Membrane potential changes induced by the ouabain-like compound extracted from mammalian brain

(Na$^+$,K$^+$-ATPase/monensin/[^{3}H]tetraphenylphosphonium ion/tissue culture)

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ABSTRACT   The electrical membrane potential ($\Delta\Psi$) of chicken embryo fibroblasts in tissue culture was determined to be -30.5 ± 2.9 mV as measured by distribution of the lipophilic [^{3}H]tetraphenylphosphonium cation (Ph$_4$P$^+$). Stimulation of the electrogenic activity of the Na$^+$,K$^+$-ATPase by the ionophore monensin induces a hyperpolarization of ≈ 47 mV and a new $\Delta\Psi$ of -77.3 ± 5.7 mV. The effects of the cardiac glycoside ouabain and an "ouabain-like compound" (OLC), which was extracted and partially purified from sheep brain, were contrasted using both the resting and hyperpolarized fibroblasts. Addition of OLC or ouabain to the incubation medium for short periods of time does not alter the cells' resting $\Delta\Psi$. However, OLC and ouabain block monensin-induced hyperpolarization. The inhibitory effects of OLC, like ouabain, are dose dependent, with half-maximal inhibition occurring at an amount of OLC equivalent to that found in 1.6 g of brain (wet weight) per ml and at 0.85 μM ouabain. In addition, the maximal actions of ouabain and OLC are not additive. These results show that the endogenous OLC specifically affects the $\Delta\Psi$ of intact cells by a mechanism analogous to that of ouabain—i.e., inhibition of the Na$^+$,K$^+$-ATPase.

The Na$^+$,K$^+$-ATPase (Na$^+$,K$^+$-pump; ATP phosphohydrolase, EC 3.6.1.3) is the enzymatic machinery for the active transport of Na$^+$ and K$^+$ across the cell membrane (1, 2). The hydrolysis of ATP by this enzyme is routinely accompanied by simultaneous movement of three equivalents of Na$^+$ out of the cell and two equivalents of K$^+$ into the cell. Thus, the activity of this pump results in the net outward movement of positive current, which may lead to the generation of an electrical membrane potential ($\Delta\Psi$). Although generally, the contribution of the Na$^+$,K$^+$-pump to the $\Delta\Psi$ of neuronal cells is small, this is not always the case. Cells that have a large portion of their resting $\Delta\Psi$ as a result of high Na$^+$,K$^+$-pump activity have been demonstrated in cat brains (3) and in molluscan neurons (4). We have demonstrated that the fraction of cell $\Delta\Psi$ that is dependent on the Na$^+$,K$^+$-pump activity can be increased artificially by the carboxylic polyether ionophore monensin (5). This ionophore catalyzes a transmembranal exchange of Na$^+$ for H$^+$. Although this electroneutral exchange does not contribute by itself to the cell $\Delta\Psi$, the resulting increase in the intracellular Na$^+$ concentration activates the electrogenic Na$^+$,K$^+$-pump (6) leading to hyperpolarization of the cell membrane (5).

Several factors that modify Na$^+$,K$^+$-ATPase activity in vitro have been proposed as physiological regulators of this system. These include intracellular Na$^+$ (7) and vanadate (VO$_4^{3+}$) (8) and extracellular Ca^{2+} (7). An additional possible site for the action of regulators of the Na$^+$,K$^+$-pump activity is the cardiac glycoside binding site on the enzyme.

There is evidence reported that cardiac glycosides specifically bind to the Na$^+$,K$^+$-ATPase and inhibit its activity (1, 2,

7). This action is generally thought to be responsible for the wide variety of pharmacological effects of this class of compounds (9). Cardiac glycosides such as ouabain are extracted from the dried leaf of the common purple foxglove Digitalis purpura and from the seeds and leaves of certain other plants (10). In the animal kingdom, structurally related compounds with cardiotonic activity have been found until recently only in the poison glands of bufonid toads (11) and in the skin (12) and plasma (13) of several species of amphibia. Recently, the presence of an "ouabain-like compound" (OLC) in mammalian brain was demonstrated in three independent studies (14–16). These studies, all of which used in vitro assays, show the ability of brain extracts to inhibit [^{3}H]ouabain binding and Na$^+$,K$^+$-ATPase activity. Furthermore, others have found that the plasma of hypertensive patients (17) and volume-expanded dogs (18–21) contains a humoral factor that also inhibits Na$^+$,K$^+$-ATPase activity. However, there have been no reports of a physiological action of OLC isolated from brain on intact viable cells nor any evidence that OLC has similar actions to ouabain in situ.

Because the Na$^+$,K$^+$-ATPase can be made to contribute significantly to the $\Delta\Psi$ of many cells (see above), a specific ouabain-like action of OLC can be established by monitoring the effects of OLC on the $\Delta\Psi$ of intact viable cells. Also, such studies would indicate clearly if OLC preparations have any general deleterious membrane effects. Therefore, in this study, we tested the effect of a partially purified OLC preparation from sheep brain on the resting $\Delta\Psi$ of cells hyperpolarized by the increased Na$^+$,K$^+$-ATPase activity.

MATERIALS AND METHODS

Cells. Primary cultures of chicken embryo fibroblasts were prepared from 11-day-old chicken embryos from SPAFAS eggs (Norwick, CT) and grown and maintained in Dulbeccos' modified Eagle's medium (GIBCO) containing 5% fetal calf serum (Seralab, Crawley Down, Sussex, England), penicillin G (100 units/ml), and streptomycin sulfate (10 μg/ml). After incubation for either 5 or 6 days, the cells were frozen in dimethyl sulfoxide or transferred. Secondary or later cultures (not more than four passages) containing 10^5 cells were plated into 16-mm-diameter multiwell dishes (Costar 3524) and incubated at 37°C for 2–3 days before the experiment.

[^{3}H]Tetraphenylphosphonium Ion ([^{3}H]Ph$_4$P$^+$) Uptake. [^{3}H]Ph$_4$P$^+$ uptake was carried out as described (22). Immediately prior to use, the medium in the wells was aspirated, and the wells were washed once with "low-K$^+$ medium" (135 mM NaCl/5 mM KCl/50 mM Tris·Hepes, pH 7.4/1.8 mM CaCl$_2$/0.8 mM MgSO$_4$/5.5 mM glucose). Reactions were initiated by aspirating the washing buffer and then adding 500 μl of assay

Abbreviations: $\Delta\Psi$, membrane potential; Ph$_4$P$^+$, tetraphenylphosphonium ion; OLC, ouabain-like compound; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

medium containing 10 μM [^{3}H]Ph$_4$P$^+$ (37 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels) to each well. Assay medium consisted of (*i*) low-K medium, (*ii*) "high-K$^+$ medium" (same as low-K$^+$ medium except that 135 mM KCl was used in place of NaCl), or (*iii*) "choline$^+$ medium" (same as low-K$^+$ medium except that 135 mM choline chloride was used in place of NaCl). After incubation at 37°C for given times, the medium was aspirated, and the wells were washed immediately with 1 ml of cold low-K$^+$ medium. The content of each well was then transferred quantitatively to scintillation vials by dissolving the material in 1 ml of 0.2 M NaOH/0.5% Triton X-100. The radioactivity was determined by liquid scintillation spectrometry. Assays were performed in triplicate, and replicate values did not vary by more than 5%. Protein was determined by the method of Lowry (23).

Calculation of Ph$_4$P$^+$ Concentration Gradients and $\Delta\Psi$. Ph$_4$P$^+$ concentration gradients were determined and used to calculate values for $\Delta\Psi$ as described (22). A value of 3.32 μl per mg of protein was used as the intracellular volume and was obtained by using ^{3}H$_2$O as a marker for total volume and [^{3}H]sorbitol for extracellular volume as described (22). Based on the assumption that the $\Delta\Psi$ across the plasma membrane of chicken embryo fibroblasts is due mainly to a K$^+$ diffusion gradient (see *Results*), Ph$_4$P$^+$ uptake in high-K$^+$ medium is considered to be unrelated to the $\Delta\Psi$ across the plasma membrane (22). Therefore, we subtracted Ph$_4$P$^+$ accumulation at high external K$^+$ to derive that component of Ph$_4$P$^+$ accumulation which is due to $\Delta\Psi$ across the plasma membrane (i.e., [Ph$_4$P$^+$]$_{in}^{corrected}$ = [Ph$_4$P$^+$]$_{in}^{low\ K^+}$ − [Ph$_4$P$^+$]$_{in}^{high\ K^+}$). Dividing the corrected [Ph$_4$P$^+$]$_{in}$ by the external Ph$_4$P$^+$ molar concentration ([Ph$_4$P$^+$]$_{out}$) yields the concentration ratio which, when inserted into the Nernst equation, gives $\Delta\Psi$ {$\Delta\Psi = -2.3\ RT/F$ log ([Ph$_4$P$^+$]$_{in}^{corrected}$/[Ph$_4$P$^+$]$_{out}$)}.

OLC Extraction and Partial Purification. OLC was extracted from 500 g of sheep brain as described (16), with minor modifications. Sheep brains were obtained from the slaughter house. Brains were removed ≈1 min after slaughtering the animals. The cerebella were discarded, and the remainder was immediately homogenized (Ultra-Torrax, IKA, Federal Republic of Germany) in acetone (5 ml/g of homogenate) containing 0.2 ml of 10 M HCl per liter. The homogenate was stirred for 2 hr at room temperature and then filtered. The filtrate was evaporated down to the aqueous phase under reduced pressure at 30°C. The aqueous extract was centrifuged at 100,000 × g for 90 min, and the supernatant was collected and dried by lyophilization. The residue was redissolved in methanol (10–20 vol) and filtered to remove salts and residual protein. The methanol was evaporated under reduced pressure at 36°C, and the residue was dissolved in 50 mM Tris·HCl (pH 7.4) and applied to a Sephadex G-25 (Pharmacia) column (glass 41 × 1 cm). The column was developed with the Tris·HCl buffer at a flow rate of 0.9 ml/min. The fractions containing OLC, measured by the ability to inhibit [^{3}H]ouabain binding and Na$^+$,K$^+$-ATPase activity (16), were stored at −20°C until used.

Materials. [^{3}H]Ph$_4$P$^+$ (2.5 Ci per mmol) was a gift of H. R. Kaback, Roche Institute of Molecular Biology, Nutley, NJ. Ph$_4$P$^+$ was obtained from K & K, ouabain from Sigma, and monensin from Eli Lilly. All other materials were of reagent grade, obtained from commercial sources.

RESULTS

$\Delta\Psi$ of Chicken Embryo Fibroblasts. Chicken embryo fibroblasts accumulated Ph$_4$P$^+$ passively against a concentration gradient in a time-dependent process (Fig. 1). A steady-state level of accumulation was achieved at 40 min and was main-
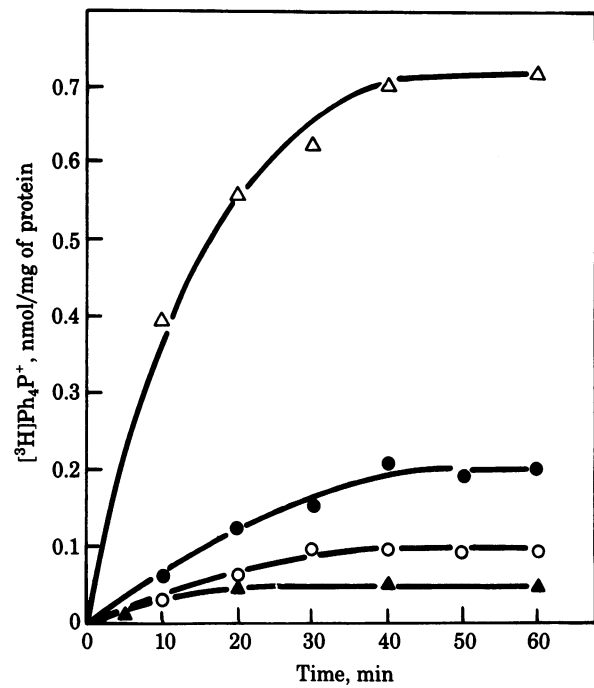

Fig. 1. Time course of [^{3}H]Ph$_4$P$^+$ accumulation into chicken embryo fibroblasts. Cells washed in low-K$^+$ medium were incubated at 37°C in low-K$^+$ medium (●), high-K$^+$ medium (○), low-K$^+$ medium containing 10 μM CCCP (▲), or low-K$^+$ medium containing 10 μM monensin (△), with 10 μM [^{3}H]Ph$_4$P$^+$ (37 mCi/mmol) for the times indicated.

tained for at least 70 min (not shown). The steady-state level of Ph$_4$P$^+$ accumulation was about twice as great in medium containing physiological concentrations of Na$^+$ and K$^+$ (i.e., 135 mM Na$^+$/ 5 mM K$^+$) as in a medium of high K$^+$ concentration (i.e., 121 mM K$^+$/13.5 mM Na$^+$). The difference in Ph$_4$P$^+$ accumulation in low- and high-K$^+$ media was used to calculate a mean value of resting $\Delta\Psi$ of −30.5 ± 2.9 mV for untreated cells (Table 1). The steady-state level of Ph$_4$P$^+$ accumulation in choline$^+$ medium (i.e., 125 mM choline$^+$/13.5 mM Na$^+$) was 0.215 ± 0.02 nmol per mg of protein, which is not significantly dif-

Table 1.   Effect of ouabain and OLC on the $\Delta\Psi$ of chicken embryo fibroblasts

Additions	[^{3}H]Ph$_4$P$^+$ uptake, nmol/mg of protein	$\Delta\Psi$, mV
In "low K$^+$ media"		
None	0.205 ± 0.012 (6)	−30.5 ± 2.9
Ouabain (100 μM)	0.187 ± 0.012 (4)	−25.5 ± 2.9
OLC (3.6 g brain/ml)	0.193 ± 0.015 (4)	−27.2 ± 3.2
Monensin (10 μM)	0.715 ± 0.04 (5)	−77.3 ± 5.7
Monensin + ouabain	0.255 ± 0.01 (5)	−40.8 ± 1.7
Monensin + OLC	0.268 ± 0.015 (3)	−42.9 ± 3.2
In "choline media"		
None	0.215 ± 0.02 (3)	−32.9 ± 4.1
Monensin (10 μM)	0.235 ± 0.06 (3)	−37.1 ± 6.2

Cells were incubated in low-K$^+$ medium or choline$^+$ medium containing 10 μM [^{3}H]Ph$_4$P$^+$ (37 mCi/mmol) in the presence of 10 μM monensin (where indicated). After a 40-min incubation at 37°C to equilibrate the Ph$_4$P$^+$ (see Fig. 1), ouabain (final concentration, 100 μM) or OLC [50 μl of extract; equivalent to 3.6 g of brain (wet weight) per ml] were added. The reactions were continued for an additional 20 min. Results are expressed as mean ± SD with the number of experiments in parentheses. $\Delta\Psi$ was calculated based on the Nernst equation $\Delta\Psi = RT/F$ ln([Ph$_4$P$^+$]$_{in}^{corrected}$/[Ph$_4$P$^+$]$_{out}$), in which [Ph$_4$P$^+$]$_{in}^{corrected}$ = ([Ph$_4$P$^+$]$_{in}^{low\ K^+}$) − ([Ph$_4$P$^+$]$_{in}^{high\ K^+}$).

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ferent from the accumulation obtained in low-K^+ medium (Table 1). This indicates that Na^+ ions do not contribute significantly to the cell $\Delta\Psi$ under these conditions. When the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) was present in the incubation medium (10 μM), only 25% of the control Ph_4P^+ accumulation was observed (Fig. 1). Because CCCP causes charge neutralization across the membrane by the enhancement of membrane permeability to H^+, the Ph_4P^+ accumulation in the presence of this ionophore is independent of the $\Delta\Psi$ and presumably represents Ph_4P^+ binding and solubility in the cell (22). The difference in Ph_4P^+ accumulation in high-K^+ medium without CCCP and low-K^+ medium in the presence of CCCP represents the accumulation of Ph_4P^+ by intracellular organelles, especially the mitochondria.

Effect of Monensin on $\Delta\Psi$ in Chicken Embryo Fibroblasts. Addition of monensin (final concentration, 10 μM) to the cells incubated in low-K^+ medium lead to a 3.4-fold increase in the steady-state accumulation of Ph_4P^+ (Fig. 1), indicating a mean $\Delta\Psi$ increase of -47 mV (Table 1). The addition of the ionophore to cells incubated in choline$^+$ medium resulted in a steady-state level of accumulation of 0.235 ± 0.06 nmol per mg of protein, a value that is not significantly different from the accumulation observed in the absence of monensin (Table 1). The dependence of the monensin effect on the presence of extracellular Na^+ and the ability of ouabain to inhibit the monensin-induced hyperpolarization (see below) are in complete accord with the results of a previous study on neuroblastoma–glioma hybrid, NG108-15, cells (5).

Effects of OLC and Ouabain on $\Delta\Psi$. Ouabain (final concentration, 100 μM) or the partially purified OLC [50 μl; equivalent to 3.6 g of brain (wet weight) per ml] had little or no effect on Ph_4P^+ accumulation during short-term incubations [i.e., up to 20 min (Table 1)]. Thus, under these conditions, the Na^+,K^+-ATPase activity does not contribute significantly to the $\Delta\Psi$. On the other hand, OLC or ouabain sharply decreased the elevation in Ph_4P^+ accumulation observed in the presence of monensin (Table 1). In the experiments shown, chicken embryo fibroblasts were allowed to accumulate Ph_4P^+ in the presence of monensin (10 μM) to a steady state, at which time various concentrations of ouabain or OLC were added. The enhancement
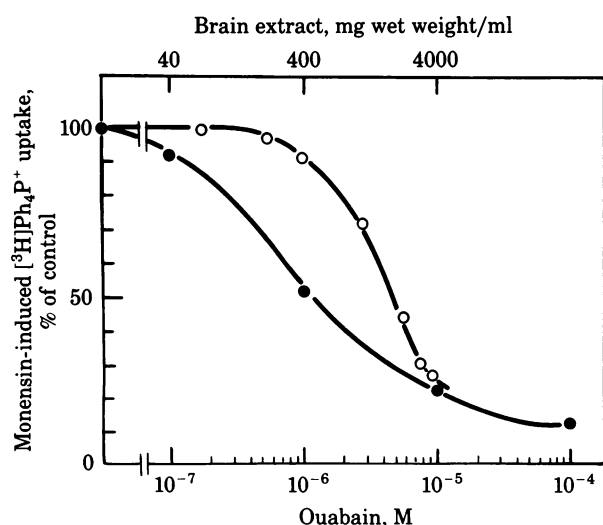

FIG. 2. Effect of OLC and ouabain on monensin-induced increase in [^{3}H]Ph_4P^+ accumulation. Chicken embryo fibroblasts were incubated in low-K^+ medium with 10 μM [^{3}H]Ph_4P^+ and 10 μM monensin for 40 min. OLC (o) or ouabain (●) were then added, and incubations were continued at 37°C for 20 min. Ph_4P^+ accumulation in low-K^+ medium containing 10 μM monensin was taken as 100%.

of Ph_4P^+ accumulation by monensin (i.e., monensin-induced hyperpolarization) was gradually decreased as ouabain or OLC concentrations were raised (Fig. 2). Half-maximal inhibitory doses were 0.85 μM and the equivalent of 1.6 g of brain (wet weight) per ml for ouabain and OLC, respectively. Neither ouabain nor OLC totally blocked the monensin-induced hyperpolarization of chicken embryo fibroblasts (Fig. 2). This observation indicates that a small portion of the monensin-induced hyperpolarization of these cells is not mediated by the Na^+,K^+-ATPase. This is clearly different from the situation in neuroblastoma–glioma hybrid NG108-15 cells in which monensin hyperpolarization was completely abolished by ouabain.

DISCUSSION

In identifying any new endogenous neurotransmitter/modulator, a number of facts need to be established: (*i*) the presence of the compound within the nervous system, (*ii*) elucidation of its metabolism, structure, and site and mode of action (on both physiological and biochemical levels), and (*iii*) a mechanism for its release into extracellular spaces. Recently, we (16) and others (14, 15) have reported that an "activity" could be extracted and partially purified from mammalian brains that competes with [^{3}H]ouabain for binding to brain membranes and inhibits a Na^+,K^+-ATPase in these membrane preparations without affecting a Mg^{2+}-ATPase, which is also present. All of these data were obtained *in vitro*, and the only action of the material (termed by us "ouabain-like compound") reported was strictly a biochemical one: i.e., inhibition of ATP hydrolysis. Nevertheless, the implication of this work is that there is an endogenous agent capable of regulating the pumping of Na^+ and K^+ across neuronal membranes. Artificial manipulation of the transmembrane fluxes of these two ions directed by the Na^+,K^+-ATPase has long been known to be an effective way to control $\Delta\Psi$. Hence, there is supportive data for a neurotransmitter/modulator-type role for such an endogenous OLC. However, until now, there has been no indication that these OLCs have a specific and ouabain-like action on intact cells. The results of this report rectify this omission.

We show here that at least one OLC truly mimics the physiological action of the cardiac glycoside ouabain when tested on intact chicken embryo fibroblasts. These fibroblasts were used in our studies simply because they provide easily accessible suspension-cultured cells that have large resting membrane potentials. Most importantly, these cells can be treated with the ionophore monensin, yielding a hyperpolarized state, in a manner dependent upon the activity of the Na^+,K^+-ATPase, an enzyme that has high affinity for ouabain and is inhibited by this glycoside. Using a method originally described for other cultured cells (22), we monitored, quantitatively the $\Delta\Psi$ of these cells based upon the equilibrium distribution of the lipophilic cation Ph_4P^+. We find that these cells have a resting $\Delta\Psi$ of -30.5 ± 2.9 mV, which is due mainly to a K^+-diffusion potential ($K^+_{in} \dashrightarrow K^+_{out}$). Removal of Na^+ ions (high choline$^+$ medium) or incubation with ouabain for short periods of time does not alter $\Delta\Psi$. OLC, like ouabain, does not contain an activity that is generally disruptive and injurious to cell membrane function. Such agents would be expected to depolarize resting cells; OLC does not. Monensin-induced hyperpolarization ($\Delta\Psi = -77.3 \pm 5.7$ mV), and its action depends upon the presence of extracellular Na^+ and is inhibited by ouabain ($IC_{50} = 0.85$ μM). The action of monensin observed here is entirely consistent with its previously described ability to hyperpolarize other tissue culture cells (5). In all cases, it appears that the ionophore increases intracellular Na^+ in an electroneutral fashion and that this rise in Na^+_{in} increases the activity of the Na^+,K^+-ATPase so that the resulting net outward Na^+ current (directed

by this enzyme) causes hyperpolarization of the cell. OLC also blocks most of this Na^+,K^+-pump-mediated hyperpolarization, and its actions are not additive to that of ouabain. The IC_{50} for OLC is equivalent to 1.6 g of brain (wet weight) per ml. With both OLC and ouabain, a portion (about 15%) of the monensin hyperpolarization is not blocked. This residual hyperpolarization could be related to increases in membrane K^+ permeability often caused by increases in Ca^{2+}_{in}, which result from increases in internal Na^+ concentrations (24).

It is perhaps worthwhile to point out that the extracted OLC does not exactly mimic the action of ouabain; OLC shows a much steeper dose–response curve than does ouabain (Fig. 2). This might result from impurities in the partially purified material or, on the contrary, could indicate that the endogenous material is quite different from ouabain. However, this would not be a novel finding. Recent work on enkephalins and endorphins clearly indicate that the endogenous opiate neurotransmitters are very different from the natural exogenous alkaloid compounds previously known to act at opiate receptor sites. Based on differences between endogenous and exogenous neurally active agents, new insights into the role and mode of action of these compounds should be forthcoming.

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