Analysis of Neurotoxin and Mitogen-stimulated Sodium Transport in Human Fibroblasts*

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Human fibroblasts contain two classes of ouabain-insensitive $Na^+$ channels. One channel is activated by alkaloid neurotoxins in combination with scorpion venom, a response which is similar to that of the voltage-sensitive $Na^+$ ionophore of nerve. The other channel is activated by fetal calf serum, several other mitogenetic agents, or low extracellular $Ca^{2+}$. The nervelike $Na^+$ channel is inhibited by tetrodotoxin but not by amiloride, while the mitogen-stimulated channel is blocked by amiloride but not by tetrodotoxin. The combined effects of neurotoxins and low $Ca^{2+}$ on $Na^+$ uptake are additive, consistent with the conclusion that these $Na^+$ pathways are different and independent. Studies of $Na^+$ efflux show the presence of two intracellular $Na^+$ compartments. When $Na^+$ flux through the ($Na^+ + K^+$)-ATPase pump is inhibited by ouabain, compartment A has a high rate constant of efflux ($k_1 = 0.30 \text{ min}^{-1}$) and constitutes 60% of the total cellular $Na^+$. Compartment B, with a markedly smaller efflux constant ($k_2 = 0.07$), contains the remaining 40% of cell $Na^+$. Stimulation of flux through the nervelike channel has a pronounced effect on the rate constants and $Na^+$ content of both compartments ($k_1 = 0.36, k_2 = 0.10$), but activation of the mitogenic channel has a pronounced effect only on the rate of efflux from compartment A and on the size of compartment B. A cytoplasmic-nuclear model for the $Na^+$ compartments is presented.

Nerve and muscle cells contain a voltage-dependent sodium channel that is responsible for the depolarization phase of the action potential (1–3). This sodium channel has been found by Catterall (4) to contain 3 binding sites for neurotoxins. One binding site is for alkaloids such as batrachotoxin, veratridine, grayanotoxin, and aconitine which cause a partial opening of the sodium channel. Scorpion venom binds at a second site, and this also results in a partial opening of the channel. When veratridine and an alkaloid neurotoxin are added simultaneously, there is a synergistic effect and the sodium channel becomes fully activated (4). A third characteristic of the $Na^+$ channel is the monovalent cation-translocating site which can be blocked by tetrodotoxin (4). Recently, a ouabain-insensitive sodium channel similar to the neurotoxin-activated sodium channel of muscle and nerve cells has been observed in human fibroblasts and glia-like cells by Munson et al. (1) and Pouyssegur et al. (5). This channel can be opened by a combination of veratridine and scorpion venom (1) or veratridine and sea anemone toxin (5). In fibroblasts, unlike muscle and nerve cells, veratridine alone cannot activate the sodium channel (1, 5). However, as in nerve cells, tetrodotoxin does block the ionophore of this sodium channel opened by the combined effects of veratridine and scorpion venom (1, 5). The function of this channel in fibroblasts is unknown.

Sodium uptake by fibroblasts can also be stimulated by mitogenic agents such as fetal calf serum and epidermal growth factor. Villereal has shown that activation of this sodium pathway represents the earliest identified event in the action of mitogens (6, 7). The pathway is fully activated within 1 min of exposure of cells to the mitogen. It is blocked by amiloride and stimulated by removing extracellular calcium (6, 7). The relationship between this sodium pathway and the neurotoxin-sensitive channel has not been studied. We report here that the alkaloid neurotoxins and mitogens stimulate sodium flux by activating different and independent pathways. Intracellular sodium accumulates in at least two discrete subcellular compartments which appear to be affected differently by the two different sodium pathways.

EXPERIMENTAL PROCEDURES

Cells—Normal human skin fibroblasts (cell line CM 2987, Institute for Medical Research, Camden, NJ) were cultured and passaged as described previously (8). Briefly, cells were grown as monolayers in Earles' minimum essential medium supplemented with 100 units/ml of penicillin, 0.1 µg/ml of streptomycin, 1% (v/v) nonessential amino acids, 20 µm Tricine, 24 mM NaHCO$_3$, and 10% fetal calf serum. Cells were passaged using 0.04% trypsin solution and received fresh medium every 3 days. Cells were grown fresh culture medium 2 days prior to use in an experiment, and all experiments were performed on cultures judged to be fully confluent by phase contrast microscopy. All cells were free of mycoplasma and between passage 10 and 35 during the course of these experiments. The volume of cell water of human fibroblasts has been determined by Villereal and Cook to be 4.3 µl/ mg of cellular protein (9), and this value was used throughout these studies.

Chemicals—Ouabain, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, veratridine, tetrodotoxin, and scorpion venom (Leiurus quinquestriatus) were all purchased from Sigma. Epidermal growth factor, fibroblast growth factor, and CR-Multiplication Stimulating Activity were obtained from Collaborative Research, Inc., Waltham, MA. Betachotoxin was the generous gift of Dr. John Daly, National Institutes of Health, Bethesda, MD. Amiloride was a gift from Merck. Scorpion toxin (L. quinquestriatus) prepared by Dr. William Catterall was the generous gift of Dr. Kenneth Blumenthal, University of Cincinnati, Cincinnati, OH. All tissue culture media and serum were purchased from Gibco Laboratories, Grand Island, NY.

"Na"-uptake Studies—Sodium uptake assays were performed by minor modifications of the procedure described by Catterall and Nirenberg (10). Cells were grown to confluence on multiwell (35 mm/ well) Costar tissue culture dishes. Thirty minutes before addition of $^{22}Na^+$, the growth medium was removed by aspiration and replaced by 1.0 ml of a preincubation solution containing toxins or mitogens, as indicated, in a buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 5.5 mM glucose, 1.0 mM Na$_2$PO$_4$, and 0.8 mM MgSO$_4$, pH 7.4. In...
experiments where the concentrations of NaCl or KC1 were altered, the ionic strength was kept constant with choline chloride. Following the preincubation period the buffer was removed. This buffer was replaced by 1.0 ml of incubation medium consisting of the same buffer solution containing 2-10 μCi of 22Na+/ml, and 10-4 M ouabain for 20 min, unless stated otherwise. 22Na+ uptake was linear over this time period (Fig. 1). Uptake was terminated by removing the buffer and rinsing the cells rapidly three times with 2.5-ml aliquots of a solution containing 164 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, and 5.0 mM Na2HPO4, pH 7.4. Experiments were done in triplicate. The rinse procedure took 1 min for each set of 3 wells. The cells were removed from the wells with 1.0 ml of 0.4 N NaOH for 30 min at room temperature and the entire sample was counted in a Packard Auto-Gamma Scintillation Spectrometer (model 5130). After counting, protein was determined by the method of Lowry et al. (11) using bovine serum albumin as standard or by absorbance at 230 and 260 nm as described by Kalb and Bernlohr (12).

Total sodium uptake was determined by flame spectrophotometry, in which case 22Na+ was not used. The cells were rinsed with 0.25 M sucrose for 30 s following the 20-min incubation period and the cells were then solubilized in 0.02 M KOH for 1 h at room temperature. The sodium content was determined on a Perkin-Elmer Flame Spectrophotometer (model 4000).

Efflux—For efflux studies, the fibroblasts were grown to confluence in individual 35-mm Falcon tissue culture plates. Before the addition of 22Na+, the growth medium was replaced by incubation buffer, as described for the uptake studies. Following a 4-h load period, the buffer containing 22Na+ was aspirated and the plate was immersed in two consecutive 200-ml volumes of Na+ free buffer and then aspirated again. This rinse procedure took 15 s. At the end of the rinse, 1 ml of efflux buffer was added to the plate on a shaking table rotating at approximately 90 rpm. The efflux buffer contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5.4 mM KCl, 1.8 mM CaCl2, 5.5 mM glucose, 0.8 mM MgSO4, 1.0 mM KH2PO4, and 130 mM choline chloride, pH 7.4. In order to check the efficiency of the wash procedure, cells were incubated in the same buffer containing 106 cpm of tritiated inulin at 37 °C for 20 min and washed. Radioactivity remaining on the plates returned to background.

The total volume of efflux buffer was removed at each time point and replaced by an equal volume of efflux buffer back to the plate. This procedure took 10-15 s. At the end of the time course for efflux, the solution was removed and the cells were dissolved from the plate by the addition of 1.0 ml of 0.4 N NaOH for 5 min at 37 °C. Radioactivity remaining in the cells was determined and an aliquot was removed for protein determination as described above. All procedures were carried out at 37 °C except as noted.

Mathematical Analysis of Data—The total amount of sodium taken up by the cells (Na) was calculated from Equation 1 and the results expressed as nanomoles of sodium/mg of protein.

\[
Na = \frac{22Na^+/mg of protein}{specific activity in medium} (1)
\]

In the sodium efflux experiments, total 22Na+ in the cells at t = 0 was determined by summing the radioactivity that had left the cells during each interval plus that remaining in the cells at the end of the experiment. The results can then be expressed as 22Na+ remaining in the cells at each time point. Analysis of the results was consistent with efflux from a two-compartment system with both compartments exhibiting first order decay patterns. For efflux from a two-compartment model, the amount of 22Na+ remaining in the cells (Y) follows the relationship

\[
Y = A_{app}e^{-kt} + B_{app}e^{-kt} (2)
\]

where \(A_{app}\) and \(B_{app}\) are the rate constants of efflux from compartments \(A\) and \(B\) respectively (13, 14). \(B_{app}e^{-kt}\) governs the slow phase of the efflux curve and \(B_{app}\) may be obtained from the extrapolated intercept of a semilog plot based on the data points in the slow phase (see for example Fig. 3). The value of \(B_{app}\) may be calculated from the half-life of that compartment (t2).

\[
k_2 = \frac{\ln 2}{t_2} (3)
\]

The term \(A_{app}e^{-kt}\) which governs the fast phase may be derived from a plot of the data points in the fast phase in which the contribution from the slow phase (\(B_{app}e^{-kt}\)) is subtracted at each point. \(A_{app}\) and \(k_2\) are then derived from this replot in the same way as \(B_{app}\) and \(k_2\).

RESULTS

Under normal steady state conditions for fibroblasts incubated in medium containing 130 mM NaCl, the addition of 22Na+ results in the rapid uptake of labeled Na+ which reaches apparent equilibrium by 10 min at a level of 70 nmol of Na+/mg of protein (Fig. 1, open circles). This represents an intracellular Na+ concentration of 17 nm which is in reasonably good agreement with the 25 nm value obtained by flame spectrophotometry. Addition of ouabain, a potent inhibitor of the sodium-potassium adenosine triphosphatase, causes an immediate increase in sodium uptake (Fig. 1, closed circles). This ouabain effect has a \(K_{1/2}\) of 1 x 10-6 M and is maximal at approximately 10-5 M ouabain (data not shown). The rate of sodium uptake in the presence of ouabain is linear for up to 60 min when the initial, rapid sodium uptake observed in the absence of ouabain is subtracted from the total (Fig. 1, triangles). Unless stated otherwise, all subsequent experiments were done in the presence of 10-4 M ouabain. Batrachotoxin is known to be a potent agonist of the sodium channel in neuroblastoma cell lines (15). Addition of batrachotoxin at 10-6 M also causes an increase in uptake of Na+ by fibroblasts (Table I). However, inclusion of 0.1 mg/ml of scorpion venom, which alone has no effect, results in a synergistic increased rate in Na+ uptake (Table I). This increase is linear for at least 20 min (Fig. 1, squares). Consequently, all further Na+ uptake measurements were done in the presence of 10-4 M ouabain. Batrachotoxin and 0.1 mg/ml of scorpion venom. Each point represents the average of two culture plates.

| Sodium uptake as a function of time in human fibroblasts. Confluent fibroblast cultures were incubated with 22Na+ at 37 °C in incubation medium and 22Na+ uptake was measured as described under "Materials and Methods." O—O, without ouabain; O, 10-4 M ouabain only; ▲—▲, cells without ouabain subtracted from cells with 10-4 M ouabain; □—□, 10-4 M ouabain plus 10-4 M batrachotoxin and 0.1 mg/ml of scorpion venom. Each point represents the average of two culture plates.

| Table I | The effects of batrachotoxin, veratridine, and scorpion venom on sodium uptake |
|---|---|
| Sodium | Uptake | Experiment condition |
| nmol/mg/min | mg/ml |
| Control | 8.6 ± 0.4 |
| Batrachotoxin (10-6 M) | 11.1 ± 0.4 |
| Batrachotoxin (10-6 M) + scorpion venom (0.1 mg/ml) | 30.3 ± 0.3 |
| Batrachotoxin (10-6 M) + scorpion venom (0.1 mg/ml) + tetrodotoxin (10-6 M) | 10.8 ± 0.5 |
| Batrachotoxin (10-6 M) + veratridine (2 x 10-4 M) | 7.6 ± 0.9 |
| Batrachotoxin (10-6 M) + veratridine (2 x 10-4 M) + scorpion venom (0.1 mg/ml) | 17.4 ± 0.8 |
| Veratridine (2 x 10-4 M) | 6.6 ± 0.1 |
| Scorpion venom (0.1 mg/ml) | 6.3 ± 0.1 |
| Veratridine (2 x 10-4 M) + scorpion venom (0.1 mg/ml) | 11.0 ± 0.5 |
uptake experiments were done by loading the cells for 20 min. It should be noted that when the ouabain control is subtracted from the neurotoxin-stimulated values in Fig. 1, the linear uptake between 1 and 20 min does not extrapolate back to zero. This suggests the possibility of a brief period of nonlinear uptake. The extra Na⁺ accumulated during this 1-min period accounts for less than 10% of the total uptake after 20 min and has not been considered further.

Munson et al. (1) have shown that veratridine in the absence of scorpion venom is without effect on sodium uptake in fibroblasts, and we have verified this (Table I). In neuroblastoma cell lines, veratridine is a relatively poor partial agonist compared to batrachotoxin (15). Unlike veratridine, batrachotoxin alone increases the rate of sodium uptake by 40% over basal in the cell line used in those studies (Table I) and by an average of 130% over basal in seven other human fibroblast lines we have examined (data not shown). Activation of the Na⁺ channel by batrachotoxin plus scorpion venom is completely inhibited by 10⁻⁶ M tetrodotoxin (Table I). This behavior is identical with that of the Na⁺ channel activated by veratridine plus scorpion venom.

In order to characterize the neurotoxin and scorpion venom effects in greater detail, we examined the kinetics of their interaction with the sodium channel(s). Increasing the concentration of veratridine in the presence of scorpion venom results in increasing rates of Na⁺ uptake up to the limits of solubility of the veratridine (2 × 10⁻⁴ M). Sodium uptake obeys Michaelis-Menten kinetics, and a double reciprocal plot of the data gives an apparent K₅₀ for veratridine = 65 μM and an apparent V_max = 29 nmol/mg-min. This K₅₀ is slightly higher than that reported by Munson et al. (1) for fibroblasts (37 μM) and by Catterall for neuroblastoma cells (50 μM). Varying the concentration of scorpion venom in the presence of 2 × 10⁻⁴ M veratridine also gives a linear double reciprocal plot (data not shown) with a K₅₀ for scorpion venom = 16 μg/ml and a V_max = 22 nmol/mg-min. These apparent values for the K₅₀ of scorpion venom in the presence of 2 × 10⁻⁴ M veratridine are also in close agreement with the value reported by Munson et al. (1) for human lung fibroblasts (K₅₀ = 22 μg/ml). Sodium uptake also follows Michaelis-Menten kinetics when the concentration of batrachotoxin is varied in the presence of 0.2 mg/ml of scorpion venom. The data give a K₅₀ for batrachotoxin of 0.2 μM and a V_max of 30 nmol/mg-min.

If batrachotoxin and veratridine activate different and independent channels, then their effects on sodium uptake should be additive. Instead, veratridine decreased the sodium uptake observed in the presence of batrachotoxin alone and batrachotoxin plus scorpion venom (Table I). This ability of veratridine to act as a partial antagonist in the presence of a more potent agonist is characteristic of the interaction of agonists which have at least some sites in common.

Since the function of the neurotoxin-sensitive channels in fibroblasts is unknown, we tested the possibility that they might respond to depolarization of the membrane. When the concentration of external KCl was increased in order to depolarize the membrane, no effect on sodium uptake was observed at concentrations up to 100 mM KCl. The concentration of Na⁺ was kept constant at 40 mM and the ionic strength was maintained at a constant level with choline chloride. High concentrations of KCl (100 mM) also displayed no synergism with any of the neurotoxins (data not shown).

Villereal (6, 7) has shown that Na⁺ uptake by fibroblasts can also be stimulated by addition of mitogens or by removal of Ca²⁺ from the incubation medium. In both cases, stimulation of Na⁺ uptake appears to occur through the same, amiloride-sensitive pathway. In our system, removal of Ca²⁺ from the medium leads to a 200–300% increase in Na⁺ uptake. The kᵢ for Ca²⁺ inhibition of this pathway is 7.5 × 10⁻⁵ M and the maximal activity, V_max, is 13 nmol/mg-min. The Na⁺ pathway activated by Ca²⁺ removal is not tetrodotoxin-sensitive (Table II), suggesting that the Ca²⁺-inhibitable channels are different from those activated by neurotoxins. This conclusion is strengthened by two additional observations. First, the increased Na⁺ uptake due to removal of Ca²⁺ from the medium is additive with the stimulation of uptake caused by batrachotoxin plus scorpion venom (Table II). Second, addition of 1 mM amiloride which has indirectly been shown to block the Ca²⁺-sensitive mitogenic pathway has no effect on the neurotoxin Na⁺ channels (Table III).

In addition to activation by fetal calf serum and by removal of Ca²⁺ as shown by Villereal (6, 7), this Na⁺ pathway is also activated by other mitogens including epidermal growth factor (7), multiplication-stimulating activity, and concanavalin A, a potent mitogen in other cell types (Fig. 2). The mitogen-

**Table II**

| Effect of CaCl₂ on sodium uptake
| --- |
| Additions | Stimulation of Na⁺ uptake over basal rate |
| --- | --- |
| Experiment 1 |  |
| Batrachotoxin | 2.8 ± 1.4 |
| Removal of Ca²⁺ | 8.9 ± 1.3 |
| Batrachotoxin and removal of Ca²⁺ | 13.6 ± 1.0 (10%) |
| Experiment 2 |  |
| Batrachotoxin + 2 μg/ml of scorpion venom | 11.0 ± 0.4 |
| Removal of Ca²⁺ | 8.9 ± 1.3 |
| Batrachotoxin + 2 μg/ml of scorpion venom and removal of Ca²⁺ | 17.2 ± 1.5 (19%) |
| Experiment 3 |  |
| Batrachotoxin + 200 μg/ml of scorpion venom | 24.5 ± 3.4 |
| Removal of Ca²⁺ | 8.9 ± 1.3 |
| Batrachotoxin + 200 μg/ml of scorpion venom and removal of Ca²⁺ | 33.9 ± 0.7 (33%) |
| Experiment 4 |  |
| Tetrodotoxin | −0.4 ± 0.5 |
| Removal of Ca²⁺ | 12.6 ± 2.0 |
| Tetrodotoxin and removal of Ca²⁺ | 14.0 ± 0.9 |

*Two by two factorial analysis of variance of the predicted versus observed values is consistent with the absence of significant interaction between the two Na⁺ channels.

**Table III**

| Effect of amiloride on sodium uptake in the presence of batrachotoxin and scorpion venom
| --- |
| Additions | Sodium uptake rate |
| --- | --- |
| None | 9.1 ± 0.3 |
| Amiloride (1 mM) | 6.1 ± 0.7 |
| Batrachotoxin plus scorpion venom | 19.7 ± 1.6 |
| Batrachotoxin plus scorpion venom plus amiloride | 18.3 ± 2.0 |
activated channel is, as expected, insensitive to tetrodotoxin. Interestingly, fibroblast growth factor did not stimulate Na+ uptake, but it is also questionable whether this factor actually induces mitosis in these cells.

We also investigated the effects of neurotoxins and serum on the efflux of 22Na from the cells in order to determine the parameters of unidirectional sodium transport. Under efflux conditions, the labeled sodium is diluted into an effectively infinite extracellular pool so that reuptake by the cells is negligible. It is then possible to calculate the rate constant of efflux. Cells were preloaded with 22Na in the presence of 10⁻⁴ M ouabain for 4 h in order to approximate steady state conditions of Na+ flux as shown by Villereal (6). Sodium efflux was then allowed to occur into Na⁺-free buffer containing 130 mM choline chloride as described under "Materials and Methods." The specific activity of intracellular 22Na remains constant throughout the experiment, so data may be expressed as nanomoles of Na⁺ left in the cells/mg of protein. The data for efflux under these conditions were found to fit the equation for first order decay in a model containing two cellular compartments:

\[ Y = A_{app}e^{-kt} + B_{app}e^{-kt} \]  

(2)

As described under "Materials and Methods," A and B are the apparent pool sizes of the two compartments at the start of the efflux period, and \( k_1 \) and \( k_2 \) are the rate constants of efflux (Fig. 3A). When only ouabain is present during the load and efflux periods, the fast compartment (A) contains 100 nmol of Na⁺/mg of protein and has a rate constant of efflux, \( k_1 = 0.30 \text{ min}^{-1} \), while the slow compartment (B) contains 570

![Graph](image-url)

**TABLE IV**

| Experimental conditions | Efflux constants | Apparent compartment | Corrected compartment | Total intracellular Na⁺ |
|-------------------------|------------------|----------------------|-----------------------|------------------------|
|                         | \( h_1 \) | \( h_2 \) | \( A_{app} \) | \( B_{app} \) | \( A_{corr} \) | \( B_{corr} \) |
| Control                 | 0.30   | 0.07  | 100    | 570    | 405 (60%)  | 265 (40%)  | 670 |
| Batrachotoxin + scorpion venom | 0.56 | 0.10  | 370    | 1090   | 795 (54%)  | 665 (46%)  | 1460 |
| Fetal calf serum (10%)  | 0.78   | 0.06  | 285    | 725    | 403 (40%)  | 607 (60%)  | 1010 |

\( ^* \) The concentration of batrachotoxin was \( 10^{-4} \text{ M} \) and the concentration of scorpion venom was 0.1 mg/ml.
nmol of Na\(^+\)/mg of protein with \(k_1 = 0.07\) min\(^{-1}\) (Table IV). The theoretical curve defined by Equation 2 closely fits the individual data points. Error bars represent differences in the curves from experiment to experiment rather than deviation from the theoretical curve within the same experiment.

Addition of 10\(^{-6}\) M batrachotoxin and 0.1 mg/ml of scorpion venom during the last hour of the 4-h \(^{22}\)Na\(^+\) loading period increases the rate of efflux from both compartments (Fig. 3B). The rate constant of efflux from the fast compartment is increased to \(k_1 = 0.56\) min\(^{-1}\), while efflux from the slow compartment is increased to \(k_2 = 0.10\) min\(^{-1}\). It is important to note that batrachotoxin thus causes an increase in the total amount of Na\(^+\) in both compartments and an increase in the efflux constants from both compartments.

During the standard 4-h preincubation and \(^{22}\)Na\(^+\) load period, cells are normally exposed to a serum-free environment. Under these conditions Villereal et al. (6, 7) have shown that the mitogenic Na\(^+\) pathway is fully inactivated. Addition of 10% fetal calf serum to the preincubation buffer (Fig. 3C and Table IV) increases the efflux rate constant from compartment A to an even greater extent than that seen with batrachotoxin, \(k_1 = 0.78\) min\(^{-1}\). The efflux rate constant for compartment B is slightly decreased to \(k_2 = 0.06\) min\(^{-1}\). On the other hand, the size of compartment B is significantly increased. Although compartment A also appears to be increased in size, this increase is real only if efflux from the two compartments occurs in parallel, thus making the two compartments essentially independent. If, however, efflux occurs in series from compartment B into compartment A and then into the extracellular space, there will be a back flux from A into B. Under these circumstances, the apparent pool sizes must be corrected as described by Solomon (13):

\[
B_{\text{corr}} = \frac{A_{\text{app}}B_{\text{app}} (k_1 - k_2)^2}{A_{\text{app}}k_1^2 + B_{\text{app}}k_2^2}
\]

\[
A_{\text{corr}} = \text{total} - B_{\text{corr}}
\]

When these corrections are made, the size of fast compartment A is found to be unaffected by the addition of fetal calf serum, while slow compartment B more than doubles in size (Table IV). These differences reinforce the nonidentity of the neurotoxin and mitogen-sensitive channels and provide a possible rationale for their serving different cellular functions.

**DISCUSSION**

The data presented here demonstrate the existence of two separate classes of ouabain-insensitive sodium channels in human skin fibroblasts. The first class is characterized by channels which are activated by alkaloid neurotoxins and blocked by tetrodotoxin. The second class is activated by mitogenic agents, is inhibited by extracellular calcium, and is not sensitive to tetrodotoxin. Munson et al. (1) first observed the presence of a neurotoxin-sensitive sodium channel in human fibroblasts. On the basis of the similarities between the fibroblast Na\(^+\) channel and the neurotoxin-activated channels of nerve cells, it has been suggested that fibroblasts contain nervelike Na\(^+\) channels. Smith and Rosengurt (16) have also described an activatable Na\(^+\) pathway in mouse 3T3 cells which is opened by mitogenic agents and inhibited by extracellular amiloride. Villereal et al. (6, 7) have further characterized this pathway in human fibroblasts. We have shown that the mitogen-activated Na\(^+\) channel (6, 7) is distinct from the neurotoxin-sensitive channel on the basis of three criteria. First, the amiloride-inhibitable mitogenic Na\(^+\) channel is not inhibited by tetrodotoxin, whereas the tetrodotoxin-inhibitable neurotoxin channel is insensitive to amiloride. Second, the rates of Na\(^+\) uptake through the two channels are additive. Third, the accumulation of sodium occurs to different extents in the two different subcellular Na\(^+\) compartments.

Previous studies of the nervelike Na\(^+\) channels of fibroblasts have used the alkaloid veratridine, a partial agonist of the Na\(^+\) channel in nerve cells. Batrachotoxin is a more potent agonist, and we have used this to further characterize the neurotoxin-sensitive Na\(^+\) channels. The interaction of batrachotoxin with the fibroblast Na\(^+\) channel differs in a number of ways from that of veratridine. Batrachotoxin is a partial agonist of the Na\(^+\) channel even in the absence of scorpion venom. In the presence of scorpion venom the action of both veratridine and batrachotoxin are potentiated, but the \(K_{1/2}\) for batrachotoxin (2 \(\times\) 10\(^{-7}\) M) is significantly lower than the \(K_{1/2}\) for veratridine (63 \(\mu\)M). These values for half-maximal activation of the fibroblast Na\(^+\) channel are very close to the values reported by Catterall for mouse neuroblastoma cells (17).

Kinetic analysis of \(^{22}\)Na\(^+\) efflux from fibroblasts revealed the presence of two Na\(^+\) compartments. There are three general models which might account for the presence of these two compartments. 1) Two physiologically distinct Na\(^+\) channels in parallel or in series or in parallel with the other compartment. Consideration of the parallel efflux model results in a difficulty. The theoretical curve defined by Equation 4 closely fits the data (Fig. 4). These differences reinforce the nonidentity of the neurotoxin and mitogen-sensitive channels and provide a possible rationale for their serving different cellular functions. 2) Two intracellular compartments (18).

The possibility that the fast phase of Na\(^+\) efflux is due to different subpopulations of cells seems unlikely for the following reasons. Since these are established fibroblast cultures containing only one morphological cell type, the only types of subpopulations to be expected are log phase growth versus resting cells or viable versus dead and dying cells. However, the two compartments of Na\(^+\) efflux continue to be seen both in subconfluent cells representing a predominant population of log phase cells and in highly confluent cultures containing a predominance of resting (G\(_0\)) cells. Trypan blue staining of cells revealed less than 1% nonviable cells in subconfluent populations and approximately 5–10% in confluent cultures. In both cases this is a much smaller number than would be required to account quantitatively for the observed size of either compartment. Thus, different subpopulations of cells are unlikely to account for the two Na\(^+\) compartments, although the possible existence of more subtle heterogeneities of fibroblast cell types within the culture cannot be ruled out.

The second model, in which one of the compartments is extracellular, also seems unlikely because the number of cell membrane or glycoalyx Na\(^+\)-binding sites required would be more than 10\(^{10}\) sites/cell, which seems excessive. Negendank and Shaller (18) have come to the same conclusion in their studies on human lymphocytes where Na\(^+\) efflux follows a pattern similar to the one we have observed in fibroblasts. They were also able to eliminate the possibility of the cell surface or glycocalyx binding and a slowly exchanging intracellular compartment; 3) two intracellular compartments (18).

In both cases this is a much smaller number than would be required to account quantitatively for the observed size of either compartment. Thus, different subpopulations of cells are unlikely to account for the two Na\(^+\) compartments, although the possible existence of more subtle heterogeneities of fibroblast cell types within the culture cannot be ruled out.
one is forced to postulate the existence of a rectifiable Na+ channel. This is not required in a series model. If a series model is assumed, the fast compartment must represent the efflux of free cytoplasmic Na+ through the plasma membrane, since if this were the slow efflux compartment, only one efflux phase would be experimentally observed. The identification of the site of fast Na+ efflux as the plasma membrane is also supported by the fact that the increases in the $k_1$ flux constant induced by the neurotoxins and by fetal calf serum are sufficient to account quantitatively for the observed net uptake of Na+, whereas the changes in $k_2$ are not (calculations not shown). Thus, the flux into compartment A must be across the first barrier encountered, and this is most likely to be the plasma membrane.

The fast cytoplasmic compartment and the slow compartment both represent large subcellular pools. In fibroblasts the only organelle which seems large enough to reasonably contain the amount of Na+ observed in the slow compartment is the nucleus. It is also known that in some tissues the nucleus maintains a Na+ concentration as much as 13-fold that of the cytoplasm (19, 20). Although nuclear accumulation of Na+ is at this time undistinguishable from the binding of Na+ to cytoplasmic macromolecules, the identification of the nucleus functioning as the second Na+ compartment might suggest a direct link between mitogen-induced Na+ uptake by the cell and the subsequent effects on DNA replication. It is this compartment which is uniquely increased by stimulation of the mitogenic pathway.

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M H Davis, C N Pato and E Gruenstein

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