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The Effect of Phloretin on the Potassium Conductance in *Aplysia* Giant Neurons

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Summary. Phloretin perfused *Aplysia* giant neurons exhibited a rapid and reversible increased potassium conductance. When the cell was voltage-clamped at the resting potential of about −40 mV, the phloretin-induced conductance increase was accompanied by an outward current, which reversed to an inward current below the potassium equilibrium potential of approximately −77 mV. The phloretin effect was not affected by variations in the sodium, chloride, calcium, or magnesium concentrations, but it was sensitive to varied potassium concentrations. The increased potassium conductance persisted at pH = 5, when pH ≪ pK, where according to a dose-response curve, only the un-ionized form of the phloretin molecule could be responsible for the effect. Phloretin increased the membrane potassium conductance as if the neuron were depolarized. The mechanism for this phloretin-induced depolarization may involve an increased surface potential due to an induction of a dipole moment or a selective increase in the number of membrane potassium sites.

Phloretin is a very interesting molecule in that it interacts with biomembranes as well as with artificial membranes. This drug is a well-known inhibitor of monosaccharide transport in human red blood cells [5, 14], and it has recently been shown that phloretin also inhibits the permeation of small hydrophilic nonelectrolytes, but it enhances the permeability of lipophilic molecules in red cells [17]. In lecithin-cholesterol bilayers, phloretin dramatically increases the valinomycin-induced cation conductance and decreases the iodine-iodide induced anion conductance [8]. Since these earlier results have led investigators to suggest that phloretin is interacting with a carrier [5, 14], a lipoprotein complex [17], or a membrane dipole [8], we studied the effect of this drug on the electrolyte permeability in an excitable membrane.

Materials and Methods

The giant cell of the abdominal [12] or left pleural [13] ganglion from the marine mollusc, *Aplysia californica* (sea hare), yielded similar results when investigated using

current and voltage-clamp techniques [7]. Phloretin was prepared by mild acid hydrolysis [2] of phlorizin which was purchased from Fluka, Buchs, Switzerland. In some cases, phloretin was directly obtained from K and K Laboratories, Plainview, New York. The ultraviolet spectrum of the prepared and purchased phloretin was used in the membrane conductance studies without further purification. Unless specified, a concentration of 2.5×10^{-4} M phloretin and 0.085 M (0.5% by vol) absolute ethanol were used in the normal artificial seawater (ASW). The ASW perfusate contained, in mM, 494 NaCl, 10 KCl, 10 $CaCl_2$, 30 $MgSO_4$, 20 $MgCl_2$, and 3 $NaCO_3$. The ASW osmolality was 1.100 osmoles/kg, and the pH was 7.5. The neuron was pinned to the bottom of a paraffin-covered 2-ml Lucite chamber and was exposed to the drug by allowing the ASW plus phloretin perfusate to flow at 1 ml/sec into the chamber to replace the ASW.

Results

When the cell was perfused with the ASW plus phloretin solution at the membrane resting potential of about -40 mV, the conductance began to increase after about a 1-sec exposure (Fig. 1). This was accompanied by an outward current when the cell was voltage-clamped (Fig. 1*A*) or a hyperpolarization when the cell was current-clamped (Fig. 1*B*). No effect was detected with an ASW plus ethanol perfusate which did not contain phloretin. The phloretin effect (Fig. 1*A*) reversed direction (Fig. 1*C*) when the membrane potential was clamped below the equilibrium potential for potassium (E_K).

From a family of curves similar to those in Fig. 1*A* and 1*C*, a reversal potential, E_R, can be measured, where E_R is the membrane potential at which the membrane current is zero (Fig. 2). In Fig. 2, the current-voltage relationship for the phloretin-exposed neuron and the control cell is shown. When the value for the control current is subtracted from the drug-treated current, a phloretin-induced current is shown. The induced current intersects the membrane potential axis at about -75 mV. A value of -75 mV for the reversal potential (E_R) for this phenomenon compared favorably with the *Aplysia* giant neuron E_K of 76.9 ± 0.5 mV. This value for E_K was assumed from the average of 111 *Aplysia* giant neurons in which the intracellular potassium activity was previously [20] measured with specific ion microelectrodes.

Variations in the external potassium but not the sodium or chloride concentration greatly altered the phloretin-induced change in membrane current and conductance (Fig. 3). When the cell was perfused with 1 mM K^+ ASW (instead of 10 mM K^+ ASW), in a sodium for potassium substitution, the E_R decreased from -77 mV (Fig. 3*B*) to about -120 mV (Fig. 3*A*). In a solution of 25 mM K^+ ASW, the E_R increased to -56 mV (Fig. 3*C*). Utilizing the measured E_R and the outside potassium concentration in the

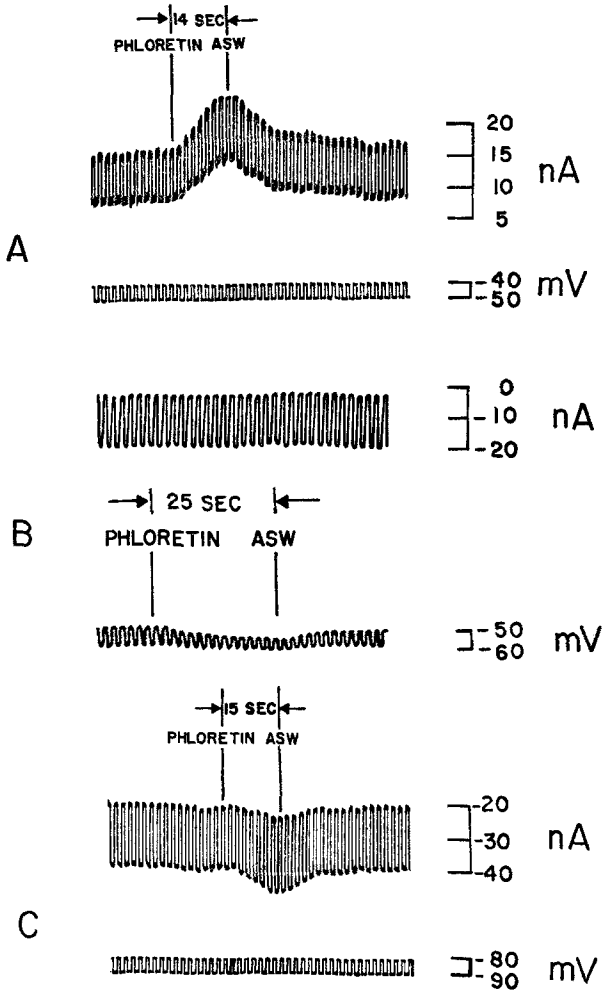

Fig. 1. (*A*) Membrane current evoked by phloretin (2.5×10^{-4} M) when an *Aplysia* giant cell, R2, is voltage-clamped at -40 mV with command 10 mV hyperpolarizing pulses superimposed. Outward current is upwards in the trace. (*B*) Potential changes produced by phloretin (2.5×10^{-4} M) when the neuron is current-clamped at -50 mV with command 18 nA current pulses superimposed. Hyperpolarization is downward. This trace represents the results from a neuron different from the cell depicted in (*A*). (*C*) Membrane current changes produced by phloretin (2.5×10^{-4} M) when cell is voltage-clamped at -80 mV with command 10 mV hyperpolarizing pulses superimposed. The induced current at this holding potential is now inward

normal ASW, a value for the inside potassium concentration, $[K^+]_i$, could be calculated from the Nernst equation, $E_R = 58 \log ([K^+]_0/[K^+]_i)$. Since $[K^+]_i$ was assumed not to vary appreciably during the rapid exposures to different outside potassium concentrations, $[K^+]_0$, a theoretical E_R as a function of $[K^+]_0$ could be calculated (Fig. 4). The slope of the line, 58,

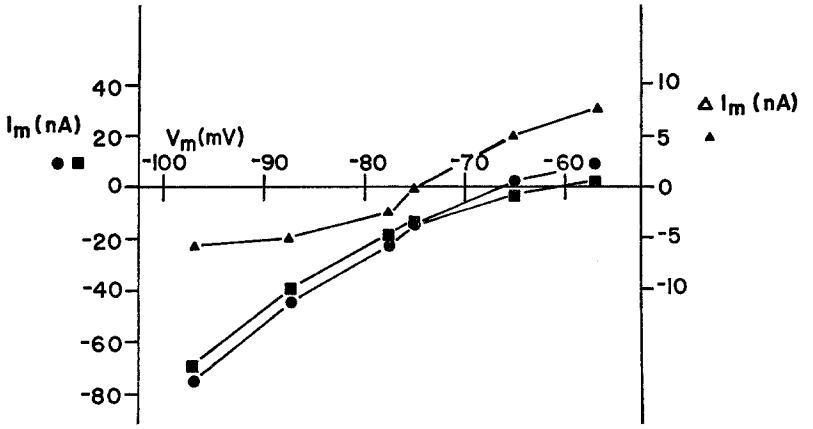

Fig. 2. Current-voltage relationship for the control *Aplysia* giant neuron (■) and the phloretin (2.5×10^{-4} M) exposed neuron (●). The phloretin-induced current (▲) is obtained by subtracting the control current from the phloretin-exposed neuron current

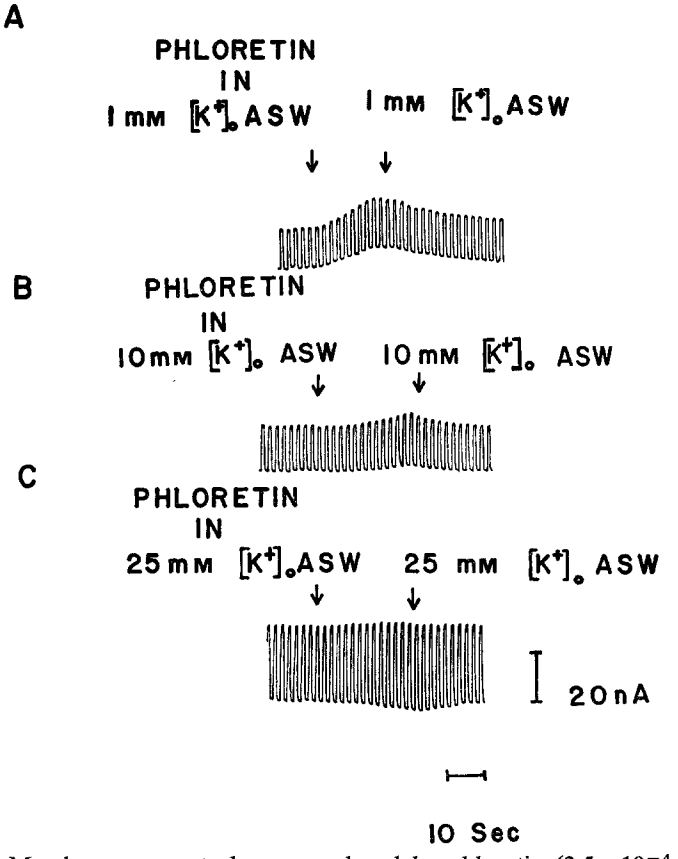

Fig. 3. (*A*) Membrane current change produced by phloretin (2.5×10^{-4} M) when the *Aplysia* cell is voltage-clamped at −50 mV with command 10 mV hyperpolarizing pulses superimposed (similar to Fig. 1*A*, but command pulses are deleted for clarity). Outward or positive current is upwards in the trace. The K_0^+ in the ASW is 1/10 the normal concentration. (*B*) Same as (*A*) except the K_0^+ is normal in the ASW. (*C*) Same as (*A*) except the K_0^+ is 2.5 times greater in the ASW

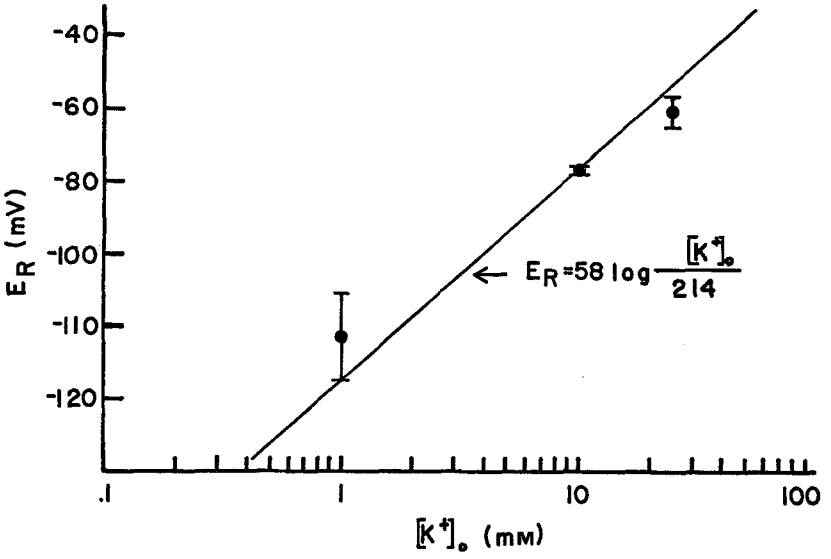

Fig. 4. 2.5×10^{-4} M phloretin-induced E_R as a function of K_0^+. The value for E_R is the mean $\pm$SEM for four different neurons. The line is not a least-squares line but is a theoretical line drawn according to the Nernst equation

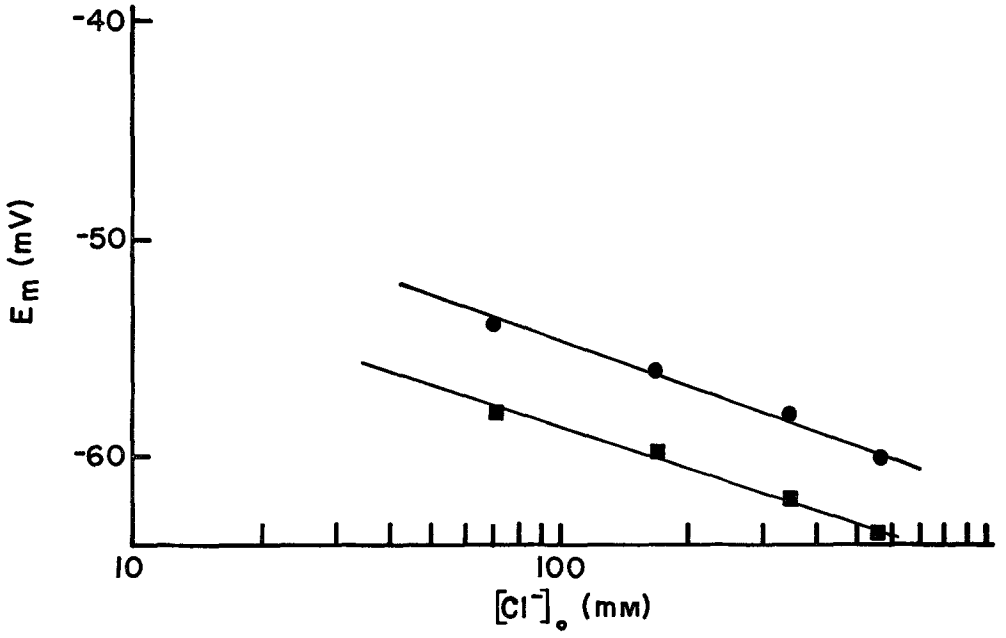

Fig. 5. The effect of phloretin (2.5×10^{-4} M) (■) on the control response (●) for E_m *vs.* Cl_0^-. The phloretin data points are consistently at a lower potential since phloretin produces a hyperpolarization as in Fig. 1*B*

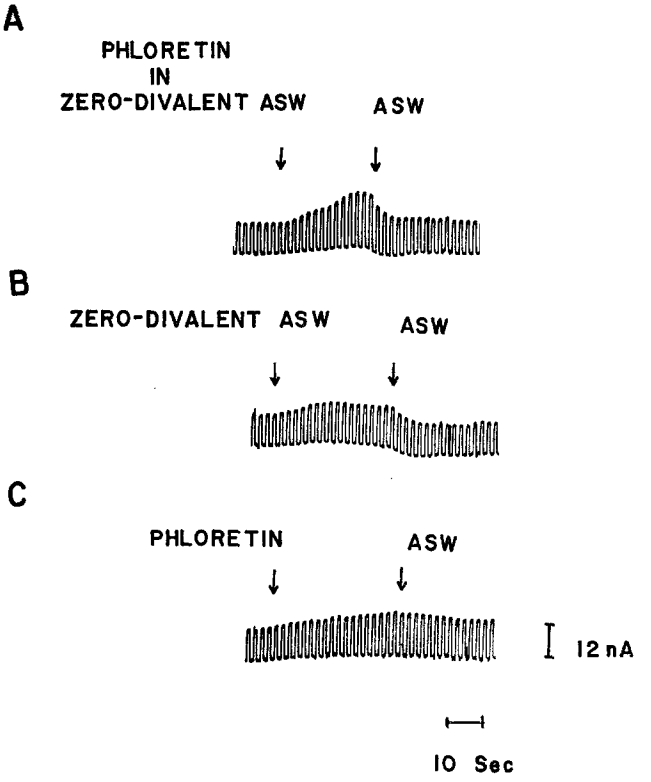

Fig. 6. (*A*) Membrane current change produced by phloretin (2.5×10^{-4} M) when cell is voltage-clamped at -50 mV with command 10 mV hyperpolarizing pulses superimposed (command pulses deleted as in Fig. 3). The calcium and magnesium ion concentrations in the ASW have been replaced by sodium. Outward current is upwards. (*B*) Same as (*A*) without phloretin. (*C*) Same as (*A*) without substituting the divalent cations for sodium

in Fig. 4 corresponds to the response in E_R per decade change in potassium ion concentration if the *Aplysia* giant cell were a theoretical potassium electrode. The response as shown by the data points is seen to be somewhat less than a K electrode (about 47 mV per 10 mM $[K^+]_0$), but this is greater than the response observed in a normal cell without phloretin (33 mV per 10 mM $[K^+]_0$) [6].

Using the procedure [6], which was previously utilized to detect an increase in chloride conductance when ASW was lowered from pH 8 to pH 5, the null effect of phloretin on the chloride conductance in the *Aplysia* giant cell is shown in Fig. 5.

When the concentration of divalent ions in the ASW was replaced by monovalent (sodium) ions, the phloretin effect was not altered as shown in Fig. 6. Although the gross phloretin effect appears enhanced in zero divalent

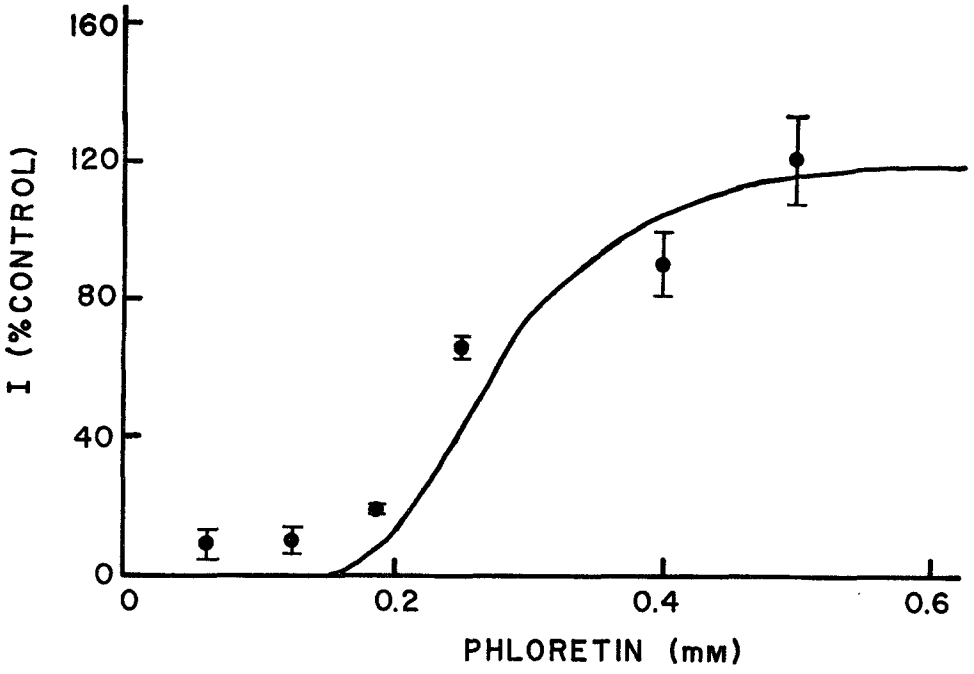

Fig. 7. The % increase in membrane current, I, when a cell is exposed to various concentrations of phloretin for 15 sec at -50 mV. The data points represent the mean $\pm$SEM for two different neurons. The line is a theoretical binding curve assuming: $K_{assoc} = 10^{14}$, cooperativity 4, the % increase in I is proportional to the number of phloretin molecules bound to the neuron and the maximum current increase is proportional to the number of hypothetical membrane receptor sites

ion-ASW (Fig. 6*A*), the subtraction of outward current due to the effect of exposing the neuron to zero divalent ion-ASW without phloretin (Fig. 6*B*) yields a net outward current similar to the control phloretin response in normal ASW (Fig. 6*C*).

The dose-response curve for the phloretin effect on the membrane current is shown in Fig. 7 for two different neurons voltage-clamped at the resting membrane potential of about -50 mV. When the neuron is perfused with phloretin (2.5×10^{-4} M) in ASW for 15 sec the outward membrane current increases by 65%, and if the cell is exposed to phloretin for 3 min the outward current and the conductance increases by almost 400%. This large increase in the membrane characteristics during long (3 min) exposure to phloretin is entirely reversible upon perfusing the neuron with ASW, but there was more reproducibility in the dose-response measurements when the effects of short (15 sec) exposures to phloretin were compared (Fig. 7).

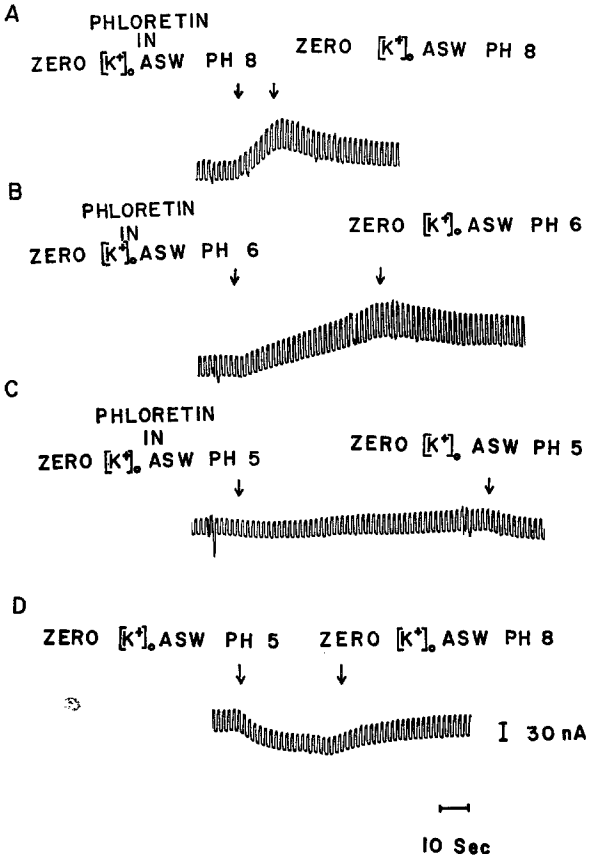

Fig. 8. (*A*) 2.5×10^{-4} M phloretin-induced membrane current trace with cell voltage-clamped at -50 mV and 10 mV hyperpolarizing command pulses superimposed. The potassium in the ASW has been substituted with sodium to maximize the effect (*see* Fig. 3). Outward current is upwards. (*B*) Same as (*A*) except the ASW pH = 6. (*C*) Same as (*A*) except the ASW pH = 5. (*D*) Same as (*A*) except lowering the pH from 8 to 5 without phloretin demonstrates an inward current accompanied by a decrease in membrane conductance

The effect of lowering the ASW pH from 8 to 5 demonstrates that the phloretin effect is still present (Fig. 8). The effect appears to be lower in magnitude at pH = 5 (Fig. 8*C*) as compared to pH = 8 (Fig. 8*A*), but this will be analyzed in further detail in the Discussion.

Some experiments were conducted in which the phloretin was injected into the neuron. When 5×10^{-4} M phloretin in 0.1 M KCl or 0.1 M KCH_3SO_3 was pressure injected (~ 20 lbs/inch^3 of compressed air) into the neuron, no effect was observed on the membrane conductance.

Discussion

The dissociation constant for phloretin can be calculated [18] from the previously published ultraviolet absorption spectrum [14]. The un-ionized and ionized forms of phloretin can be represented by

$$HA \underset{pH<pK}{\overset{pH>pK}{\rightleftharpoons}} A^- + H^+. \tag{1}$$

The dissociation constant, K, for phloretin is

$$K = \frac{a_{H^+}\, a_{A^-}}{a_{HA}} = \frac{[H^+] f_{H^+} [A^-] f_{A^-}}{[HA] f_{HA}} \tag{2}$$

or by taking the negative log and rewriting according to the appropriate general chemical assumptions yields

$$pK = pH - \log \frac{x}{1-x} - \log f_{A^-} \tag{3}$$

where

$$x = \frac{a - a_1}{a_2 - a_1}. \tag{4}$$

x is the fraction of phloretin present in the ionized form, and a, a_1 and a_2 are the absorbances of the phloretin solution at a known pH, a low pH and at a high pH, respectively, all at a single wavelength. The activity coefficient, f_{A^-}, can be calculated from the Davies equation [18]

$$\log f_{\pm} = \frac{A |Z_1 Z_2| \sqrt{I}}{1 + \sqrt{I}} + bI \tag{5}$$

where $A = 0.5$, $I = 5 \times 10^{-2}$ [14], $Z_1 = +1$, $Z_2 = -1$, and $b = 0.2$ [18]. From Eq. (3) and the absorbance values in the phloretin ultraviolet spectrum (Fig. 2*b*, Ref. [14]), a $pK \simeq 7.5$ is obtained.

Organic anions such as salicylate derivatives with high octanol/water partition coefficients, k, are known to increase the membrane conductance of *Navanax* neurons [3, 4, 15, 16]. These authors concluded that these organic anions absorbed onto the cell and directly increased the field strength of the anionic sites in the membrane, but an increase in the number of anionic sites in *Navanax* with salicylate was ruled out since the intrinsic cation selectivity was altered. The $\log k$ for phloretin can be estimated by summing up the $\log k$ values for the various phloretin substituents. An estimated

value of $\log k = 2.16$ for phloretin [1] is similar to the $\log k = 2.21$ for salicylate. The mechanism for the phloretin effect in *Aplysia* neurons does not appear to be due to the ionized form of phloretin, since the effect is still present at pH $\ll$ pK. At pH = 5 and a total phloretin concentration equal to 5×10^{-4} M, there is less phloretin present in the ionized form than at pH = 7.5 and a total phloretin concentration of 1×10^{-4} M. Since essentially no effect at 1×10^{-4} M total phloretin concentration at pH $\simeq$ 7.5 was detected, as shown in the dose-response curve (Fig. 7), the fact that an effect appears at pH = 5 with 5×10^{-4} M phloretin (Fig. 8*C*) indicates that the un-ionized form of the drug causes the increase in the *Aplysia* neuron membrane conductance. The magnitude of the phloretin (pH = 5, 5×10^{-4} M) effect on the membrane conductance appears to be less (Fig. 8*C*) than at pH = 8 and 5×10^{-4} M (Fig. 8*A*), but as shown in Fig. 8*D*, the effect of lowering just the ASW without phloretin from pH = 8 to pH = 5 causes an inward current and a decrease in conductance. In the control state (Fig. 8*D*) this decrease in membrane conductance is presumably due to a decrease in potassium conductance. Therefore, the net effect of phloretin at pH = 5 is an outward current accompanied by an increase in membrane conductance.

The effect of the un-ionized form of the phloretin molecule at pH = 5 on cholesterol-enriched lecithin bilayers has recently been reported [8]. These authors proposed that a dipole field just inside the membrane orients the phloretin molecules at the interface which decreases the positive or surface potential of the membrane. If a dipole field in the *Aplysia* neuron similarly altered the configuration of the phloretin molecules on the cell membrane, an increase in cation conductance could be expected. These authors also found a decrease in anion-mediated conductance with phloretin, which is not observed in the *Aplysia* giant cell.

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1 The estimated $\log k$ (phloretin) = $\log k$ (Phenol)[a] + $\log k$ (ethylene)[a] + $\log k$ (gallic acid)[b,c] = 1.46 + 1.0 − 0.3 = 2.16.

(a) Hansch, C. 1971. *In:* Drug Design. E. M. Adriends, editor. Vol. 1, p. 271. Academic Press Inc., New York.

(b) The $\log k_{\text{octanol}}$ for gallic acid was not available so the $\log k_{\text{ether}}$ (*see* footnote d below) was utilized. The agreement between octanol and ether $\log k$ values is good for similar compounds, i.e., for phenol $\log k_{\text{octanol}} = 1.46$ while $\log k_{\text{ether}} = 1.64$ and for salicylate acid $\log k_{\text{octanol}} = 2.26$ while $\log k_{\text{ether}} = 2.10$.

(c) In the estimation of the $\log k$ for phloretin, a 2,4,6-trihydroxybenzaldehyde substituent would be more ideal than gallic acid (2,4,6-trihydroxybenzoic acid), but the $\log k_{\text{ether}}$ for an acid or an aldehyde group appears comparable, e.g., $\log k_{\text{ether}}$ (propionic acid) = 0.24 and $\log k_{\text{ether}}$ (propionaldehyde) = 0.30.

(d) Collander, R. 1949. Die Verdeckung organischer Verbindungen zwischen Äther und Wasser. *Acta Chem. Scand.* **3**:717.

The increase in membrane conductance produced by phloretin is mainly due to an increase in potassium permeability, as seen by the close agreement between E_K and the E_R for the phloretin effect. Also, one can compare the experimental membrane current elicited by phloretin while the neuron is voltage-clamped with the change in potassium current (ΔI_K) calculated from the measured change in the membrane conductance (Δg_M). This is shown by

$$\Delta I_K = \Delta g_M (E_m - E_K) \tag{6}$$

where E_m and E_K are the membrane potential and the potassium equilibrium potential, respectively. The slope conductance was utilized to measure Δg_M since the current-voltage relationship for the phloretin-induced current was almost linear in the range investigated. For the neuron in Fig. 1*A*, the conductance change due to phloretin was 2.7×10^{-7} mhos, E_K was assumed to be -77 mV [20], and the membrane potential was the voltage-clamp holding potential (-40 mV). If the membrane conductance increase was due to only potassium conductance, then the phloretin-mediated current calculated from Eq. (6) would be 10.0 nA. This compares well with the value of 10.2 nA, which is the measured phloretin-induced current from Fig. 1*A*.

The change in the *Aplysia* giant cell resting membrane potential produced by an increase in potassium permeability, P_K, can be calculated from the constant field equation [11]:

$$E_m = \frac{RT}{F} \ln \left[\frac{P_K(K)_0 + P_{Na}(Na)_0 + P_{Cl}(Cl)_i}{P_K(K)_i + P_{Na}(Na)_i + P_{Cl}(Cl)_0} \right] \tag{7}$$

where E_m = membrane potential, R, T and F have their usual meanings, P_K, P_{Na} and P_{Cl} are the ion permeabilities, $(K)_0$, $(Na)_0$ and $(Cl)_0$ are the external ion activities, and $(K)_i$, $(Na)_i$ and $(Cl)_i$ are the internal ion activities. Recently, P_K and P_{Cl} have been reported [19, 20] to be (2.6 to 18.5) $\times$ 10^{-8} cm/sec and (4.0 to 36) $\times$ 10^{-8} cm/sec, respectively. The sodium permeability can be calculated since P_K/P_{Na} is approximately 10 (H. M. Brown, *unpublished data*). The various internal and external ion activities have been measured [19, 20] with ion specific microelectrodes, e.g. $a_K^0 = 7$ mM, $a_K^i = 165.3 \pm 3.4$ mM (mean $\pm$ SEM) [20], $a_{Cl}^0 = 341 \pm 3.5$ mM and $a_{Cl}^i = 36.7 \pm 0.8$ mM [19]. The sodium activities were calculated [4] assuming that the overshoot of the action potential ($+48 \pm 1.2$ mV) equals E_{Na}, the equilibrium potential for sodium [10], and $a_{Na}^0 = 337$ mM [6]. This yielded $a_{Na}^i = 50$ mM [6].

Although a reasonable value (-40 mV) for the resting membrane potential can be calculated with Eq. (7), it is difficult to demonstrate effectively that an increase in P_K produced by phloretin yields a hyperpolarization in the *Aplysia* giant cell membrane potential. This is mainly due to the wide range in the P_K and P_{Cl} values reported [19, 20]. Since the effect of phloretin on the *Aplysia* giant cell is independent of external chloride concentration (Fig. 5), we can simplify Eq. (7) to

$$E_m = \frac{RT}{F} \ln \left[\frac{P_K(K)_0 + P_{Na}(Na)_0}{P_K(K)_i + P_{Na}(Na)_i} \right]. \tag{8}$$

Utilizing Eq. (8) and substituting in the ion activities listed above along with $P_K/P_{Na} \simeq 10$ (H. M. Brown, *unpublished data*) yields a resting membrane potential, E_m, equal to -36 mV. If we postulate that the 40% increase in membrane conductance elicited upon a rapid exposure to phloretin (Fig. 1*B*) is due to a 40% increase in P_K, then $P_K/P_{Na} = 14$ and Eq. (8) yields a phloretin hyperpolarized membrane potential equal to -43 mV. This is qualitatively similar to what is experimentally observed as exemplified by Fig. 1*B*, although the neuron in Fig. 1*B* is original, i.e., before exposure to phloretin at a lower membrane potential than the theoretically calculated resting membrane potential.

In summary, phloretin rapidly and reversibly increases the potassium conductance of *Aplysia* giant neurons. This effect appears to be due to the un-ionized form of the phloretin molecule which, as pointed out by one reviewer, increases the potassium conductance as if the neuron were depolarized. Potassium conductance in *Aplysia* neurons is known to increase upon depolarization [1, 9], but the voltage-dependence or independence of chloride conductance is not known in this preparation. Thus, the negative results obtained for the effect of phloretin on chloride conductance (Fig. 5) cannot preclude a mechanism for the effect of phloretin on *Aplysia* neurons which involves an alteration in the membrane dipole moment. The phloretin-induced depolarization effect on the membrane conductance suggests that this un-ionized chemical is increasing the external surface potential via a dipole potential or selectively increasing the number of potassium channels, since: (1) The reversal potential for the phloretin effect is at the equilibrium potential for potassium (Fig. 2); (2) The increase in membrane current produced by phloretin is approximately accounted for by assuming that potassium is the only ion carrying the current [*see* Eq. (6)]; and (3) The hyperpolarization of the *Aplysia* giant cell membrane can be predicted by

the constant field equation by postulating that the effect of phloretin is an increase in potassium permeability.

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