EFFECTS OF THE DIPOLAR FORM OF PHLORETIN ON POTASSIUM CONDUCTANCE IN SQUID GIANT AXONS

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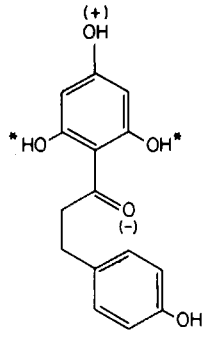
ABSTRACT The effects of phloretin on membrane ionic conductances have been studied in the giant axon of the squid, *Loligo pealei*. Phloretin reversibly suppresses the potassium and sodium conductances and modifies their dependence on membrane potential (E_m). Its effects on the potassium conductance (G_K) are much greater than on the sodium conductance; no effects on sodium inactivation are observed. Internal perfusion of phloretin produces both greater shifts in $G_K(E_m)$ and greater reductions in maximum G_K than does external perfusion; the effect of simultaneous internal and external perfusion is little greater than that of internal perfusion alone. Lowering the internal pH, which favors the presence of the neutral species of weakly acidic phloretin (pK_a 7.4), potentiates the actions of internally perfused phloretin. Other organic cations with dipole moments similar to phloretin's have little effect on either potassium or sodium conductances in squid axons. These results can be explained by either of two mechanisms; one postulates a phloretin "receptor" near the voltage sensor component of the potassium channel which is accessible to drug molecules applied at either the outer or inner membrane surface and is much more sensitive to the neutral than the negatively charged form of the drug. The other mechanism proposes that neutral phloretin molecules are dispersed in an ordered array in the membrane interior, producing a diffuse dipole field which modifies potassium channel gating. Different experimental results support these two mechanisms, and neither hypothesis can be disproven.

INTRODUCTION

The ionic conductances in excitable membranes are controlled by the membrane potential through the motion of "gating" molecules, which are charged or dipolar entities within the membrane that change their position of configuration in response to changes of the membrane's electric field (Hodgkin and Huxley, 1952 *b*; Armstrong and Bezanilla, 1975). The electric field strength at a gating molecule can be modified by the presence of negatively charged groups located at the membrane surface. Such negative charges are intrinsic features of certain phospholipids and the conductances in bilayer lipid membranes (BLM) which contain these phospholipids are modified by the same conditions which modify excitable membrane conductances, i.e., changes in ionic strength, H^+, and divalent ion concentration (Gilbert and Ehrenstein, 1969; Hille et al., 1975; McLaughlin et al., 1971). Thus electrostatic potentials at membrane surfaces can modify ionic conductances in both artificial bilayers and



PHLORETIN
(μ=5.6)

* pK_a = 7.4

FIGURE 1 The structure of phloretin.

biological membranes, and this result provides evidence for the phospholipid bilayer character of excitable membranes.

Ionic conductances through bilayer lipid membranes are also affected by dipole potentials within the membrane. A variety of small organic compounds, of which phloretin (Fig. 1) is the prototype, enhances the cation permeability but decreases the anion permeability in BLM and in red cells (Andersen et al., 1976; de Levie et al., 1979; Melnik et al., 1977; Wieth et al., 1973). Phloretin also lowers nonelectrolyte transport and facilitated hexose transport in red cells (LeFevre, 1961; Czech et al., 1973; Owen and Solomon, 1972). The effects of phloretin have also been studied in the soma of *Aplysia* giant neurons, where high concentrations ($>10^{-4}$ M) applied externally for brief periods increase the resting potassium conductance. Phloretin and the other organic molecules which effect permeabilities in red cells and BLM have large dipole moments and are active in their uncharged form. These compounds are proposed to act on lipid bilayers by introducing an additional dipole potential within the membrane interior (Andersen et al., 1976; Melnik et al., 1977). The profile of this dipole potential is not known, but the ion conductance changes may be equivalent to a change in the membrane surface potential as large as 200 mV.

We have investigated the effects of phloretin and four other dipolar compounds on the ionic currents in squid giant axons to determine how exogenous dipolar molecules would modify the ionic conductances of excitable membranes. Of the five compounds tested, only phloretin produced significant modifications, and certain aspects of its effects differ from those seen in BLM and in *Aplysia* neurons.

METHODS

Experiments were performed on single giant axons isolated from *Loligo pealei* obtained at the Marine Biological Laboratory, Woods Hole, Mass. The axons were cleaned of adhering connective tissue and smaller nerve fibers, canulated with a fire-polished glass capillary at the caudal end, and the axoplasm squeezed out with a tiny rubber roller, after the technique of Baker et al. (1961). The axons were reinflated with a standard internal solution (SIS, see below) using a micrometer syringe and transferred

to a plexiglas chamber similar in design to that of Armstrong et al. (1973). Unless otherwise noted, the internal and external perfusion solutions were continuously flowing during drug application and testing. The axons were voltage-clamped using a piggyback axial wire electrode and conventional techniques as described previously (Wu and Narahashi, 1973; Oxford et al., 1978).

The voltage clamp was compensated for voltage errors arising from most of the measured series resistance. Unless otherwise noted, the membrane potential was held at −80 mV between clamp steps and depolarized in either 5, 10, or 20 mV step increments at a maximum frequency of 1 s^{-1}. Capacitive and leakage currents were assumed to be linear and eliminated by analog electronic subtraction (see Armstrong and Bezanilla, 1975). The potassium conductance was calculated by dividing the peak or steady-state potassium current by $E_m - E_K$, the driving force on potassium ions; potassium currents were assumed to remain linear with driving force in the presence of phloretin. In cases where there was a noticeable decline of I_K with time, the peak currents for each potential step were taken for G_K determinations. This approximation leads to underestimates for G_K, particularly at larger depolarizations, and effectively reduces the calculated $\overline{G_K}$ and the slope of the G_K–E_m relationship. However, the decline of I_K in time is due to potassium accumulation outside the axon (Adelman et al., 1973) and will require a smaller correction when the potassium currents are reduced, as they are by phloretin. (Even for the largest currents, shown by the control currents at pH 7.3 in Fig. 6, the maximum theoretical change in the reversal potential at the peak of the current trace, after ~2 ms at an 80-mV depolarization, is only 15 mV (Adelman et al., 1973), which leads to an underestimate of G_K of ~9%.) In addition, in three axons the reversal potential (E_K) was determined directly by repolarization to different levels at the end of several large test steps before and after phloretin. The change in E_K was observed to be <10 mV in each case at the largest depolarizing step (+100 mV) and was indeterminable at test potentials more negative than −20 mV due to the small current magnitudes. Even if there were large changes in E_K, the effects of K^+-accumulation will tend to minimize the phenomena we observe here, by making control currents resemble phloretin-modified currents, so artifacts arising from using peak I_K values rather than correcting for K^+-accumulation cannot account for the observed effects of phloretin.

The axons were normally bathed in flowing artificial seawater (ASW) which contained: 450 mM Na^+, 10 mM K^+, 50 mM Ca^{++}, 576 mM Cl^-, and either 30 mM Tris-hydroxymethyl amino methane or 5 mM HEPES buffer, at pH 8.0. Tetrodotoxin (3×10^{-7} M) was used in many of the experiments to eliminate the ionic current contribution of sodium channels.

The axons were internally perfused with an SIS containing: 350 mM K^+, 50 mM Na^+, 320 mM glutamate$^-$, 50 mM F^-, 310 mM sucrose, and 15 mM phosphate buffer, at pH 7.3. Internal pH changes were accomplished by substituting either 30 mM PIPES (pK_a = 6.8) or 30 mm *z*-(cyclohexylamino)ethanesulfonic acid (CHES) (pK_a = 9.3) for the phosphate and titrating to the desired pH.

Phloretin was obtained from K & K Laboratories, Plainview, N.Y., and also as a kind gift from Josh Zimmerberg, Albert Einstein College of Medicine. 2,6-dihydroxy-acetophenone, *p*-nitrophenol, *m*-nitrophenol, and phloroacetophenone were obtained from ICN Pharmaceuticals, Plainview, N.Y. All of the compounds are sparingly soluble in water and were kept as stock solutions (mM) in dimethylsulfoxide (DMSO). New stock solutions were made every 2 or 3 d and the final experimental solutions made immediately before their addition to the axon. DMSO at concentrations <2% (vol:vol) has no effect alone on membrane ionic conductances or on the voltage clamp electrodes. Temperature in all experiments was maintained between 6° and 10°C by a Peltier device and electronic feedback circuitry. The temperature could be maintained to within ±0.2°C of the set point.

RESULTS

Phloretin Modifies G_K More than G_{Na}

The effects of phloretin added to the internal solution on membrane ionic currents under voltage clamp are illustrated in Fig. 2. Both the early transient sodium currents and delayed steady-state potassium currents are decreased by phloretin. The amplitude of the potassium currents is considerably more sensitive to phloretin than that of the sodium currents. The

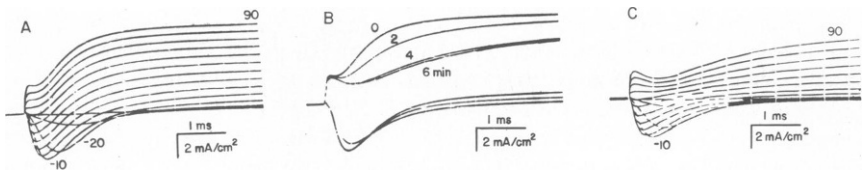


FIGURE 2 Effects of internal phloretin on ionic currents under voltage clamp. Current-time families before (*A*) and during (*C*) exposure to 5×10^{-5} M phloretin. Voltage steps were applied in 10 mV increments to the indicated potentials from a holding potential of −80 mV. The time course of phloretin action is shown in *B* for voltage steps to −10 and +90 mV. Axon 071776*a*.

potassium currents also appear to be slowed, taking longer to reach steady-state values during all depolarizing voltage steps. The average decrease in the maximum peak sodium conductance produced by internal phloretin was 32% ($n = 4$), whereas the average potassium conductance declined by 51%; (50 μM, $pH_{in} = 7.3$, see Table I).

The most striking effect of phloretin was on the voltage-dependence of both sodium and potassium conductances. The curves relating each conductance to membrane potential (E_m) are shifted along the voltage axis in the positive direction, such that the axon membrane must be depolarized further than in the control to achieve the same degree of activation of the remaining, drug-modified conductance. Fig. 3 illustrates this shift in two axons. In both cases the sodium (G_{Na}) or the potassium (G_K) conductances were normalized to their maximum values and their logarithms plotted against the absolute membrane potential. Under these conditions the effect of phloretin is to shift the curves in parallel with no measureable change in their slope, although changes in slope are observed at lower internal pH (see below). The shift, as determined at 20% of the maximum conductance ($V_{0.2}$), was +8 mV for G_{Na} and +31

TABLE I
EFFECTS OF PHLORETIN ON MAXIMUM POTASSIUM CONDUCTANCE

Concentration	pH_0	pH_i	$\frac{\bar{G}_K \text{ (test)}}{\bar{G}_K \text{ (control)}}$	n
(μM)				
Internal phloretin				
50	8.0	7.3	0.54 ± 0.07	5
10	8.0	7.3	0.68 ± 0.01	2
1	8.0	7.3	0.85	1
50	8.0	8.7	0.69 ± 0.04	2
50	8.0	6–6.2	0.34 ± 0.08	2
10	8.0	6.3	0.58	1
External phloretin				
50	8.0	7.3	0.73	1
50	6.9	7.3	0.81	1
10	6.3	7.3	0.67	1
Phloretin, both sides				
50 (inside)	7.3	7.3	0.44	1
50 (both)	7.3	7.3	0.42	1
10 (inside)	8.0	7.3	0.68	1
10 (both)	8.0	7.3	0.67	1

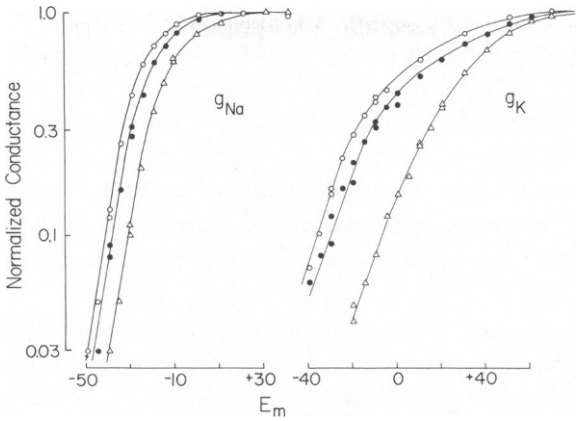


FIGURE 3 Voltage shifts of sodium and potassium conductance by phloretin. Conductances are normalized (G/G_{max}) and represent values before (○), during (△), and following (●) exposure to phloretin internally. Sodium conductance measurements are from axon 071677*b* exposed to 6×10^{-5} M phloretin, pH 7.3. Maximum G_{Na} values were 55.5, 33, and 47 mS/cm^2 before, during, and after phloretin, respectively. Potassium conductance measurements are from axon 071877*a* exposed to 1×10^{-5} M phloretin, pH 7.3. Maximum G_K values were 34, 23, and 29 mS/cm^2 before, during, and after phloretin, respectively.

mV for G_K. The larger shift of the G_K–E_m relation seen when comparing these two experiments is even more striking considering the six-fold lower phloretin concentration used in the G_K determination (see figure legend). Shifts of both conductances were usually 80–90% reversed upon washing with phloretin-free solution. The lower sensitivity of G_{Na} to phloretin treatment is also reflected in measurements of sodium channel inactivation. Several determinations of the voltage-dependence of sodium channel inactivation using conventional double-pulse protocols (Hodgkin and Huxley, 1952 *b*) revealed a negligible effect of phloretin on this parameter.

Examination of many axons treated with tetrodotoxin to eliminate sodium current consistently demonstrated a pronounced slowing of potassium current kinetics (see Fig. 6). In axons perfused with potassium-free internal solutions to eliminate outward potassium current, no significant changes in sodium current kinetics were observed upon phloretin treatment beyond those expected from response of the activation rates to the small voltage shift observed in the peak conductance-voltage curve.

In view of the greater sensitivity of the potassium conductance to phloretin we chose to examine this interaction in more detail. The remainder of this paper deals only with the effects of phloretin on the potassium conductance. These effects are categorized as the three phenomenological changes introduced above: (*a*) reduction of the maximum potassium conductance $\overline{G_K}$. (*b*) depolarizing shift of the voltage-dependence for the reduced G_K, and (*c*) slowing of the activation of G_K upon step membrane depolarization.

Phloretin Slows the Rate of G_K Activation

The effect of phloretin on the rate of G_K activation is further analyzed in Fig. 4. The kinetics of control (I_K) and phloretin-modified currents (I_K^*) are compared by first scaling up the I_K^* values at all times by the ratio of the steady-state I_K values, $I_K^\infty / I_K^{*\infty}$ (= 1.63) to yield the

dashed lines. The slowing of I_K^* is apparent; the time required to reach half the steady-state value ($t_{1/2}$) is 1.9 ms for I_K and 4.8 ms for I_K^*. However, the relative delay in the rise of the activation curve is not modified by phloretin. This is demonstrated by multiplying the time axis for the phloretin curve by the factor $t_{1/2}/t_{1/2}^* = 0.40$, and thus condensing $I_K^*(t)$ in time, as shown for discontinuous points noted by the open circles in Fig. 4. The circles closely overlay the curve for $I_K(t)$, showing that the time course of the phloretin modified current is equivalent to that of the control potassium current with an increased relaxation time, τ_n, for the transition between closed and open states of the potassium channel (Hodgkin and Huxley, 1952 *c*).

The voltage-dependence of the activation rate of G_K is shifted by phloretin. In the membrane potential range of -20 to $+120$ mV the internal perfusion of 50 μM phloretin ($pH_{in} = 6.4$) produces a shift in the half-time for G_K activation (see Fig. 4) of 24–31 mV in the depolarizing direction. These shifts are about the same size as those of the steady state G_K–E_m relationship, and are consistent with the concept that phloretin inhibits the activation of potassium channels.

Phloretin Acts from Both Membrane Surfaces

Phloretin applied to the external axon surface produced qualitatively the same effects on G_K as internal phloretin; however, greater drug concentrations were required to produce quantitatively equal effects. In axons bathed in normal ASW and continuously perfused with SIS, 50 μM internal phloretin reduced $\bar{G}_K$ by 46% while the same concentration of external phloretin reduced $\bar{G}_K$ by only 27% ($pH_o 8$, pH_i 7.3; see Table I). The depolarizing shift of $V_{0.2}$ averaged 32.4 ± 2.8 mV for internal phloretin (50 μM) but only 26.7 ± 1.8 mV for external phloretin (see Table II). As with internal phloretin, all the effects of external phloretin on $\bar{G}_K$ were almost completely reversible.

The apparent greater sensitivity of the axon to internal phloretin application is probably a consequence of the relative internal and external perfusion rates, and should in no way be construed as evidence for locating the site of action of this drug. During external application of

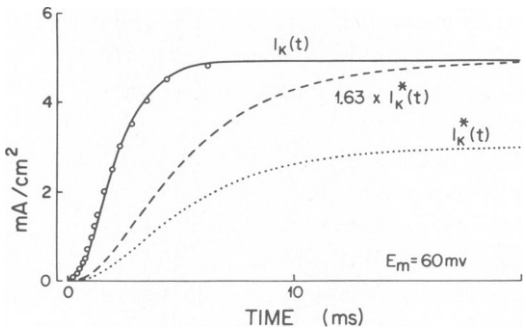


FIGURE 4 Analysis of kinetic changes in potassium current produced by phloretin. The solid line shows the control current (I_K), the dotted line the current with 10 μM phloretin inside (I_K^*), both in response to a depolarizing step to +60 mV. When the $I_K^*(t)$ curve is scaled vertically by the ratio of the steady-state currents, $I_K(\infty)/I_K^*(\infty) = 1.63$, the dashed-line curve is generated. The circles are values from the dashed-line curve scaled horizontally by the ratio of the half-times, $t_{1/2}/t_{1/2}^* = 0.405$. $T = 9°C$. ASW + $3 \cdot 10^{-7}$ M TTX.

TABLE II

SHIFTS OF G_K vs. E_m ($V_{0.2}$) BY PHLORETIN

Experiment	Concentration ($\times 10^{-6}$ M)	pH_o	pH_i	$V_{0.2}$ control	$V_{0.2}$ test	$V_{0.2}$
				(mV)	*(mV)*	*(mV)*
Internal phloretin						
071976*a*	50	8.0	7.3	−17	+9	+26
071976*b*	50	8.0	7.3	−17	+10	+27
	50	8.0	8.7	(0)*	−3	−3
	50	8.0	6.2	(−17)	+17	+34
072976*b*	50	8.0	7.3	−9	+23	+32
	50	8.0	8.7	+2	+14	+12
	50	8.0	6.2	−13	+30	+43
071177*a*	50	8.0	7.3	−31	−1	+30
071877*a*	10	8.0	7.3	−26	+5	+31
	10	6.3	6.3	−30	+14	+44
External phloretin						
073176*a*	50	8.0	7.3	−4	+21	+25
	50	6.15	7.3	−5	+21	+26
071177*a*	50	6.9	7.3	−9	+21	+30
030477*a*	50	8.0	7.3	−35	−14	+21
	50	8.0	6.3	−41	−12	+29
Phloretin, both sides						
071277*a*	50 (inside)	7.3	7.3	−30	+20	+50
	50 (both)	7.3	7.3	−30	+26	+56
071477*a*	10 (inside)	8.0	7.3	−32	−1	+31
	10 (both)	8.0	7.3	−32	+6	+38
071877*b*	10 (outside)	6.3	7.3	−38	−9	+29
	10 (both)	6.3	6.3	−49	−4	+45
Mean ± SEM values						*(mV)*
	10–50 (inside)		7.3			+32.4 ± 2.8
	10–50 (inside)		6–6.3			+41.0 ± 2.5
	10–50 (inside)		8.7			+4.5 ± 3.3
	10–50 (outside)		7.3			+26.7 ± 1.8

*Values in parentheses are estimates of control $V_{0.2}$ at the indicated pH_i using the value at pH 7.3 and assuming +7.15 mV shift/pH unit.

phloretin the axon was continuously perfused internally with phloretin-free SIS; if internal perfusion was temporarily stopped, the effects of phloretin are increased, reversibly. Such a result shows that, under the conditions of drug application used here, the phloretin concentration was at some steady-state value, but not in equilibrium between the membrane phase and either external or internal aqueous solutions.

When phloretin was present in solutions bathing both sides of the axon there was little greater effect than if phloretin were present only in the internal solution. The results of an experiment to test the effects of phloretin on both sides of the membrane are illustrated in Fig. 5. The axon was first exposed to normal internal and external solutions (SIS and ASW), both adjusted to pH 7.3, and then perfused internally with 50 μM phloretin. The maximum potassium conductance was reduced by 56% and the curve shifted along the voltage axis ($V_{0.2}$) by +50 mV. (The slope of the curve was also decreased, an effect described in detail below.) These steady-state effects were reached after 5 min of exposure to phloretin. While continuing

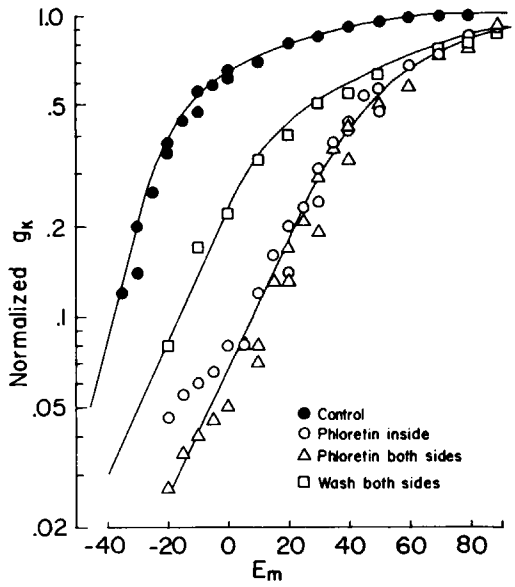


FIGURE 5 Internal and external phloretin act at a common site. Normalized G_K values are from axon 071277*a* before phloretin (●), during 5×10^{-5} M phloretin applied internally (○), during 5×10^{-5} M phloretin applied to both sides simultaneously (△), and following removal of phloretin from both sides for 26 min. (□). Maximum G_K values under the above conditions were 28.4, 12.6, 12.0, and 21.5 mS/cm^2, respectively. The pH was maintained at 7.3 on both sides in each case.

the internal phloretin perfusion, 50 μM phloretin was perfused externally for another 15 min. The additional shift of $V_{0.2}$ was <5 mV and the maximum potassium conductance was reduced only an additional 2%. In the absence of internal phloretin, the application of 50 μM phloretin in the external bathing medium produced a decline of ~20% in $\bar{G}_K$ and a shift of ~+23 mV in $V_{0.2}$ (not shown). The very limited additional effects of external phloretin in the presence of internal phloretin suggest that the drug has a common site(s) of action for modifying G_K regardless of the side of application. Phloretin is known to permeate red cell membranes (Jennings and Solomon, 1976) so its access to a membrane site from either surface is not unexpected, although the rate of penetration is not known for the squid axon.

When the experimental sequence was reversed and the axon was first exposed to external phloretin, followed by exposure to phloretin in the internal solution, a large, additional positive shift (+16 mV) was observed. However, the total shift was approximately equal to the shift from internal phloretin alone. As in the previous example, the effects of internal plus external exposure to phloretin were not additive. Therefore, while application from either side produces the same qualitative effects, application at the inner axon surface is more effective than at the outer surface and, in fact, dominates the response when the drug is applied simultaneously to both sides. The results are consistent with a membrane site of phloretin action which is accessible to the drug in the axoplasmic solution through an internal unstirred layer significantly thinner than the unstirred layer which controls the diffusion of phloretin to the membrane from the external solution (see Discussion).

Phloretin Is More Active in the Neutral Form

Because phloretin is weakly acidic (pK_a 7.4), both negatively charged and neutral species will be present at physiological pH. The relative potencies of these two drug species were investigated at different internal and external pH values. In Fig. 6, families of potassium current versus time are shown before and during internal perfusion with 50 μM phloretin at three different values of internal pH. As the internal pH is lowered, favoring the neutral form of phloretin inside the axon, all three drug effects on G_K are enhanced. At pH 8.7, where phloretin is primarily in the anionic form (5% neutral, 95% charged), a small decline in the amplitude, some slowing of activation kinetics, and a negligible voltage shift of G_K is observed. At the standard internal pH of 7.3, where neutral and anionic species are nearly equal (56% neutral, 44% charged), the magnitude of $\bar{G}_K$ is decreased by nearly 40%, the activation of G_K is obviously slowed and a shift in the voltage-dependence of G_K is evidenced by the relative amplitudes of potassium current at 0 mV with and without phloretin. Decreasing the internal pH to 6.2 (94% neutral, 6% charged) results in a much larger decrease in current (note the change in ordinate scale) and a substantial shift of the voltage-dependence for G_K activation (Fig. 7 and Table II). A fourth effect of phloretin becomes apparent at low pH, a change in the slope of the G_K–E_m curves (Fig. 8). This effect is described further below.

Changing internal pH alone does result in some changes in G_K. $\bar{G}_K$ is reduced by 55% upon lowering the pH from 7.3 to 6.3 (Fig. 7). In several control experiments the shift of $V_{0.2}$ was determined to be +7.15 mV/pH unit (pH 6–9.4 in phloretin-free internal solution). This value was used to correct measurements in Table II for which a pH control on the same axon was not available.

The neutral species of internal phloretin is evidently the more effective inhibitor of $\bar{G}_K$, although the anionic form may also affect this parameter weakly. In contrast, the anionic species does not appear to effect $V_{0.2}$ at all. The shift of $V_{0.2}$ is much smaller (12 mV) in the

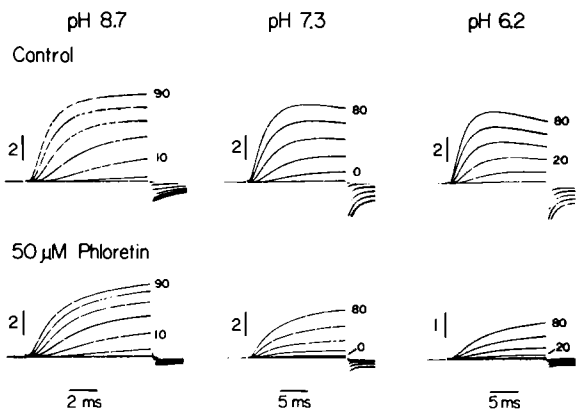


FIGURE 6 Effect of phloretin at different internal pH. Potassium current-time families before and during internal exposure to 5×10^{-5} M phloretin at pH 8.7, 7.3, or 6.2 as indicated above each set of curves. Voltge steps were applied in 20 mV increments from a holding potential of −70 mV. Families at pH 8.7 are from axon 072976*b*, while those at pH 7.3 and 6.2 are from axon 071976*b*. Vertical scales are in units of mA/cm^2. Note change of vertical scale in phloretin at pH 6.2.

presence of 2.5 μM neutral +47.5 μM anionic phloretin (pH_{in} 8.7) than in 5.6 μM neutral +4.4 μM anionic phloretin (pH_{in} 7.3) (31 mV). In this regard it is interesting that only the neutral form of phloretin binds to red cells to inhibit glucose transport (LeFevre and Marshall, 1959).

It is not possible, however, to determine precisely what fractional activity should be ascribed to the anionic species. One reason for this limitation is our uncertainty about possible modifications of a hypothetical phloretin "receptor" brought about by changes in pH. Although the effects of lowering internal pH on $\bar{G}_K$ are not different in the presence or absence of internal phloretin (Fig. 7), there still may be modifications of the drug "receptor." A second limitation in assigning activity to the anionic species is our uncertainty about the pK_a of phloretin in the membrane. If phloretin is bound in a region of relatively low dielectric constant, but still available for protonation, then its true pK_a at this site will probably be lower than that in aqueous solution. This shift in the equilibrium between neutral and protonated species results from the stabilization of the neutral phloretin molecule relative to the charged form in the low dielectric membrane interior, as well as the lower concentration of H_3O^+ in this milieu.

Neutral Phloretin Decreases K Channel Voltage Sensitivity

In addition to the shift of the G_K–E_m relation in the depolarizing direction by phloretin, the limiting slope of the relation is decreased. This phenomenon is most pronounced at low internal pH where neutral phloretin molecules predominate (Fig. 8). The activation of the potassium conductance is apparently less sensitive to voltage in the presence of phloretin at pH 6.3 than in the control situation. The maximum voltage sensitivity of the potassium

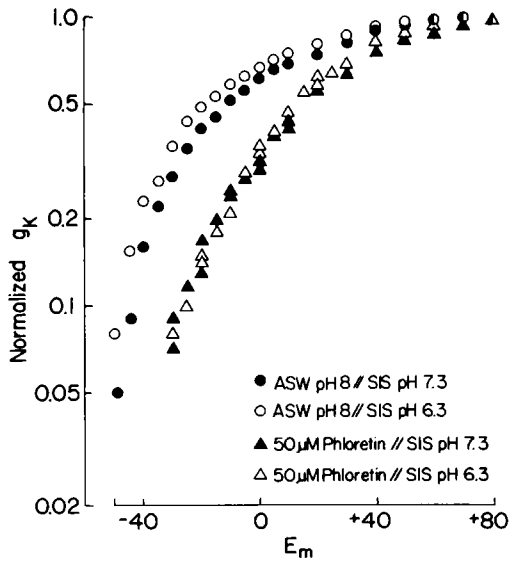


FIGURE 7 Phloretin does not modulate K-channel pH sensitivity. Normalized G_K values from axon 080477*a* before phloretin at internal pH 7.3 (●) and 6.3 (○), and then during external application of 5×10^{-5} M phloretin at internal pH 7.3 (▲) and 6.3 (△). Maximum G_K values under the above conditions are 32, 14.4, 24.9, and 12.8 mS/cm^2, respectively.

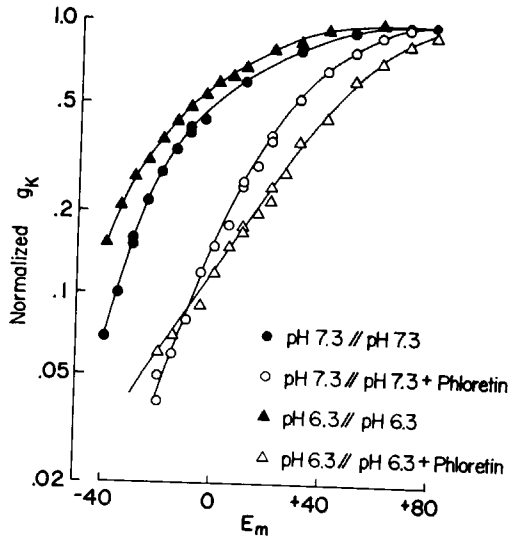


FIGURE 8 Neutral phloretin decreases G_K voltage sensitivity. Normalized G_K values from axon 071877*a* before (●) and during (○) internal phloretin (1×10^{-5} M, pH 7.3) then measured before (▲) and during (△) internal phloretin (1×10^{-5} M, pH 6.3). Maximum G_K values under the above conditions were 34, 23, 20.7, and 8.6 mS/cm^2, respectively. The indicated pH values represent outside/inside.

conductance, taken from the limiting slopes of ln G_K vs. E_m curves, averaged 11.2 mV/*e*-fold G_K in control experiments; in the presence of internal $5 \cdot 10^{-5}$ M phloretin the sensitivities averaged 10.9, 14.6, and 22.3 mV/*e*-fold G_K at internal pH 8.7, 7.3, and 6.3, respectively. It thus appears that the neutral form of phloretin is also more effective in decreasing the slope of the G_K–E_m function.

At very negative membrane potentials the maximum slope of the ln G_K–E_m function is proportional to the total number of charges moved in the membrane (Q_K) or to the size of the dipole moment change ($\Delta\mu$), resulting in the transition of closed potassium channels to the fully opened state, regardless of the number of intermediate states (Almers, 1978). Reductions in the maximum slope of ln G_K–E_m can be produced by reductions in either $\Delta\mu$ (or, equivalently, Q_K) or in the electric field strength at the dipole, $\vec{\epsilon}$, (or, equivalently, the potential difference between the locations of the gating particles corresponding to fully closed and fully opened channel states). The first factor, the gating dipole moment or charge, could be reduced by either a decrease in the total charge on the gating molecule, or by some alteration in conformation, or by the binding of a dipolar compound. The second factor, the local electric field, could be reduced by raising the dielectric constant of the medium around the gating molecule. The second mechanism is probably less specific for drug structure than the first. Accordingly, we sought to discriminate between the two by investigating the effects of molecules that were structurally dissimilar from phloretin but which had about the same dipole moment.

Effects of Related Dipolar Compounds

Four compounds possessing dipole moments only slightly lower than phloretin ($\mu = 5.6$) were compared regarding their ability to alter G_K. Phloroacetophenone ($\mu = 5.5$), 2, 6-dihydroxy-

acetophenone (μ = 5.5), *p*-nitrophenol (μ = 5.4), and *m*-nitrophenol (μ = 4.5) (dipole moment values were taken from Fig. 1 of Andersen et al., 1976) produced only marginal changes in the magnitude and voltage dependence of G_K when perfused internally, even at concentrations as high as 10^{-3} M.

These compounds produce conductance changes in BLM, but only at concentrations of 10^{-4}–10^{-2} M in solutions of pH 3–3.5 (Andersen et al., 1976). The absence of an effect on the squid axon could well be due to the relatively low concentration of the effective, unionized form of these compounds (pK_a = 7.15, *p*-NO_2-phenol). Among the compounds tested, phloretin alone produced changes of axonal membrane conductance, but it is unclear whether this selective potency arose from differences in membrane solubility or from the selective binding of phloretin to ionic channels in the axon.

DISCUSSION

Mechanisms of Phloretin Action

Phloretin at concentrations up to 10^{-4} M modifies the potassium conductance of squid giant axons. The maximum membrane potassium conductance, $\overline{G}_K$, is reduced and the remaining conductance is kinetically modified in a uniform manner. The rate of G_K activation is slowed at all potentials and the function relating steady-state G_K to the membrane potential is both shifted to more depolarized potentials and reduced in steepness, i.e., G_K becomes less sensitive to voltage in the presence of phloretin. Each of these effects is greater under conditions which favor the neutral form of phloretin, a molecular species shown to have a large dipole moment, μ = 5.6 D.

The kinetic modifications of G_K may be explained by several hypotheses. Slowing of the activation rate could result from a specific effect on the processes of potassium activation. In lipid bilayers containing cholesterol, phloretin appears to produce a small increase in fluidity (Andersen et al., 1976). As this result is opposite to the effect expected in the axon, it is improbable that the slowing of G_K activation arises from a decrease in fluidity.

Both the slowing of G_K activation and the modification of the voltage-dependence of steady-state G_K can be explained by specific effects of phloretin on the gating of potassium channels. This explanation can be accomplished only so far as we have detailed information about the mechanism of G_K activation. The sigmoid time course of the onset of potassium conductance can be ascribed to a linear sequence of transitions between conformational states separating fully closed and open channels. Alternatively, G_K activation could result from the oligomerization of plural channel subunits which orient themselves independently in the membrane before forming a conducting potassium channel. In either case, the total dipole moment change associated with the transition of channel subunits from fully closed to the open state is proportional to the limiting slope of the ln G_K vs. E_m function at large negative potentials. This proportionality holds independently of the number of intermediate states or gating subunits (Almers, 1978).

Now, the effect of phloretin is to reduce this limiting slope by ½ to ⅓ (Fig. 8). The control dipole moment change associated with G_K activation is 500–1,000 D (Levitan and Palti, 1975), depending on whether one adopts a membrane thickness of 75 or 40 Å, the latter being

determined from x-ray analysis of nerve membranes (Blasie et al., 1972). Therefore, reducing the limiting slope of ln G_K–E_m to ½ requires the reduction of the total dipole moment change by 250–500 D. Since each neutral phloretin molecule carries a dipole moment of 5.6 D, many molecules would be required to act in concert to effect the changes observed in G_K. For the scheme which postulates independent subunits, these molecules could be distributed equally among the subunits: ten subunits, for example, would each bind a minimum of four to five phloretin molecules. And for the scheme with a linear sequence of transitions of one gating particle, the total dipole change reduction requires the binding of 45–90 phloretin molecules. Such calculations assume that phloretin binding changes the dipole moment only by subtracting the dipole of the drug molecule from that of the gating particle, but changes in gating dipoles due to conformational changes and/or modifications of the pKs of acidic and basic groups on the gating particle could also be induced by phloretin binding. Such induced effects would necessitate major changes in the gating particles to account for the large decrease in dipole moment change, however, and we consider this mechanism, as well as the one requiring binding of many phloretin molecules, rather unlikely.

A somewhat similar and perhaps more reasonable mechanism is that phloretin molecules selectively bind near the gating particles of potassium channels and produce a local dipole field which directly modifies gating. At a distance of 5 Å from one phloretin dipole (5.6 D) electric fields of 10^7–10^8 V/cm will exist in low dielectric media, easily large enough to produce the phenomena we observe.

The third mechanism to explain the effects of phloretin on potassium activation invokes the same changes in membrane dipole potential which explain the effects seen in BLM. Phloretin molecules distribute symmetrically in the membrane, decreasing the internal positive dipole potential due to intrinsic membrane lipids (see Fig. 3 of Andersen et al., 1976). In Fig. 9 the transition of a potassium channel between fully closed and open states has been schematized as the movement of a single gating charge (or equivalently a dipole reorientation) between two free energy minima separated by a barrier. The free energy profile illustrated by the solid curve includes contributions from surface charge asymmetries, dipole potentials, and an imposed transmembrane potential sufficient to produce equal probabilities of occupancy of the closed and open channel states. The gating particle is assumed to be negative by analogy with the effects of phloretin on lipophilic anions in bilayers, although a positive gating charge could have been incorporated as easily. The dipole potentials before (solid line) and after phloretin (dashed line) are illustrated using the trapezoidal approximation (Hall et al., 1973). The modification of this dipole potential by phloretin changes the free energy profile for the gating transition of a potassium channel from the closed to the open state (Fig. 9 *A*). The activation energy, represented by the difference between the energy of the closed state and the peak energy of the transition, as well as the difference between free energies of closed and open states, are both increased by phloretin, producing, respectively, a slower rate of activation and a shift in the voltage required to activate the channels. In addition, under certain conditions, for example at low internal pH with sufficient neutral phloretin in the membrane, the polarizability of the membrane environment around the potassium gating molecule might also be altered, resulting in a decrease in the slope of the ln G_K–E_m function. Thus, the mechanism which proposes a modified dipole potential in the membrane can explain all the observed effects of phloretin on axonal potassium conductance, except for the reduction

in $\bar{G}_K$. This last effect has a different pH dependence than the kinetic effects (see Table I) and may be due to a second, independent mechanism.

Objections to this mechanism stem from apparent inconsistencies between our data and that previously reported in bilayers. The other dipolar compounds tested which are active on bilayers have no noticeable effect on G_K in squid axons, and G_K is much more sensitive to phloretin than G_{Na}, both observations appearing to require a specific interaction. However, the ineffectiveness of other dipolar compounds could be due to their relative insolubility in the squid axon near the potassium channel. As mentioned in Results, the low pH values (3–3.5) used in the bilayer experiments promoted a high concentration of the neutral form of these compounds. Such pH extremes are not tolerated by the squid axon potassium conductance (Wanke et al., 1979) prohibiting an analogous experiment and thus reducing the concentration of the neutral form present in our experiments (pH 7.3).

Also, inequalities may exist between the exact dipole field changes generated by the other molecules and that generated by phloretin; the former could have little influence on the gating of potassium channels, the latter much greater influence, and this difference would be more likely if potassium gating were restricted to a relatively narrow region of the membrane. One such extreme case is depicted in Fig. 9 *A* where the rate limiting energy barrier for opening the channel is arbitrarily positioned entirely within the transition zone of the dipole potential approximation. For the alternative extreme example, an identical free energy profile is instead located entirely within the "plateau" region of the dipole potential (Fig. 9 *B*). In this case the energies of both the closed and open states of the channel as well as the activation energy are increased equally as phloretin lowers the positive dipole potential. Thus no voltage shifts or kinetic changes of the gating mechanism would be apparent with phloretin exposure. Likewise, the axonal sodium conductance might also be insensitive to the dipole change, not because phloretin molecules are absent near G_{Na} gates, but because these gates undergo transitions which are only marginally responsive to the phloretin-induced dipole change.

Phloretin Mechanisms and Binding

There is experimental evidence for the specific binding of phloretin to biological membranes. Neutral phloretin molecules bind to red cell membranes at pH 6.6, exhibiting two classes of sites, a high affinity site, K_D = 1.5 μM, and a low affinity site, K_D = 54 μM (Jennings and Solomon, 1976). However an extract of red cell membrane lipids only binds phloretin with low affinity; and the physiological inhibition of glucose transport in red cells by phloretin at pH 7.4 reaches 50% of maximum effect at 2.5 μM phloretin (LeFevre and Marshall, 1959). Both these results suggest that neutral phloretin binds to a protein in the cell membrane with K_D = 1.25–1.29 μM. Examination of Tables I and II shows that the effects on G_K of the squid axon of phloretin at 10 μM are almost as large as those at 50 μM, which is consistent with saturable binding to a membrane receptor with a K_D well below 10 μM phloretin. In comparison, in studies of lipid bilayers the effects of phloretin also show saturation effects at 10^{-6}–10^{-4} M phloretin (Melnik et al., 1977; de Levie et al., 1979). Thus, it appears that the concentration dependence of phloretin on the axon agrees with both the specific binding to a membrane protein and with the modification of the membrane dipole field by lipid-bound drug, and neither mechanism can be ruled out.

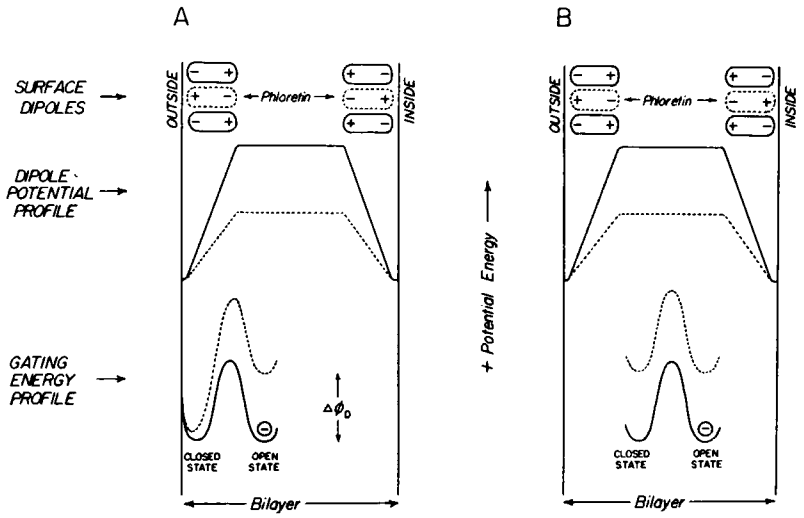


FIGURE 9 Diagrammatic representation of effects of phloretin-induced dipole potential change in membranes on potassium channel gating. Phloretin molecules (dashed dipoles) are interdigitated at opposite polarity with intrinsic membrane dipoles. The resultant positive dipole potentials before (solid trapezoid) and after phloretin (dashed trapezoid) are conventional approximations. For a specified membrane potential a charged gating particle, here arbitrarily chosen to be negative, is distributed between open and closed states depending upon the free-energy difference (ΔG^{o}) between states. The control membrane potential was specified to depict $\Delta G^{o} = 0$. The rate of activation depends on the energy difference between the closed state and the maximum energy of transition, the activation energy, E_a. Phloretin modifies the gating energy profile (dashed line) by the addition of a trapezoidal energy component corresponding to the reduction in positive dipole potential across the bilayer, $\Delta\phi_D$. (*A*) The closed state energy well and E_a are positioned in the dipole potential transition region. Phloretin increases both ΔG^{o} between closed and open states and E_a for the transition of a negative gating particle. (*B*) The same gating profile is positioned in the potential plateau between both dipole transition regions. In this case phloretin has no effect on either ΔG^{o} or E_a despite an increase in total free energy. Although gating of ion channels cannot be described accurately by a single energy barrier model, as in Fig. 9, phloretin could affect several transition steps or only the rate limiting step by the mechanism schematized here.

Site of Phloretin Action

Phloretin produces qualitatively the same effects on G_K whether applied in the internal or external perfusing solution, but internal application produces greater effects. This is probably because the unstirred layer adjacent to the inner membrane surface is thinner than the one adjacent to the outer surface. During drug application we assume that the uncharged form of phloretin can permeate the membrane relatively freely so that the gradient of drug falls linearly across both unstirred layers and the intervening membrane (Gutknecht and Tosteson, 1973; McLaughlin, 1975). However, because of the differences in thickness of the unstirred layers we would expect the drug concentration in the membrane to be greater for internal than for external drug application. This hypothesis was tested by stopping the flow of internal perfusing solution during external phloretin application. In the absence of flow the inner unstirred layer should grow thicker, the drug concentration gradient become flatter, and the phloretin concentration in the membrane should rise. In fact, the block from external phloretin was reversibly enhanced when the internal flow stopped, in agreement with the

prediction just made. In general, differential potencies which occur for internal vs. external application of membrane permeable compounds indicate only the side of the thicker unstirred layer and not the location of the drug receptor. Asymmetrical unstirred layers in squid axons are probably a consequence both of the Schwann cells outside the axon, which limit the velocity of flow next to the axon membrane and may provide some resistance to drug permeation, and of the faster volume flow of internal compared to external perfusion.

An alternative explanation for the greater effect by internally added phloretin involves an asymmetrical distribution of phloretin in the membrane to produce an asymmetric change in the trapezoidal profile of Fig. 9 *A*. If we suppose that the gating particle undergoes transitions at the inner surface of the membrane, and that phloretin equilibrates within the membrane slowly compared to the duration of the current measurements, then internally added phloretin will produce a large change in dipole field affecting gating, whereas externally added phloretin may produce a very small change in this dipole field. This model is also consistent with the minimal additional affect produced by external phloretin added in the presence of internal phloretin. However, since we know neither the rate of diffusion of phloretin nor the position of the gating transition(s) with respect to the assumed trapezoidal profile, this mechanism, like the previous ones, remains hypothetical.

Comparison with Effects on Other Membranes

Effects of phloretin have been reported for lipid bilayer membranes doped with current carriers and lipophilic ions and for *Aplysia* giant neurons. The effects on axons reported here are different from those in bilayers, where addition of phloretin and other dipolar compounds decreased anion conductances, but increased cation conductances. It is curious that the cation conductance increases seen in bilayers are not observed in squid axons, but no clear reason for these negative results is available from these experiments. Direct comparisons of anion and cation conductances between the two systems is, however, probably inappropriate. Previous studies in bilayers assayed the translocation of cation-carrier complexes (e.g., nonactin-K^+) or lipophilic cations (e.g., tetraphenylarsonium) which can cross the bilayer at any region of appropriate lipid composition, whereas the sodium and potassium conductances in the axon are mediated by membrane specializations (i.e., channels) which presumably provide an aqueous pore for ion transport in which bilayer lipid dipole fields are likely much weaker. Rather the equivalent gating charges of these channels are more closely analogous to the ionic conductances induced in BLM studies. In this regard more complete studies in bilayers of the effects of phloretin on extinsic voltage-dependent pore-forming molecules (e.g., alamethicin, EIM) would be useful.

Owen (1974) has reported that phloretin applied externally to giant neurons of *Aplysia* increased the resting potassium conductance of the soma membrane. The results are the opposite reported here for the squid axon; the discrepancy may represent either differences in the method of drug application or between the mechanisms of G_K activation in the two species. The *Aplysia* neurons were unaffected by 10^{-4} M external phloretin, pH 7.5, and pressure-injected internal phloretin had no effect. In contrast, in squid, 10^{-5} M phloretin is effective from either surface. However, the increased G_K observed in *Aplysia* was recorded during brief drug exposures (15–25 s usually) and is a response which might be expected from a transient, asymmetric adsorption of phloretin molecules at the external membrane surface. Such

adsorption would transiently lower the external dipole potential more than the internal and modify the membrane electric field similarly to depolarization. No transient conductance increases from phloretin were seen in squid axons, but a different concentration range was studied. Most of the *Aplysia* experiments used $2 \cdot 10^{-4}$ M phloretin, whereas in squid phloretin concentrations $>5 \cdot 10^{-5}$ M were used infrequently (although 10^{-4} M only reduced G_K). Perhaps under the same conditions of application, similar effects of phloretin on G_K would be observed in *Aplysia*.

In summary, these experiments show that the potassium conductance in squid axons is selectively modified by neutral molecules of phloretin. Both the maximum conductance and channel gating are modified and the changes in the latter function are far greater than those produced in sodium channels. Phloretin may be a useful pharmacological tool for discriminating potassium gating currents in excitable membranes.

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