{2+} -free ASW containing tetrodotoxin (TTX), using brief depolarizing clamp pulses. External TEA blocks the delayed K^+ current reversibly in a dose-dependent manner. The experimental results are well fitted with a Michaelis-Menten expression, assuming a one-to-one reaction between TEA and a receptor site, with an apparent dissociation constant of 6.0 mM. The block depends on membrane voltage and is reduced at positive membrane potentials. The Ca^{2+} -activated K^+ current was measured in Ca^{2+} -free artificial seawater (ASW) containing TTX, using internal Ca^{2+} ion injection to directly activate the K^+ conductance. External TEA and a number of other quaternary ammonium ions block the Ca^{2+} -activated K^+ current reversibly in a dose-dependent manner. TEA is the most effective blocker, with an apparent dissociation constant, for a one-to-one reaction with a receptor site, of 0.4 mM. The block decreases with depolarization. The Ca^{2+} -activated K^+ current was also measured after intracellular iontophoretic TEA injection. Internal TEA blocks the Ca^{2+} -activated K^+ current (but the block is only apparent at positive membrane potentials), is increased by depolarization, and is irreversible. The effects of external and internal TEA can be seen in measurements of the total outward K^+ current at different membrane potentials in normal ASW.

The quaternary ammonium ion, tetraethylammonium (TEA), blocks voltage-dependent potassium channels in nerve and muscle cells (Hagiwara and Saito, 1959; Armstrong and Binstock, 1965; Hille, 1967*a*; Koppenhöffer, 1967; Stanfield, 1970; Fink and Wettwer, 1978). In myelinated axon, quaternary ammonium ions block the voltage-dependent K^+ current (the delayed outward K^+ current or the "delayed outward rectifier") when applied to either the external or the internal membrane surface (Koppenhöffer and Vogel, 1969), but the properties of the block depend on the side of the membrane to which the drug is applied (Armstrong and Hille, 1972). There is general agreement that external or internal TEA also reduces the total outward K^+ current of the molluscan nerve cell soma membrane (Hagiwara and Saito, 1959; Neher

and Lux, 1972; Kostyuk et al., 1975), but their effect on different components of the K^+ current is less understood. Meech and Standen (1975) have reported that external TEA reduces both the delayed outward and the Ca^{2+} -activated K^+ currents with equal efficiency. This is consistent with reports (Hagiwara and Saito, 1959; Smith and Zucker, 1980) that high external TEA almost completely blocks outward membrane currents. There is, however, data (Thompson, 1977; Aldrich et al., 1979; Connor, 1979) that suggest that in some molluscan neurons external TEA may block the delayed outward K^+ current with greater efficiency. The effects of internal TEA on different components of the K^+ current have also been studied (Heyer and Lux, 1976 b). These effects indicate that TEA may block the delayed outward K^+ but not the Ca^{2+} -activated K^+ current. In a previous study (Hermann and Gorman, 1979a), we showed that external TEA reduces both the voltage-dependent and Ca^{2+} -activated components of the K^+ current in molluscan pacemaker neurons, whereas internal TEA affects only the voltage-dependent component. There is evidence from axonal membranes (Armstrong and Binstock, 1965; Armstrong and Hille, 1972), however, that the reduction of the delayed outward K^+ current by internal TEA depends upon membrane potential as well as drug concentration. Our previous comparisons of the effects of TEA on different components of the K^+ current were made at different potentials and thus provide no information about the possible effects of membrane voltage on the block. In this paper we show that both the delayed outward K^+ and the Ca^{2+} -activated K^+ currents can be blocked by external or internal TEA but that the properties and the efficiency of the block depends on membrane potential. Aspects of this work have been presented in preliminary form (Hermann and Gorman, 1978 and 1979a).

METHODS

All experiments were performed on the pacemaker neurons R-15 and L-6 in the abdominal ganglion of *Aplysia californica*. The experimental procedures, artificial seawater (ASW) solutions, recording, voltage clamp, and electrophoretic injection circuitry were identical to those reported in the preceding paper (Hermann and Gorman, 1981). In some experiments, in addition to the internal Ca^{2+} microelectrode containing 0.1 M $CaCl_2$, a separate microelectrode containing 0.5 M KEGTA was inserted into the neuron so that EGTA (ethylene glycol tetraacetic acid) could be injected iontophoretically in the cytoplasm. Unless otherwise specified, all ASW solutions contained 5×10^5 M tetrodotoxin (TTX), which was added directly to the solution just before its use. When tetraethylammonium or other quaternary ammonium ions were used in the external bathing solution, an equal quantity of Na^+ ions was removed, so that the ionic strength of the solution was unchanged. In addition to tetraethylammonium chloride (Aldrich Chemical Co., Milwaukee, Wis., Eastman Kodak Co., Rochester, N. Y., or Fluka, A.G., W. Germany), the following quaternary ammonium ions were used: tetramethylammonium chloride, trimethylethylammonium bromide, dimethylethylpropylammonium bromide, diethylmethylpropylammonium bromide, triethylmethylammonium bromide, triethylphenylammonium iodide, triethylpentylammonium bromide, triethyloctylammonium bromide, tetrapropylammonium chloride, and tetrabutylammonium bromide. Triethylpentylammonium (C-5) and triethyloctylammonium (C-8) were synthesized from triethylamine and brom-

opentane or bromooctane with a procedure outlined by Armstrong (Armstrong [1975] and personal communication).

In experiments in which effects of internal tetraethylammonium were tested, a separate intracellular microelectrode containing 0.5 M TEACl was used. All ions were electrophoretically injected into cells in the voltage-clamp mode, so that there was no net change in the flow of current across the membrane during injection. An estimate of the amount (mol/l) of TEA injected can be obtained from the relation $M = nit/zF$, where n is the transport number of the electrode, i is the injection current, z is the valence ($z = 1$), and F is the Faraday constant. Typically, an intracellular injection of TEA⁺ for 8 min, using a current of 500 nA was sufficient to produce a maximum effect on the K⁺ current. Further injection had very little additional effect (see Fig. 10). If a transport number of 0.26 is used for the TEA electrodes (Nicholson et al., 1979), then the internal TEA concentration of the R-15 soma (radius of ~125 μM) could theoretically increase by ~79 mM. To maintain osmotic equilibrium, however, an intracellular concentration of this magnitude only can occur if TEA replaces an intracellular cation that is free to move across the membrane. At a holding potential near the resting potential, the most likely candidate is K⁺, but, if a large number of K⁺ ions leave the cell during intracellular TEA injection, the K⁺ equilibrium will change. We have shown (Hermann and Gorman, 1979 *a*) that the reversal potential for the Ca²⁺-activated K⁺ current, which provides a reasonable estimate of the K⁺

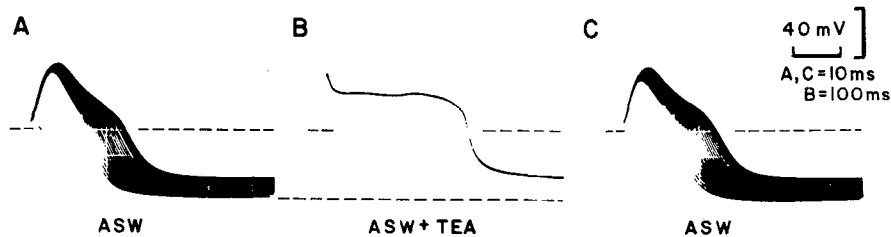


FIGURE 1. Effects of TEA on the action potential of cell R-15. (A and C) Superimposed spontaneous action potentials during pacemaker discharge in normal ASW before (A) and after (C) TEA. (B) Action potential in ASW containing 50 mM TEA. The time scale in A and C is 10 ms, and in B is 100 ms.

equilibrium potential (Gorman and Hermann, 1979), is unaffected by prolonged internal TEA injection. These data suggest that the internal TEA concentration is probably <79 mM. TEA⁺ is a small, mobile cation, whose diffusion coefficient in axoplasm is estimated to be only slightly less than that for Na⁺ (Koppenhöffer and Vogel, 1969), and it is possible that much of the TEA injected migrates rapidly from the soma to axonal regions (see Bryant and Weinreich [1975]).

RESULTS

Effects of External TEA on the Action Potential

The effects of high external TEA (50 mM) on the action potential of cell R-15 are shown in Fig. 1. The multiple discharge of action potentials (Fig. 1 A) during pacemaker activity was reduced to a single action potential (Fig. 1 B), which was of similar amplitude but about 10 times longer in duration than an individual action potential during the burst. The prolonged action potential in TEA was characterized by a peak and a long-lasting plateau at about +30

mV and was terminated by a slow repolarization to more negative potentials. The response was readily reversed when the cell was returned to normal ASW (Fig. 1 C). The effects of external TEA on pacemaker activity and the sizable increase in internal Ca^{2+} concentration that occurs in cell R-15 during the prolonged action potential have been shown previously (Gorman and Thomas, 1978). The effect of TEA on the action potential is quite different from the approximately 2.2-fold increase in duration produced by 4-aminopyridine (see Fig. 10 of Hermann and Gorman [1981]).

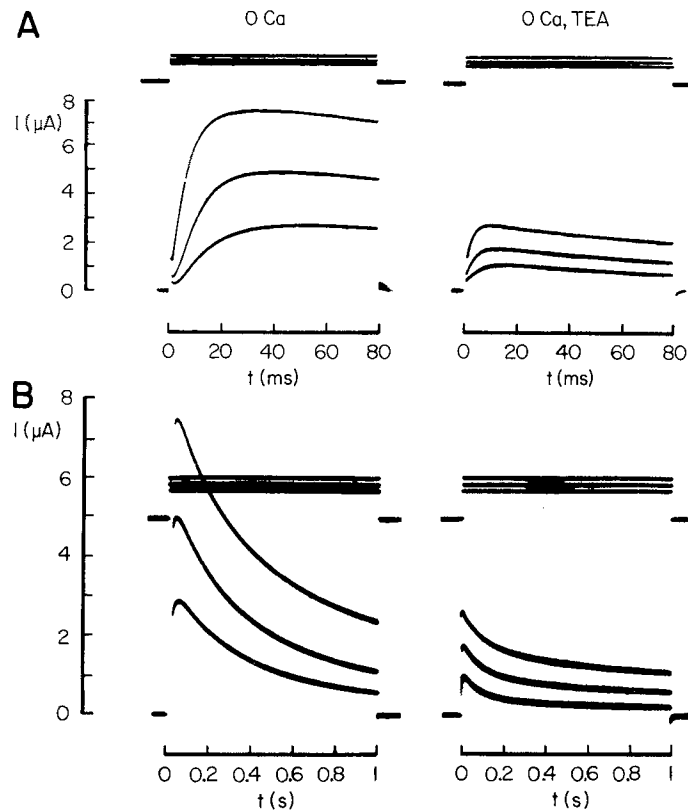


FIGURE 2. Effects of external TEA on R-15 membrane outward K^+ currents. The left side shows outward currents measured in Ca^{2+} -free ASW containing 5×10^{-5} M TTX, and the right side shows currents measured in 50 mM TEA. The cell was injected with EGTA from 5 min at 500 nA. In A the pulse duration was 80 ms, and in B 1 s (both to +20, +35, and +50 mV from a holding potential of -45 mV).

Inhibition of the Delayed Outward K^+ Current by TEA

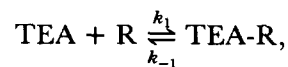
Fig. 2 illustrates the effect of 50 mM external TEA on the K^+ outward currents measured with depolarizing clamp pulses of short and long durations and of various amplitudes in a cell injected with EGTA in Ca^{2+} -free ASW

containing 5×10^{-5} M TTX. TTX was used to eliminate the fast, early inward current carried by Na⁺ ions, and Ca²⁺-free ASW was used to eliminate the Ca²⁺ current (see Hermann and Gorman [1981]). The Ca²⁺-free ASW, however, contained $\sim 4 \times 10^{-6}$ M Ca²⁺ (Gorman and Hermann, 1979). EGTA was intracellularly injected by iontophoresis (500 nA for 5 min) to abolish any residual Ca²⁺-activated K⁺ current that might result from Ca²⁺ influx from an external solution that is only nominally Ca²⁺ free. This procedure was preferable to the use of the external EGTA to chelate Ca²⁺ ions, since external EGTA also progressively increases the leakage current. By contrast, internal EGTA had very little effect on membrane leakage. It has been shown (Tillotson and Gorman, 1980) that an internal injection of EGTA at this duration and intensity is more than sufficient to abolish all absorbance changes produced by Ca²⁺ influx (in cells containing the Ca²⁺ indicator dye arsenazo III) during 500-ms voltage-clamp pulses to all positive potentials. Finally, the membrane potential was held at -45 mV to inactivate the fast outward K⁺ current. Under these conditions, the residual outward current (Fig. 2) is composed primarily of the delayed outward K⁺ current and the leakage current. As described in the preceding paper (Hermann and Gorman, 1981), the leakage current can be estimated in each record from the instantaneous jump in current that occurs after the capacitive discharge of the membrane. It is approximately linear between -100 and about $+50$ mV. In the absence of TEA, the delayed outward K⁺ current rose to a peak with a delay and showed a slow decay during maintained depolarization. The maximum delayed outward K⁺ current, at any potential, was smaller than the comparable current in the absence of TEA. By contrast, in high external TEA concentrations (~ 50 mM), the leakage current was increased slightly.

The rise of the outward current was increased slightly and its decay slowed by the presence of external TEA (Figs. 2 and 3A). The decay of the delayed outward K⁺ current occurs in two phases. The second, slower phase was affected by TEA, whereas the early, faster phase was unchanged. The changes in current kinetics were small and were not further investigated.

Concentration Dependence of TEA Inhibition

The blocking effect of TEA on the delayed outward K⁺ current depends on its external concentration and was greatest at higher concentrations (Fig. 3A). This concentration dependence can be seen in dose-response plots of the current ratio ($I_{K,TEA}/I_K$) vs. the logarithm of the external TEA concentration (Fig. 3B). If one TEA molecule binds to a single receptor site R, the reaction can be represented by



where k_1 and k_{-1} are the forward and reverse rate constants and $K_{\text{TEA}} = k_{-1}/k_1$ is the apparent dissociation constant of the reaction. The experimental values shown in Fig. 3B were reasonably well fitted with a theoretical curve,

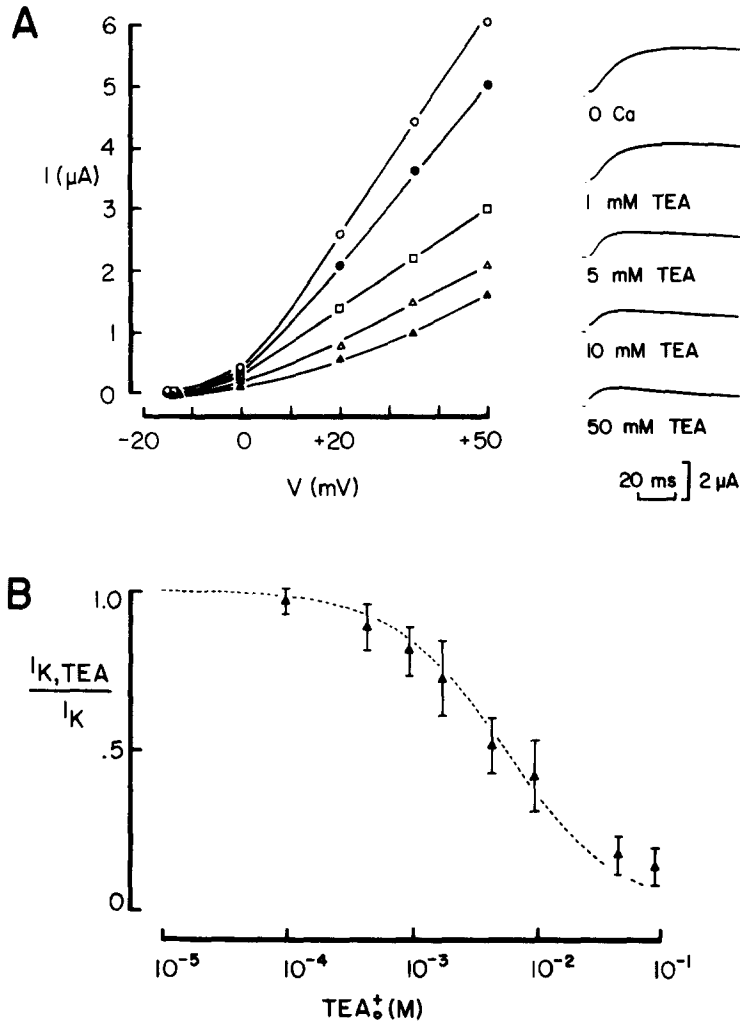


FIGURE 3. Effects of TEA concentration on the delayed K⁺ current. (A) Plot of K⁺ current measured at the end of 85-ms pulses in Ca²⁺-free ASW containing 5×10^{-5} M TTX before (open circles) and in four external TEA concentrations (1 mM, closed circles; 5 mM, open squares; 10 mM, open triangles; 50 mM, closed triangles). The data are corrected for leakage current. Individual current responses to pulses to +20 mV from a holding potential of -45 mV at the indicated TEA concentration are shown to the right of the current-voltage plot. The cell was injected intracellularly with EGTA for 5 min at 500 nA. (B) Dose-response plot of delayed K⁺ current ratio, $I_{K,TEA}/I_K$ at +20 mV for five cells vs. external TEA concentration. The theoretical curve drawn through the experimental points was calculated from Eq. 1 (see text). The bars drawn through each point represents 1 SEM.

using the equation

$$\frac{I_{K,TEA}}{I_K} = \frac{K_{TEA}}{K_{TEA} + [TEA]_o}, \quad (1)$$

where I_K is the peak delayed outward K⁺ current in Ca²⁺-free ASW, $I_{K,TEA}$ is the peak K⁺ current at the same potential in Ca²⁺-free ASW containing TEA, and $[TEA]_o$ is the external TEA concentration. The average value for K_{TEA} for the six cells shown in Fig. 3B was 6.0 ± 1.4 mM (SEM). In four experiments, Ca²⁺-free ASW plus TTX was used, and, in the other two experiments, EGTA was injected (see Fig. 2) or 3 mM La³⁺ was added to the Ca²⁺-free ASW to block any accumulation of intracellular Ca²⁺ caused by Ca²⁺ influx from a bathing medium that is only nominally free of Ca²⁺. The effects of external TEA were similar in each condition.

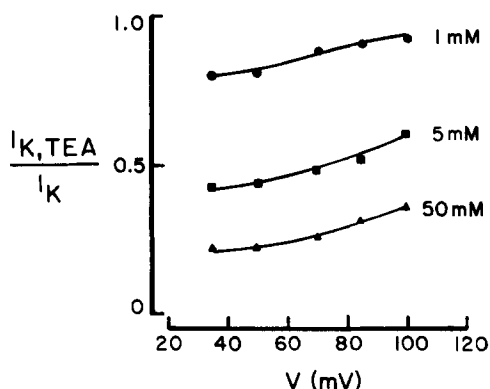


FIGURE 4. Effect of membrane potential on the block of the delayed K⁺ current by external TEA. Plot of the current ratio $I_{K,TEA}/I_K$ at the indicated external TEA concentrations vs. membrane potential. The currents were measured in Ca²⁺-free ASW at the end of 200-ms pulses to the indicated membrane potentials from a holding potential of -45 mV.

External TEA increases membrane resistance (Fig. 3A) and therefore reduces the proportion of the change in voltage that is imposed across the resistance in series with the membrane during a depolarizing pulse. This causes an error in the measurements of the current ratio (particularly at high external TEA concentration), which is reflected in the dose-response plots shown in Fig. 3B and in Figs. 7 and 9C. The current ratio was corrected at each concentration, using an average series resistance of 1.5 k Ω (see Hermann and Gorman [1981]). The corrected experimental values shown in Fig. 3B were shifted slightly to the left but were reasonably well fitted with a theoretical curve from Eq. 1. Our findings suggest that we overestimate slightly the apparent dissociation constant because of the series resistance error.

Voltage Dependence of the External TEA Block

The effect of external TEA on the delayed outward K⁺ current was examined at various membrane potentials in three cells. Fig. 4 shows a plot of the

current ratio ($I_{K,TEA}/I_K$) in three external TEA concentrations vs. membrane potential, using data from one of these cells. There was a progressive recovery of the delayed outward K^+ current during depolarizing steps to positive membrane potentials that occurred at all concentrations. The results were similar in all three cells and suggest that the block produced by external TEA is partially removed by membrane depolarization. The effect of membrane depolarization to positive potentials on the block produced by external TEA is similar to, but smaller than, its effect on the block produced by 4-aminopyridine in cell R-15 (see Fig. 3 of Hermann and Gorman [1981]) and is opposite to the increase in the effectiveness of the block of the delayed K^+ current produced by *internal* TEA ion derivatives in squid axon at positive membrane potentials (Armstrong, 1969). The effects of membrane depolarization can also be seen in dose-response plots of current ratio at different membrane potentials vs. the logarithm of the external TEA concentration (data not shown), where there is a small but progressive increase in the apparent dissociation constant at more positive membrane potentials (greater than about +35 mV). The effects of voltage on the current ratio shown in Fig. 4 were slightly altered but not abolished when the current ratio was corrected for the estimated series resistance error.

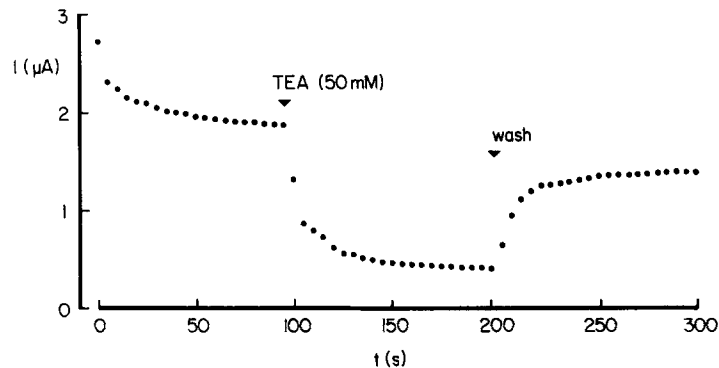


FIGURE 5. Time-course of onset and removal of TEA block of delayed K^+ current. Plot of the delayed outward K^+ current measured with 75-ms pulses from -45 to $+35$ mV in Ca^{2+} -free ASW containing 5×10^{-5} M TTX. The pulses were repeated at a rate of 0.2 Hz. The cell was injected with EGTA for 5 min at 500 nA. The data are corrected for leakage current. The times at which TEA was introduced and removed from the bath are indicated by the arrows.

Time-Course of the TEA Block of the Delayed K^+ Current

TEA is a small molecule that might be expected to diffuse rapidly to the exposed neuronal membrane surface when added to the bathing medium, and it has been shown in myelinated axon that externally applied TEA reaches its blocking site in <50 ms (Vierhaus and Ulbricht, 1971). The onset of the block produced by 50 mM external TEA was studied with 75-ms test pulses to +35 mV applied once every 5 s. The time-course of the inhibitory action of the drug on the delayed K^+ current measured at the end of the test pulse in Ca^{2+} -free ASW was fast (Fig. 5). The time necessary to produce a 50% block in two

cells was 6 and 10 s, respectively, but since a complete exchange of the bath took at least 5 s (Hermann and Gorman, 1981) much of this time is consumed in getting the drug to the membrane. A steady-state block occurred in ~225–240 s; however, the exposed cell R-15 is covered by a layer of glia and contains relatively deep invaginations that are likely to slow the diffusion of TEA to the different regions of the membrane. Reversibility of the drug effect was very good (40% complete in 8–10 s and 90% complete in 40–50 s) (Fig. 5). The reversible effect of external TEA is very different from the apparent irreversibility of internal TEA (Hermann and Gorman, 1979*a*) and suggests that there may be different receptors for the TEA molecule on the inner and outer membrane surfaces (Vierhaus and Ulbricht, 1971; Armstrong and Hille, 1972).

Effects of External TEA on the Ca²⁺-activated K⁺ Current

Injection of Ca²⁺ ions into molluscan neurons activates a current carried by K⁺ ions (Meech, 1974) whose amplitude depends on the amount of Ca²⁺ injected and on the holding potential (Gorman and Hermann, 1979). At membrane potentials positive to its reversal potential (about –70 mV) the K⁺ current is outward. Ca²⁺ ions were iontophoretically (100 nA) injected until the outward K⁺ current reached a quasi-steady state, at which time the Ca²⁺-free bathing medium was rapidly replaced with a Ca²⁺-free medium containing 50 mM TEA (Fig. 6*A*). The onset of the block of the Ca²⁺-activated K⁺ current by external TEA was as fast or faster than its inhibitory effect on the delayed outward K⁺ current. A 50% block was reached in ~5 s, and the block was 90% complete in ~14 s. The block was fully reversible and had a similar time-course.

The effects of external TEA on K⁺ currents activated by a constant Ca²⁺ ion injection (200 nA for 10 s) once every 2–3 min were studied at various membrane potentials between –80 and +50 mV (Fig. 6*B*). The difference between the peak K⁺ current activated by Ca²⁺ injection and the holding current at each potential in Ca²⁺-free ASW is plotted in Fig. 6*B*. External TEA blocked both outward and inward K⁺ currents and at high external concentrations (~50 mM) the block was complete between –80 and about –30 mV, but at more positive potentials (–10 mV to +50 mV) there was a small fraction of the Ca²⁺-activated K⁺ current (1–2%) that was not blocked. These results are consistent with those given above for the delayed outward K⁺ current (see Fig. 4) and indicate that at positive membrane potentials there is a partial removal of the block caused by external TEA.

The relief of the external TEA block of the Ca²⁺-activated K⁺ current at positive membrane potentials may help to explain the repolarization of the prolonged action potential (see Fig. 1). Armstrong and Binstock (1965) have pointed out that an action potential plateau at positive membrane potentials can be maintained only as long as there is no *net* current flow across the membrane at that potential. In molluscan neurons there is an inward current carried by Ca²⁺ ions that is only partially inactivated at positive potentials (Eckert and Lux, 1976) and that is present during the plateau phase of the prolonged action potential (Horn and Miller, 1977). This current must be

balanced by outward currents flowing through unblocked K^+ and leakage channels. It has been shown (Gorman and Thomas, 1978) that in the presence of 50 mM external TEA there can be an increase as great as 13-fold in the intracellular Ca^{2+} concentration at the end of a prolonged action potential for

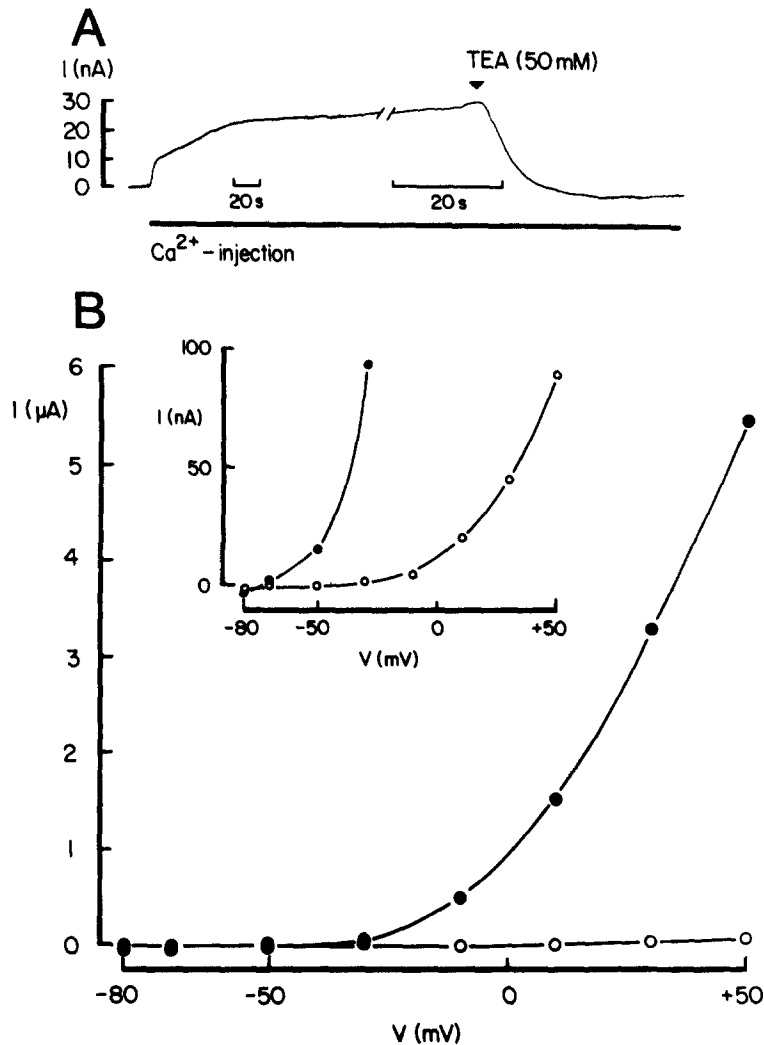


FIGURE 6. The effects of external TEA on the Ca^{2+} -activated K^+ current. (A) K^+ current produced by a 100-nA internal Ca^{2+} injection for the time shown by the line beneath the current trace at a holding potential of -50 mV in Ca^{2+} -free ASW. The time at which external TEA (50 mM) was introduced is indicated above the response. (B) plot of K^+ current in Ca^{2+} -free ASW vs. membrane holding potential before (closed circles) and in (open circles) 50 mM external TEA. The current was measured as the difference between the holding current at each potential and the peak current activated by an intracellular Ca^{2+} injection for 10 s at 200 nA. The insert shows a plot of the K^+ current on a different current scale.

a 10-fold increase in the action potential duration. At the plateau potential (about +30 mV) a small fraction of the Ca²⁺-activated K⁺ current is available for activation and is likely to be activated once the intracellular Ca²⁺ concentration reaches sufficient magnitude, thereby causing the repolarization of the action potential. It has been shown that the prevention of Ca²⁺ influx in TEA-treated neurons by Ca²⁺ channel inhibitors decreases action potential duration (Horn and Miller, 1977) and that prevention of intracellular Ca²⁺ accumulation by internal Ca²⁺ chelation prolongs the action potential duration (Ehile and Gola, 1979). Both of these effects occur in cell R-15 and are consistent with the behavior of the Ca²⁺-activated K⁺ current and its control of the duration of the prolonged action potential in TEA-treated cells.

Concentration Dependence of TEA Inhibition

The effects of various concentrations of TEA on the Ca²⁺-activated K⁺ current are shown in Fig. 7. Two methods were used to determine the apparent dissociation constant for the reaction of external TEA with the Ca²⁺-activated K⁺ current. Fig. 7A shows the effect of TEA concentration on the outward K⁺ current activated by constant internal Ca²⁺ injection (200 nA for 10 s) at a holding potential of -30 mV. Averaged data for seven cells are shown in the dose-response plot. The experimental points are reasonably well fitted with a theoretical curve, using Eq. 1 with $K_{\text{TEA}} = 0.4$ mM, which is ~15 times smaller than the value determined above for the delayed outward K⁺ current. The two types of experiments are, however, not directly comparable because of the different protocols (long internal Ca²⁺ ion injection vs. brief depolarization pulses) and the difference in membrane potential at which measurements were made. A different type of experiment was used to determine the effect of TEA on the Ca²⁺-activated K⁺ current at potentials more positive than ~30 mV (see Hermann and Gorman [1981]). The membrane was depolarized briefly to +20 mV in Ca²⁺-free ASW before and at the end of a 10-s internal Ca²⁺ ion injection that activated an outward K⁺ current. The current difference was used as an index of the additional K⁺ current activated by the change in internal Ca²⁺. This protocol was repeated in various external TEA concentrations (Fig. 7B) in three cells, and the averaged results are plotted on a dose-response plot in Fig. 7B. External TEA reduced the delayed and the Ca²⁺-activated outward K⁺ currents but had a greater inhibitory effect on the Ca²⁺-activated component. The experimental points in Fig. 7B are reasonably well fitted with a theoretical curve, using Eq. 1 with $K_{\text{TEA}} = 0.5$ mM, which is only slightly greater than the value determined for the dissociation constant at more negative potentials.

Effects of TEA Ion Derivatives on I_{K,Ca}

Several quaternary ammonium (QA) ions besides TEA block the delayed outward K⁺ current when applied to the external surface of myelinated axon (Hille, 1967b; Armstrong and Hille, 1972). A number of these ions were tested externally to determine their effect on the Ca²⁺-activated K⁺. In each case, Ca²⁺ was injected iontophoretically for 10 s at a holding potential (-30 mV)

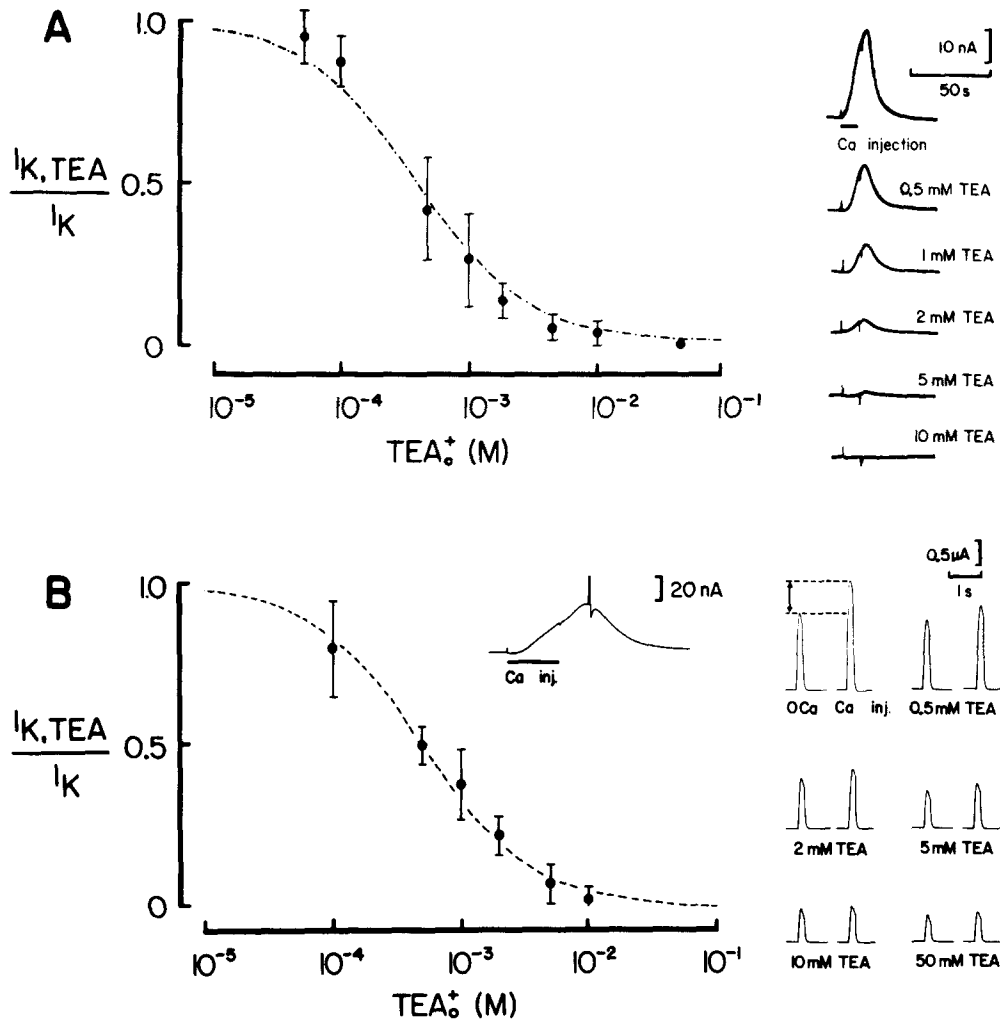


FIGURE 7. Effects of external TEA concentration on the Ca^{2+} -activated K^+ current. (A) dose-response plot of Ca^{2+} -activated K^+ current $I_{K,TEA}/I_K$ at -30 mV for seven cells vs. external TEA concentration. The experimental points were measured as the difference between the holding current at -30 mV and the peak current activated by an intracellular Ca^{2+} injection for 10 s at 200 nA. The theoretical curve fitted to the points was calculated from Eq. 1, using a dissociation constant $K_{TEA} = 0.4$ mM. The right side of A shows individual outward current responses at a holding potential of -30 mV to a 10-s, 200-nA Ca^{2+} injection at the indicated external TEA concentration. (B) dose-response plot of the outward current difference at $+20$ mV produced by Ca^{2+} injection $I_{K,TEA}/I_K$ for four cells vs. external TEA concentration. The outward current was measured as the difference between the outward current response to a $+20$ -mV pulse (holding potential, -50 mV) before and at the end of a 10-s, 500-nA Ca^{2+} injection in various external TEA concentrations. The theoretical curve fitted to the experimental points was calculated from Eq. 1, using a dissociation

at which the K⁺ current was outward, and the effect of various quaternary ammonium ions were determined (see Fig. 7A). The results are summarized in Table I, where the apparent dissociation constants for those ions that blocked the Ca²⁺-activated K⁺ current are listed (column 2). Other compounds that were tested and that had no effect on the K⁺ current up to concentrations of 50 mM are also included. Table I also lists the apparent dissociation constants for those QA ions that block the delayed outward K⁺ current of vertebrate myelinated axon (column 4) when applied externally (Hille, 1967*b*; Armstrong and Hille, 1972). The data presented in Table I demonstrate

TABLE I

Quaternary ammonium ion	K_D	n	K'_D	Diameter
	<i>mM</i>		<i>mM</i>	
Tetramethylammonium	>500.0	3	500.0	6.94
Trimethylethylammonium	>50.0	1	300.0	6.94 (7.47)
Dimethylethylpropylammonium	>50.0	1	—	(8.52) (6.94), (7.99) (7.47)
Diethylmethylpropylammonium	18.0	1	—	(8.52) (7.47), (8.00) (7.99)
Triethylmethylammonium	14.0	1	15.0	8.00 (7.47)
Tetraethylammonium	0.4	7	0.4	8.00
Triethylphenylammonium	0.5	2	—	8.00 (8.90)
Triethylpentylammonium	5.5	2	~1.0	8.00 (>10.00)
Triethyloctylammonium	3.4	1	—	8.00 (>10.00)
Tetrapropylammonium	>50.0	1	60.0	9.04
Tetrabutylammonium	>50.0	1	—	9.88

The apparent dissociation constant (K_D) for the reaction of various QA ions with a receptor site associated with the Ca²⁺-activated K⁺ channel and the number of experiments (n) are listed in the second and third columns. Mean values are given where more than one experiment is involved. The fourth column lists the apparent dissociation constant (K'_D) for the reaction of external QA ions with the delayed outward K⁺ channel of myelinated axon (data from Hille [1967] and Armstrong and Hille [1972]). The last column lists the estimated ionic diameters. The estimates are based on measurements of ionic radii of symmetrical QA ions given by Robinson and Stokes (1959, Table 6.2). The numbers in parentheses refer to the asymmetrical ionic diameter. The two sets of values given for dimethylethylpropylammonium and diethylmethylpropylammonium correspond to their possible ionic configurations. The procedure for estimating the asymmetrical diameter of triethylphenylammonium is given in the text.

the following:

1. TEA inhibits the Ca²⁺-activated K⁺ current at the lowest concentrations, i.e., it has the strongest affinity for the external receptor site.
2. Increasing or decreasing the diameter for a QA ion (relative to the diameter of TEA) decreases its ability to bind to the receptor.
3. The substitution of a phenyl for an ethyl group (triethylphenylammonium) has very little effect on the binding ability of a QA ion.

constant $K_{TEA} = 0.5$ mM. The right side of *B* shows responses (pen-recorder records) from a single cell at the indicated external TEA concentrations. The top pair of records shows how the measurements were made. Ca²⁺-free ASW was used in all cases. The bars drawn through the experimental points in *A* and *B* represent 1 SEM.

4. The addition or subtraction of a single methyl group has a large effect on the ability of a QA ion to bind to the receptor.

5. Increasing the hydrocarbon chain length of one of the side chains (triethylpentylammonium and triethyloctylammonium) decreases the binding ability of a QA ion.

The effects of external QA ions on the Ca^{2+} -activated K^+ current are very similar to their effects on the delayed outward K^+ current of vertebrate myelinated axon (compare the data given in columns 2 and 4 of Table I) but differ from the effects of *internal* TEA ion derivatives on the delayed K^+ current of squid and of myelinated axon (Armstrong, 1969; Armstrong and Hille, 1972), where increasing the hydrocarbon chain length of one of the side chains of a QA ion increases the effectiveness of the block.

A rough estimate of the size of the external binding site can be obtained from the diameters of the symmetrical and assymetrical QA ions given in Table I (column 5). TEA has an estimated diameter of 8.0 Å. The effectiveness of the block diminishes markedly when symmetric QA ions are less than ~7.0 Å (tetramethylammonium) or greater than ~9.0 Å (tetrapropylammonium or tetrabutylammonium). Triethylphenylammonium is assymetric but is as effective as TEA. Its assymetrical radius (4.9 Å) can be estimated from crystallographic data using a N—C internuclear distance of 1.43 Å for the aromatic N—C bond, a distance of 1.08 Å for the C—H bond, and a distance of 2.4 Å between nonbonded carbon atoms in the benzene ring (Kitaigorodskii, 1961). Its assymetric diameter is, therefore, 8.90 Å (4.00 Å + 4.90 Å). The results suggest that the blocking site can accommodate a QA ion whose diameter is greater than ~7.0 Å, but less than ~9.0 Å. It is unlikely, however, that the size of a QA ion is the sole determinant of its ability to block a K^+ channel. Triethylmethylammonium, diethylmethylpropylammonium, and dimethylthylpropylammonium ions differ in size from TEA only slightly (Table I, column 5) but are very different in their binding affinity. It is possible that the ability of a QA ion to reach the binding site is determined by steric factors, whereas its ability to remain at the site is determined by the groups attached to the nitrogen atom.

Effects of Internal TEA on the Ca^{2+} -activated K^+ Current

We have reported (Hermann and Gorman, 1979 *a*) that internal TEA blocks the delayed outward K^+ current measured with brief depolarizing pulses to positive potentials but that at potentials more negative than the zero membrane potential it has no effect on the K^+ current produced by internal Ca^{2+} injection. There is evidence from axons (Armstrong 1969 and 1971; Armstrong and Hille, 1972), however, that the block of the delayed outward K^+ current by internal quaternary ammonium ions depends on membrane voltage as well as on concentration, i.e., the block at any concentration is greater at more positive membrane potentials, presumably because TEA or similar molecules are driven into open channels (Armstrong, 1969). To determine whether it is possible to produce a similar voltage-dependent block of the Ca^{2+} -activated K^+ current, a constant internal Ca^{2+} injection was used (100 nA for 10 s). The

injection was repeated every 2–5 min at holding potentials between -80 and $+50$ mV (Fig. 8). TEA was injected iontophoretically for 12 min and the protocol was repeated. This quantity of internal TEA is more than sufficient to block the delayed outward K^+ current in these cells (Hermann and Gorman, 1979 *a*). The results (Fig. 8) show that there is a substantial reduction of the Ca^{2+} -activated K^+ current at potentials more positive than ~ 0 mV, indicating that internal TEA blocks the Ca^{2+} -activated K^+ channel at positive potentials and suggesting that the blocking site is within the membrane electrical field. Injection of internal TEA usually produced a slight increase in the Ca^{2+} -activated K^+ current at potentials more negative than ~ 0 mV (Fig. 8). Commercial TEA contains $\sim 5\%$ triethylamine as an impurity, which alters

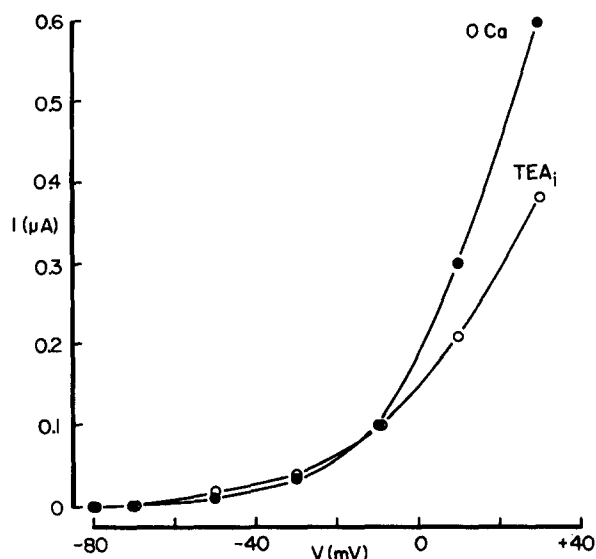


FIGURE 8. Effects of internal TEA on the Ca^{2+} -activated K^+ current. Plot of K^+ current in Ca^{2+} -free ASW vs. membrane holding potential before (closed circles) and after (open circles) four 3-min intracellular injections of TEA at 500 nA. The current was measured as the difference between the holding current and the peak current at the end of a 10-s Ca^{2+} injection at 100 nA.

intracellular pH and, therefore, reduces the buffering capacity of the cytoplasm for Ca^{2+} ions (Zucker, 1981). The small increase in the Ca^{2+} -activated K^+ current under these conditions is as expected. No correction was made for this change.

Effects of External TEA on the Outward Current in Normal ASW

The membrane current measured at the end of depolarizing clamp steps (50-ms or longer) to potentials more positive than about -20 mV in normal ASW is carried predominantly by K^+ ions (Heyer and Lux, 1976 *a*). The outward current vs. voltage relation is N shaped at positive membrane potentials (Fig. 9A). The reduction of the current between about $+80$ and $+120$ mV has

been related to the reduction of the Ca^{2+} -activated component of the total outward K^+ current as the membrane potential approaches the Ca^{2+} equilibrium potential (Meech and Standen, 1975; Heyer and Lux, 1976 *a* and 1976 *b*). The influx of Ca^{2+} ions from normal ASW and, thus, the Ca^{2+} -activated K^+ current is reduced in this region, whereas the delayed K^+ and the leakage currents continue to increase in amplitude. In addition, for cells such as R-15, in which the Ca^{2+} -activated K^+ current represents a substantial fraction of

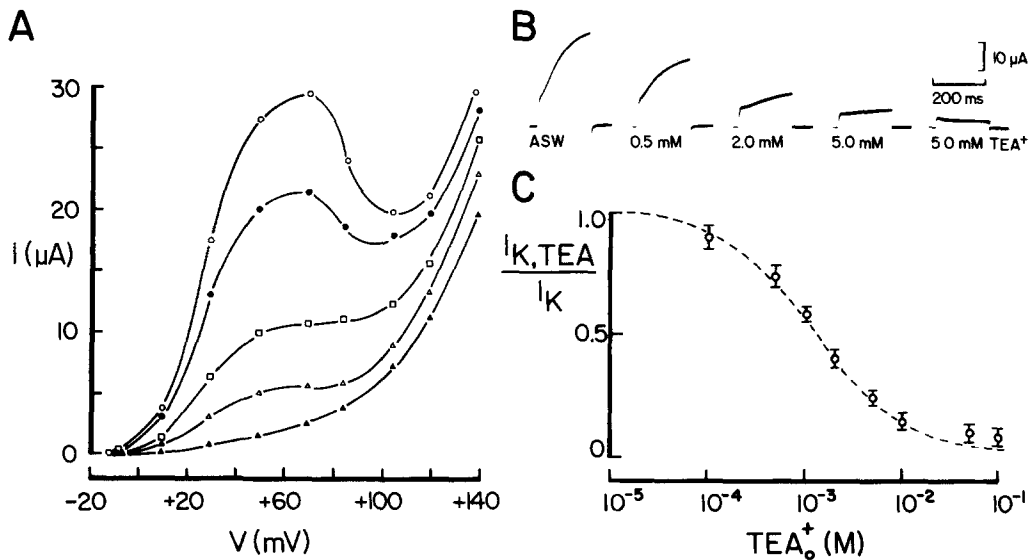


FIGURE 9. Effects of external TEA on the outward current in normal ASW. (A) Plot of the outward current measured at the end of 200-ms pulses (holding potential, -50 mV) before and in different external TEA concentrations (open circles, ASW; closed circles, 0.5 mM; open squares, 2.0 mM; open triangles, 5 mM; closed triangles, 50 mM) vs. membrane potential. (B) Individual currents measured at $+70$ mV at the indicated external TEA concentrations. The holding potential was -50 mV in A and B. (C) Dose-response to plot of outward current measured in normal ASW at the end of 200-ms pulses to $+20$ mV, $I_{K,TEA}/I_K$, from four cells vs. external TEA concentration. The holding potential was -45 mV in all cases. The theoretical curve fitted to the experimental points was calculated from Eq. 1, using a dissociation constant $K_{TEA} = 1.3$ mM. The lines through the experimental points represent 1 SEM.

the total outward current (Hermann, 1978), the current wave form (at membrane potentials negative to the Ca^{2+} equilibrium potential) is substantially different in the presence of external Ca^{2+} ions. The outward current measured with depolarizing pulses of moderate duration (~ 200 ms) is not only larger than the current measured at the same potential in Ca^{2+} -free ASW but reaches a peak at the end of the pulse (Fig. 9B) rather than near the beginning and shows none of the decline during the pulse that characterizes the delayed outward K^+ current (see Fig. 2).

The effects of external TEA on the current-voltage relation in *normal* ASW are consistent with its action on the individual component of the K⁺ current (Fig. 9A and B). In normal ASW, the outward current is dominated by the Ca²⁺-activated K⁺ current at potentials between -20 and about +80 mV, but at more positive potentials the delayed outward K⁺ and outward leakage currents become more important. External TEA reduced the outward current at all potential ranges in a dose-dependent manner, but the degree of reduction was greatest at those potentials at which the contribution of the Ca²⁺-dependent component is most significant (Fig. 9A). For example, the N shape of the current-voltage relation, which depends on the Ca²⁺-activated K⁺ current, is completely eliminated by high external TEA concentrations (50 mM). Similarly, the current at +70 mV (Fig. 9B) is changed from a slowly rising wave form that reaches a peak at the end of the voltage clamp pulse to a much smaller wave form that declines during the pulse. These effects are consistent with the results given in previous sections, which show that at an external TEA concentration of 50 mM the block of the Ca²⁺-activated K⁺ current is complete (Fig. 7), whereas only 75–85% of the delayed K⁺ current is blocked (Fig. 3B).

Fig. 9C shows a dose-response plot of the current ratio at +20 mV for four cells in normal ASW vs. the external TEA concentration. The experimental points are reasonably well fitted with a theoretical curve, using Eq. 1 with $K_{\text{TEA}} = 1.3$ mM. The value for the dissociation constant in normal ASW is less than the values estimated previously for cell R-15 (Adams and Gage, 1979) and for some other molluscan neurons (Neher and Lux, 1972; Meech and Standen, 1975; Thompson, 1977) under similar external ionic conditions but is close to the value estimated for isolated *Helix* neurons (Kostyuk et al., 1975). The apparent dissociation constant (K_{TEA}) for the net outward current in normal ASW, however, is not a constant but depends upon the relative contribution of the Ca²⁺-activated and the delayed K⁺ currents at any potential (see Hermann [1978]) as well as upon their respective dissociation constants.

Effects of Internal TEA on the Outward Currents in Normal ASW

The effects of internal TEA on the outward currents measured with depolarizing pulses in normal ASW are also consistent with its effect on the individual components of the K⁺ current. Fig. 10 shows the current-voltage relation and representative currents at +20 mV before and after several intracellular TEA injections. Internal TEA reduced the outward current, but this action differed from that produced by external TEA in several important respects. First, its effect was great at positive membrane potentials and minimal at potentials more negative than the zero membrane potential. Second, the N shape of the current-voltage relation was reduced but never completely eliminated. Finally, the rising phase of the outward current (measured with depolarizing pulses to +20 mV) was slowed by internal TEA. We (Hermann and Gorman, 1979 a) have shown that the internal TEA injection of the magnitude used to obtain the results shown in Fig. 10 produce a substantial block of the delayed outward K⁺ current. The Ca²⁺-activated K⁺ current is also reduced by internal TEA,

but this reduction is only apparent at positive membrane potentials (see Fig. 8). The reduction of the outward current at +130 mV can be accounted for primarily by the effect of internal TEA on the delayed outward K^+ current, because the Ca^{2+} -activated K^+ current is minimal at this potential. The shift of the initial peak of the current-voltage relation, from about +70 mV to less positive values, as well as its reduction after injection of TEA can be accounted for by the blocking effect of internal TEA on the Ca^{2+} -activated K^+ current

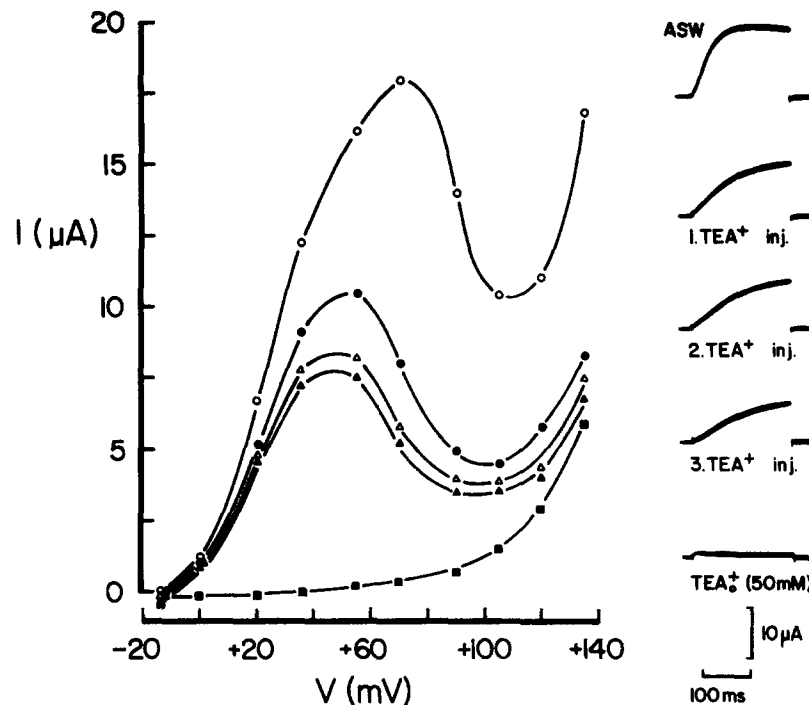


FIGURE 10. Effects of internal TEA on the outward current in normal ASW. Plot of outward current measured at the end of a 200-ms pulse before (open circles) and after three internal TEA injections (closed circles, 54 μ C; open triangles, 90 μ C; closed triangles, 117 μ C) vs. membrane potential. The effect of 50 mM external TEA (closed squares) is also shown. The right side shows individual responses measured at +20 mV under the indicated conditions.

at positive voltages. Fig. 10 also provides a comparison between the effects of internal and external TEA on the outward K^+ current in the same cell. The addition of 50 mM external TEA after the final intracellular injection of TEA had a minimal effect on the outward current at +130 mV but completely eliminated the N shape of the current-voltage relation.

DISCUSSION

Two major conclusions can be drawn from the effects of TEA on the molluscan neuronal soma membrane. First, TEA blocks the Ca^{2+} -activated as

well as the delayed outward K⁺ current. Second, the properties of the block depend upon membrane potential and upon the side of the membrane to which TEA is applied.

The effects of TEA have been studied best in squid axon (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965; Armstrong, 1966, 1969, and 1971), in which it affects the K⁺ channel only from the inside membrane surface, and in vertebrate myelinated axon (Hille, 1967*a*; Koppenhöffer, 1967; Koppenhöffer and Vogel, 1969; Armstrong and Hille, 1972), in which it is effective when applied to either side of the membrane. In both systems there is strong evidence that TEA does not cross the membrane through the K⁺ or other channels (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965; Hille, 1967*a*). Although it is not possible to completely exclude a single receptor site whose properties depend on the direction of approach of the blocking ion, the available data suggest that there are separate internal and external TEA receptors for the K⁺ channel (Koppenhöffer and Vogel, 1969; Armstrong and Hille, 1972). In molluscan neurons there is evidence that suggests that TEA does not cross the membrane and that there are separate receptor sites. First, the effect of internal TEA is irreversible, whereas the effect of external TEA is readily reversed. Second, the effects of changes in membrane potential on the block produced by TEA depend upon the side of the membrane to which it is applied.

In both squid and vertebrate axons *internal* TEA depresses the delayed outward K⁺ current more strongly at higher depolarizations without altering appreciably current kinetics (Armstrong, 1966; Armstrong and Hille, 1972). The voltage dependence of the block and changes in current kinetics are more marked when asymmetrical QA ions are used. The axonal data suggest that once the delayed K⁺ channel is activated, and thus open, TEA (or other QA ions) can be driven by membrane depolarization into a region of the channel where it occludes the outward movement of K⁺ ions (Armstrong, 1969 and 1971). In molluscan neurons, the block of the Ca²⁺-activated K⁺ current by internal TEA also depends on membrane voltage and suggests that the two currents respond in much the same way to internal TEA.

In frog myelinated axon *external* QA ions reduce the delayed outward K⁺ current without altering its kinetics, and more hydrophobic QA ions are less effective than TEA (Hille, 1967*a*; Koppenhöffer, 1967; Koppenhöffer and Vogel, 1969; Armstrong and Hille, 1972). External TEA has a similar effect on the delayed outward K⁺ current of the molluscan neuronal soma membrane, with the important difference that the apparent dissociation constant for the reaction is about 15 times greater than the value determined for myelinated axon (Hille, 1967*a*). In contrast, the dissociation constant for the reaction of TEA with the Ca²⁺-activated K⁺ current is identical to the value given for the axonal delayed K⁺ current. The two results are not directly comparable because the dissociation constant also depends on the external Ca²⁺ concentration (Mozhayeva and Naumov, 1972), which was normal (2.0 mM) in previous studies on myelinated axon but <10 μM in the present experiments. The values given for the dissociation constant for the delayed

outward K^+ current of myelinated axon would be smaller if determined in Ca^{2+} -free conditions.

The block by external TEA of both the delayed and the Ca^{2+} -activated K^+ currents is sensitive to changes in membrane potential, but their voltage dependence is opposite to that for internal TEA, i.e., membrane depolarization *decreases* rather than increases the effectiveness of the block. There is also a small decrease in the effectiveness of external TEA on the delayed outward K^+ current of myelinated axon at very positive potentials, which is apparent in data reported by several groups (Hille, 1967*a*; Koppenhöffer, 1967; Mozhayeva and Naumov, 1972). The voltage dependence of the reaction of external TEA with K^+ channels suggests that the receptor site is within the membrane electric field. Armstrong (1969, 1971, and 1975) proposed that TEA can fit into the *internal* mouth of the K^+ channel because it has about the same diameter as a hydrated K^+ ion (both of which are estimated to be ~ 8.0 Å). It is possible that both the inner and the external mouths of the voltage-dependent and the Ca^{2+} -activated K^+ channels are large enough to accommodate a fully hydrated K^+ ion or a QA ion of the proper diameter but are separated by a narrower region that allows only the passage of K^+ and ions of similar size in their unhydrated form and excludes the passage of QA ions (see Armstrong [1975] and Hille [1975]). A QA ion in the external mouth of a K^+ channel could be driven out of the channel by membrane depolarization or by electrostatic repulsion from a K^+ ion moving out of the cell in the presence of an electrical field of sufficient strength. The weak effect of depolarization on the block produced by external TEA, however, indicates that a QA ion, if it enters the external mouth of the channel, must be held firmly to the blocking site. Mozhayeva and Naumov (1972) have suggested that the energy of the channel-QA ion complex depends upon both the chemical properties of the receptor site and the density of the distribution of negatively charged groups in the vicinity of the channel. If the charge density of these groups in the vicinity of the Ca^{2+} -activated and the delayed K^+ channels differ, then the higher binding energy of the Ca^{2+} -activated K^+ channel for TEA ions, as indicated by the approximately 15-fold difference in the dissociation constants, might be explained.

The shape of the external TEA dose-response plot for the K^+ current (both the delayed and the Ca^{2+} -activated K^+ currents) suggests that one external TEA ion blocks a single K^+ channel and is consistent with previously reported data from other molluscan neurons (Meech and Standen, 1975) and with data from myelinated axon (Hille, 1967*a*; Mozhayeva and Naumov, 1972). The approximately 15-fold difference in the dissociation constants for the two components of the K^+ current indicates that it is possible to distinguish between different components of the K^+ current on the basis of their sensitivity to external TEA. This finding, however, differs from results from *Helix* neurons reported by Meech and Standen (1975), who conclude that TEA blocks both components of the outward current equally, with a dissociation constant of ~ 10 mM, and from results from *Tritonia* neurons reported by Thompson (1977), who concludes that TEA blocks the delayed outward K^+ current, with

a dissociation constant of 8 mM, but has a much weaker effect on the Ca²⁺-activated K⁺ current. The values for the dissociation constants agree reasonably well with our value for the reaction of TEA with the delayed K⁺ current ($K_D = 6.0$ mM) but are considerably greater than our value for the Ca²⁺-activated K⁺ current ($K_D = 0.4$ mM). The results from *Helix* neurons were obtained from an analysis of tail currents in Ca²⁺-containing saline, and it is possible that this method is less sensitive because of overlapping inward and outward currents that decay with a similar time-course or that the difference between the two currents under Ca²⁺-free conditions is much less obvious in the presence of Ca²⁺. The results from *Tritonia* neurons were also obtained in Ca²⁺-containing media but were based on an analysis of outward currents measured during depolarizing pulses to various potentials. Thus, they can be more directly compared with the measurements presented in the preceding sections. The effect of high external TEA on the shape of the outward current

TABLE II

Drug	Delayed outward K ⁺ current				Ca ²⁺ -activated K ⁺ current				Reference
	Effect	Site	K_D	ΔV	Effect	Site	K_D	ΔV	
			<i>mM</i>				<i>mM</i>		
TEA	-	e or i	6.0	yes	-	e or i	0.4	yes	This paper and Hermann and Gorman (1979a)
4-amino-pyridine	-	i	0.4	yes	+	i	-	-	Herman and Gorman (1981)
Ba ²⁺	-	i	-	-	-	i	-	-	Gorman and Hermann (1979); Hermann and Gorman, (1979b)
Quinine	-	i	-	no	-	i	-	no	Unpublished results
Caffeine	-	i	16.0	yes	+	i	-	no	Hermann and Gorman (1980)

The effects of various agents on the delayed outward and on the Ca²⁺-activated K⁺ currents of cell R-15 are signified by an increase (+), or decrease (-) along with their presumed site of action on the external (e) or internal (i) membrane surface or both. Data concerning the dissociation constant for the reaction (K_D) and whether the reaction depends on membrane potential (ΔV) are also indicated.

—, indicates cases in which the necessary data were not determined.

response in *Tritonia* cells is quite different from its effect on *Aplysia* neurons under similar conditions; e.g., 50 mM TEA blocked ~50% of the outward current and spared a current component with slower kinetics (see Fig. 6 of Thompson [1977]), whereas in cell R-15 and identical TEA concentration blocked almost completely the outward current and left a very small current with much faster kinetics (see Fig. 9). This difference is interesting and suggests that in some molluscan neurons there may be a qualitative rather than a quantitative difference between the effects of external TEA on the voltage-dependent and on the Ca²⁺-activated components of the K⁺ current.

One of the goals of the studies presented in this and in the preceding paper (Hermann and Gorman, 1981) is the identification and definition of agents (both biological and nonbiological) that can be used to selectively block various current components. Table II summarizes data, primarily from cell R-15, relating to the effects of various agents on different components of the K⁺ current. Data concerning the presumed site of action of each agent (inside

(i) or outside (o) the cell or both), the dissociation constant for the reaction, (K_D) based on external concentration measurements, and whether the reaction depends on membrane voltage are also given. The agents that block the K^+ currents are diverse, ranging from small ions (Ba^{2+}) and molecules (TEA, and 4-aminopyridine) to larger molecules (quinine and caffeine). All block the delayed outward K^+ current, but two of the agents, 4-aminopyridine and caffeine, have the opposite effect on the Ca^{2+} -activated K^+ current. Most of these agents appear to work inside the cell, which makes their mode of action difficult to evaluate fully. This is particularly true of agents that alter the Ca^{2+} -activated K^+ current. In this case, it is possible that changes in the K^+ current reflect changes in the ability of the cytoplasm to sequester Ca^{2+} rather than a direct effect on the K^+ channel. There is evidence (Hermann and Gorman, 1981), however, that the effect of 4-aminopyridine on the Ca^{2+} -activated K^+ current may be direct, and this evidence, when taken together with the other pharmacological results summarized in Table II and those given previously (Gorman and Thomas, 1980), suggest that K^+ ions move through separate, voltage-dependent, and Ca^{2+} -activated K^+ channels.

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