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Evidence for a role of calmodulin in serum stimulation of Na^+ influx in human fibroblasts

(calcium/transport/trifluoperazine/ionophore A23187)

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ABSTRACT **Sodium influx in serum-deprived human diploid fibroblasts can be stimulated by addition of serum (5-fold) or the divalent cation ionophore A23187 (3-fold). The possible involvement of calmodulin in serum or A23187 stimulation of Na^+ influx has been investigated by using six psychoactive agents that are known to bind calmodulin and inhibit calmodulin-sensitive enzymes. Each agent inhibited serum- and A23187-stimulated Na^+ influx in a dose-dependent manner. Furthermore, the K_i for inhibition of serum-stimulated Na^+ influx correlates directly with the Ca^{2+}-specific calmodulin binding previously determined in a cell-free system [Levin, R. M. & Weiss, B. (1979) *J. Pharmacol. Exp. Ther.* 208, 454–459]. None of the agents tested had any effect on the serum-insensitive component of net Na^+ influx in these cells. These data support the concept that serum and A23187 stimulate Na^+ influx in human diploid fibroblasts via an increase of intracellular Ca^{2+} and a subsequent calmodulin-mediated activation of the amiloride-sensitive transport pathway.**

When human fibroblasts are deprived of certain essential growth factors they are arrested in the G_1/G_0 phase of the cell cycle. Growth arrest is relieved by addition of 10% fetal bovine serum, after which cells undergo a complex series of biochemical events culminating in DNA synthesis and cell division. The earliest events after serum stimulation are membrane events, including increases in hexose influx (1), uridine influx (2), K^+ influx (3), Ca^{2+} influx (4), and Na^+ influx (5–8). Recent studies have suggested that the influx of Na^+ may serve as a trigger for the initiation of mitogenesis (9). We have previously investigated the serum-stimulated Na^+ influx in human fibroblasts (HSWP) and found it to be amiloride sensitive (6) and to display linear kinetics (7). The effect of serum in stimulating net Na^+ influx can be mimicked by the divalent cation ionophore A23187 (6), which is known to increase cytosolic Ca^{2+} in many cell types (10). Furthermore A23187-stimulated Na^+ influx can also be inhibited by amiloride (6). Because serum itself has been demonstrated to increase Ca^{2+} influx, we suggest that one mechanism by which serum-stimulated net Na^+ influx may be regulated is through an increase of intracellular Ca^{2+}.

If increased intracellular Ca^{2+} is associated with Na^+ influx, then a Ca^{2+}–calmodulin complex may be the actual mediator (11, 12). The presence of calmodulin has been demonstrated in many cultured cell types, including NIL-2 (a hamster fibroblast), 3T3 cells (mouse fibroblasts) (13), and MRC-5 (a human fibroblast line) (14). Furthermore, inhibition of calmodulin has been shown to inhibit DNA synthesis, cell proliferation (15), and cell spreading in cultured cells (14). In order to evaluate the role of calmodulin in serum-stimulated Na^+ influx, we used a series of psychoactive agents that have been demonstrated by Levin and Weiss to specifically bind to calmodulin and inhibit calmodulin-dependent phosphodiesterase activity (16). Other workers have shown that these agents also inhibit other calmodulin-dependent enzymes such as adenylate cyclase (17), Ca^{2+},Mg^{2+}-ATPase (18), and protein kinases (19). Our results provide evidence that psychoactive drugs that bind calmodulin inhibit both serum-stimulated and A23187-stimulated net Na^+ influx in a dose-dependent fashion. More importantly, the K_i for inhibition of serum-stimulated Na^+ influx correlates directly with the K_i for inhibition of phosphodiesterase activity (16) and Ca^{2+}-specific calmodulin binding activity (16) of these agents.

MATERIALS AND METHODS

Cells. Human fibroblasts (HSWP) derived from human foreskin were obtained from James Regan (Oak Ridge National Laboratory). The cells were cultured in Eagle's minimal essential medium (Eagle's medium, GIBCO) containing 10% fetal bovine serum (GIBCO). Cells were grown at 37°C in a 95% air/5% CO_2 atmosphere and were used between the 10th and the 25th passages. Na^+ influx in both serum-deprived and serum-stimulated cells was observed to be constant over this range of passages. Cells were removed from stock flasks by trypsinization and were seeded onto 60-mm culture dishes for use in transport experiments. The cells were used 3–5 days after subculturing while they were still in the logarithmic phase of growth.

Drugs. Drugs were obtained from the following sources: trifluoperazine (TFP), Smith Kline & French; haloperidol, McNeil Laboratories (Fort Washington, PA); diazepam and chlordiazepoxide, Hoffmann–La Roche; imipramine, CIBA–Geigy; chlorpromazine, Sigma; and A23187, Calbiochem. All agents were dissolved in ethanol with the exception of A23187 and imipramine, which were dissolved in dimethyl sulfoxide and normal saline, respectively. Vehicle concentration in each assay was 0.5% or less, and controls demonstrated that vehicle addition alone had no effect on Na^+ influx.

Transport Studies. Net Na^+ influx was determined after equilibrating cells in amino acid-free, 10 mM Tris-buffered Eagle's medium for 4 hr at 37°C in an air atmosphere in the presence of 0.1% fetal bovine serum. We have previously shown that such treatment reduces Na^+ influx to the same level as measured in quiescent, serum-deprived cells (6). The preincubation medium was aspirated at time $t = 0$ and 3 ml of amino acid-free, Tris-buffered Eagle's medium containing 20 μM digitoxin was added to prevent active Na^+ efflux from the cells. Digitoxin was added from an ethanol stock with final alcohol concentration of 0.2%. At a concentration of 20 μM, digitoxin inhibits Na/K pump activity immediately (unpublished observations). Dishes were incubated at 37°C in a gyrorotary shaker bath with a specially adapted platform so that dishes could be shaken at 100 rpm. Assay times were 5 min unless otherwise designated. Uptake was terminated by aspirating the assay

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Abbreviation: TFP, trifluoperazine.

medium and rapidly dipping the dishes four times in each of 4 1-liter beakers containing ice-cold isotonic $MgCl_2$ (pH = 7.0 with KOH). Controls for assessing the effectiveness of the wash procedure are described in a previous publication (7). In the present studies $MgCl_2$ was used as a wash medium instead of the previously used choline chloride because the removal of surface-bound Na^+ is slightly more effective and the $MgCl_2$ salts were less contaminated by Na^+. After washing, the dishes were aspirated free of wash medium and inverted to dry. The cells were extracted with 3 ml of 0.2% sodium dodecyl sulfate for fluorometric protein determination by methods previously described (20) or with 3 ml of 5% trichloroacetic acid for Na^+ determination. Na^+ concentration of the trichloroacetic acid extract was measured by using a Varian model 275 atomic absorption spectrometer. Initial net Na^+ influx was taken as the slope of the linear phase of a plot of Na^+ content (μmol/g of protein) versus time. In previous studies (6–8) Na^+ influx was measured by using ^{22}Na tracer influx. The finding that serum-insensitive Na^+ flux values obtained by using the tracer technique are higher than values obtained by using net Na^+ influx suggests that a significant portion of ^{22}Na influx was due to Na^+/Na^+ exchange. Due to the lower serum-insensitive flux the extent of serum stimulation of net Na^+ flux in the present studies is approximately 5-fold rather than the 2.5-fold reported previously (6–8).

RESULTS

Effect of TFP on Serum-Stimulated Na^+ Influx. HSWP cells incubated in Eagle's medium containing 0.1% fetal bovine serum for 4 hr (referred to as serum-deprived cells) have Na^+ influx levels equal to the level of cells arrested in the G_1/G_0 phase of the cell cycle, and the Na^+ influx in these cells can be rapidly stimulated by the addition of serum (6). In order to determine the involvement of calmodulin in the serum stimulation of Na^+ influx, serum-deprived cells were preincubated with the calmodulin antagonist TFP prior to serum stimulation. Assaying serum-deprived cells in the presence of 10% fetal bovine serum results in a marked stimulation of net Na^+ influx (which is linear for 5 min) over that seen in serum-deprived cells. The initial net Na^+ influx is 5.5 μmol/g of protein per min in serum-deprived cells (Fig. 1 *Inset*, lower line) and 26.6 μmol/g of protein per min in serum-stimulated cells (Fig. 1 *Inset*, upper line). On the other hand, when serum-deprived cells are preincubated with 25 μM TFP prior to addition of 10% fetal bovine serum, the initial net Na^+ influx is significantly inhibited (Fig. 1). The serum-dependent component (serum-stimulated minus serum-deprived) of the initial net Na^+ influx is only 47% of the control value after a 2-min preincubation with TFP and only 32% after a 5-min preincubation. The inhibition by TFP is complete after 5 min of preincubation.

Effect of Other Calmodulin Inhibitors on Serum-Stimulated Na^+ Influx. In order to establish that the TFP inhibition of Na^+ influx was not due to a nonspecific membrane effect (see *Discussion*), we investigated five other reported calmodulin antagonists. These agents represent four drug classes of psychoactive agents that have been characterized by Levin and Weiss relative to Ca^{2+}-specific calmodulin binding activity as well as inhibition of calmodulin-dependent phosphodiesterase activity (16). Fig. 2 shows the dose–response curves for inhibition of net Na^+ influx over a range of drug concentrations. Each of these agents inhibited initial net Na^+ influx in response to serum in a dose-dependent manner. In all cases the control serum-stimulated

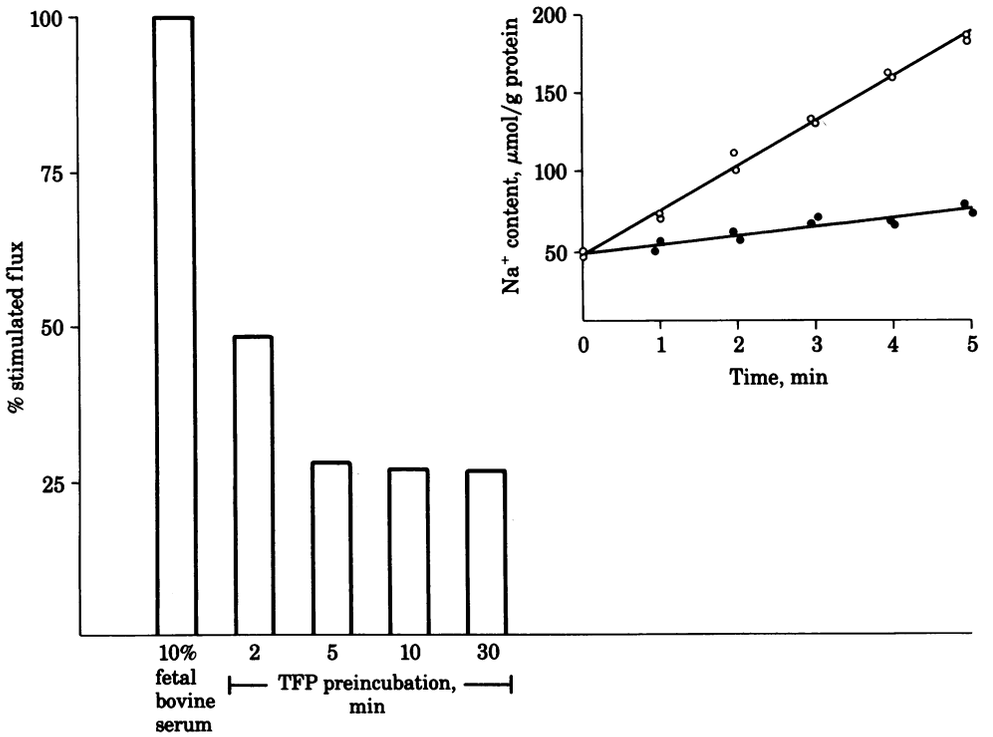

FIG. 1. Serum-stimulated net Na^+ influx in HSWP cells: Effect of TFP. Cells were serum deprived for 4 hr in amino acid-free Tris-buffered Eagle's medium + 0.1% fetal bovine serum and then preincubated with medium containing ethanol (control) for 30 min or 25 μM TFP for 2, 5, 10, or 30 min. Uptake of Na^+ was then assayed after 5 min in the presence of 10% fetal bovine serum, 20 μM digitoxin, and ethanol (control) or 10% fetal bovine serum, 20 μM digitoxin, and 25 μM TFP. Each value represents the mean of six samples. Stimulated flux is defined as flux in the presence of 10% fetal bovine serum minus serum-deprived flux. (*Inset*) Time course of net Na^+ influx in serum-deprived and serum-stimulated HSWP cells. Cells were serum deprived for 4 hr as described above and uptake of Na^+ was assayed for 5 min in the presence of 20 μM digitoxin (●) or 10% fetal bovine serum and 20 μM digitoxin (○).

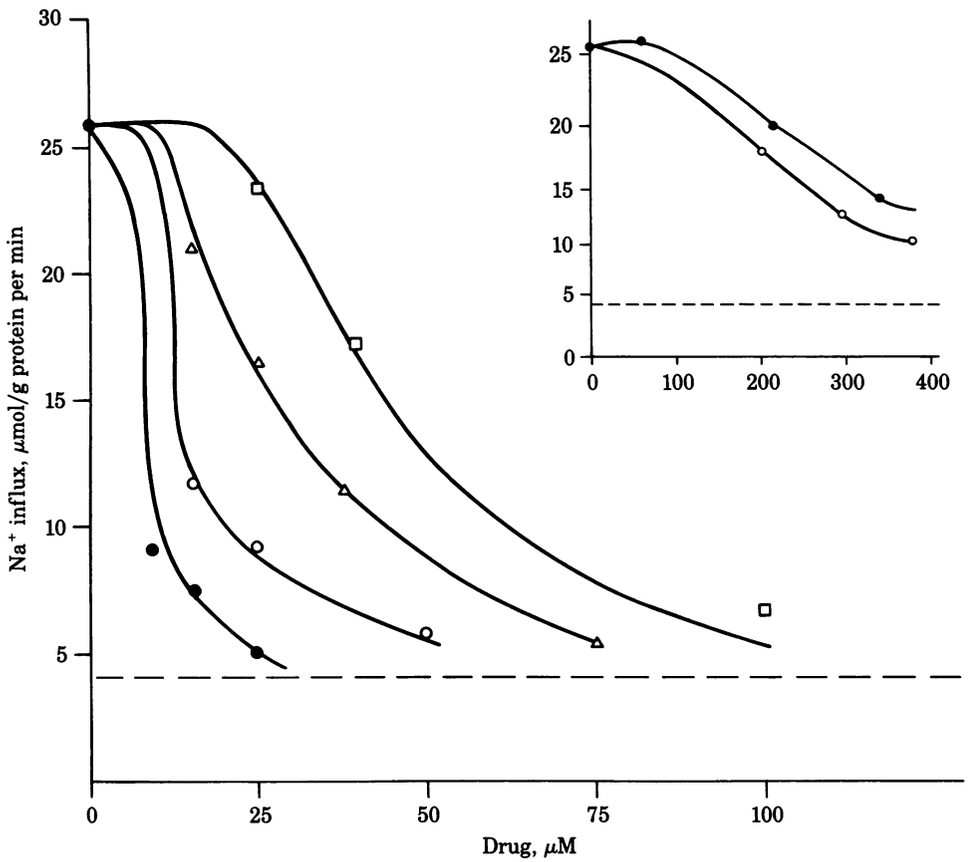

FIG. 2. Dose–response curves for calmodulin-antagonist inhibition of Na^+ influx in the presence of 10% fetal bovine serum. Cells were serum deprived for 4 hr in amino acid-free Tris-buffered Eagle's medium with 0.1% fetal bovine serum and then preincubated for 30 min with medium containing various concentrations of calmodulin antagonists: TFP (●), chlorpromazine (○), haloperidol (△), and imipramine (□) and (*Inset*) diazepam (○) or chlordiazepoxide (●). The initial rate of Na^+ influx was measured after 5-min incubation in medium containing 20 μM digitoxin, 10% fetal bovine serum, and various concentrations of calmodulin antagonists. The broken line represents the level of flux in serum-deprived cells for this series of experiments. Each point represents the mean of eight samples.

flux was determined in the presence of the drug vehicle. Drug vehicle alone was found to have no effect on Na^+ influx. When values for 50% inhibition of Na^+ influx (I_{50}, determined from fractional inhibition plots) were plotted vs. calmodulin-specific binding as determined in a cell-free system (16), a close correlation was observed (Fig. 3).* These results provide support for the concept that these agents are acting specifically by binding to calmodulin and thereby inhibiting Na^+ influx.

Time Dependence of Calmodulin Inhibition. Although the K_i for inhibition of serum-stimulated Na^+ influx correlates closely with the ability of various drugs to bind calmodulin, these agents vary widely in solubility characteristics. Consequently, it is possible that the relationship observed in Fig. 3 is fortuitous and based on differences in access of drugs to calmodulin—i.e., perhaps agents such as TFP with a low K_i have easier access to calmodulin than do agents such as diazepam with a high K_i. In order to eliminate this possibility the time course of inhibition was established for a representative drug with a low K_i (i.e., TFP) and a representative drug with a high K_i (i.e., diazepam). It was determined that both TFP and diazepam are effective in inhibiting serum-stimulated Na^+ influx within 2 min, indicating that each agent has ready access to calmodulin (data not shown). These results demonstrate that the differences in inhibition of Na^+ flux observed with these drugs are not due to differences in membrane solubility and therefore accessibility to calmodulin.

Effect of Calmodulin Inhibitors on Serum-Independent Na^+ Influx. The effect of TFP on the initial net Na^+ influx in serum-deprived cells was difficult to determine directly (see *Discussion*). Therefore, in order to determine whether the drug had an effect on the serum-independent component of Na^+ influx, TFP inhibition of net Na^+ influx in the presence of 10% fetal bovine serum was analyzed by a fractional inhibition plot (Fig. 4) (21). The inhibition data are plotted as the reciprocal of the fractional inhibition, $[(v_o - v_i)/v_o]^{-1}$, in which v_o = control flux and v_i = the flux in the presence of TFP, versus the reciprocal of the TFP concentration. This plot yields a y intercept of 1 if all of the Na^+ influx in the presence of serum is TFP sensitive. However, if one assumes that only the component of Na^+ flux activated by serum will be inhibited by TFP, then the kinetic plot should have a y intercept that is not equal to 1, but equal to 1 + (TFP-insensitive flux/TFP-sensitive flux) (see ref. 4 for details). The y intercept in Fig. 4 is 1.15 ± 0.03, which yields a TFP-sensitive flux of 21.9 μmol/g of protein per min and a TFP-insensitive flux of 3.9 μmol/g of protein per min, which indicates that TFP has very little effect on the serum-independent component of Na^+ influx (4.7 μmol/g of protein per min). The y intercepts of the fractional inhibition plots for the other calmodulin antagonists were 1.18 ± 0.18 for chlorpromazine, 1.19 ± 0.05 for imipramine, 1.49 ± 0.16 for diazepam, and 1.39 ± 0.13 for chlordiazepoxide (values are y intercept

* Chlordiazepoxide was omitted in the calculation of the correlation coefficient (r = 0.982) because calcium-specific binding was high relative to inhibition of phosphodiesterase activity (16); when it is included the correlation coefficient = 0.956.

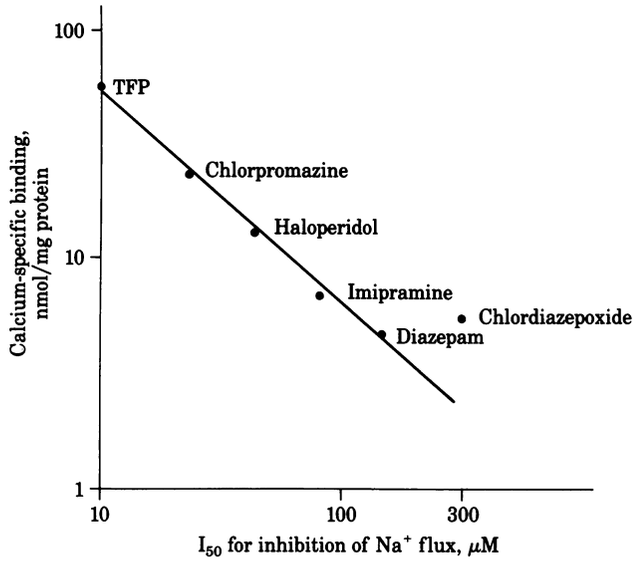

FIG. 3. Relationship between binding of psychoactive drugs to calmodulin and inhibition of serum-stimulated Na^+ influx in HSWP cells. Cells were serum deprived for 4 hr in amino acid-free Tris-buffered Eagle's medium with 0.1% fetal bovine serum and then preincubated for 30 min with medium containing calmodulin antagonists. The initial rate of Na^+ influx was measured in medium containing 20 μM digitoxin, 10% fetal bovine serum, and various concentrations of antagonists. I_{50} values were determined from fractional inhibition plots (see text and ref. 4 for details). Calmodulin-binding data are from Levin and Weiss (16). Correlation coefficient $r = 0.982$ (excluding chlordiazepoxide).

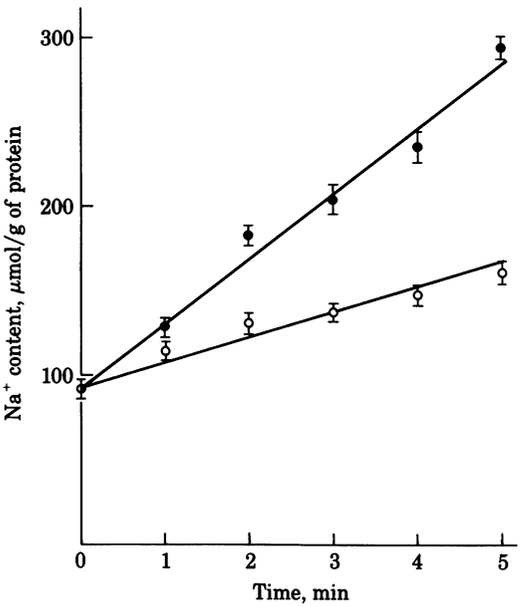

FIG. 5. Time course of A23187-stimulated net Na^+ influx in HSWP cells: Effect of imipramine. Cells were serum deprived for 4 hr in amino acid-free Tris-buffered Eagle's medium with 0.1% fetal bovine serum and then preincubated for 30 min with medium containing ethanol (control) or 20 μM imipramine. Then 10 μM A23187 was added to dishes for 5 min and initial rate of net Na^+ uptake was measured for 5 min in medium containing 20 μM digitoxin and 10 μM A23187 (control) (●) or 20 μM digitoxin, 10 μM A23187, and 20 μM imipramine (○). Each point represents the mean ± SEM of six samples.

± standard error), indicating that none of the agents tested had any significant effect on the serum-independent component of Na^+ influx.

Effect of Calmodulin Antagonists on A23187-Stimulated Na^+ Influx. If the pathway for serum-stimulated Na^+ influx is regulated through a Ca^{2+}–calmodulin complex, then one would predict that A23187-stimulated Na^+ influx should also be inhibited by calmodulin antagonists. The results of pretreating serum-deprived cells with a representative calmodulin antagonist (imipramine) prior to A23187 stimulation are shown in Fig. 5. Cells were incubated with 10 μM A23187 for 5 min and then Na^+ influx was assayed in the presence of 20 μM digitoxin. A23187 treatment causes a dramatic stimulation of Na^+ influx—i.e., 18.0 μmol/g protein per min (Fig. 5, upper line) versus 6.2 μmol/g of protein per min in control (data not shown). When serum-deprived cells were pretreated with 20 μM imipramine for 30 min and then treated with 10 μM A23187 prior to flux measurements, Na^+ influx was dramatically inhibited—i.e., 7.9 μmol/g of protein per min (Fig. 5, lower line). Inhibition of Na^+ influx in A23187-treated cells was also observed when cells were pretreated with other known calmodulin antagonists—e.g., TFP, chlorpromazine, haloperidol, diazepam, or chlordiazepoxide† (data not shown). These results provide further support for the suggested role of intracellular Ca^{2+} and calmodulin as regulators of Na^+ influx.

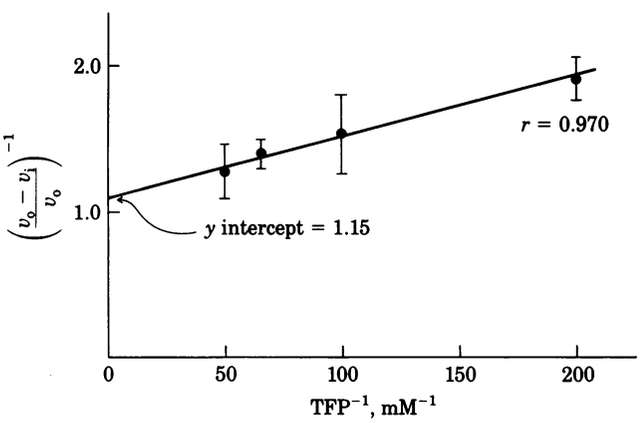

FIG. 4. Fractional inhibition for TFP inhibition of Na^+ influx in the presence of 10% fetal bovine serum. Cells were serum deprived for 4 hr in amino acid-free Tris-buffered Eagle's medium with 0.1% fetal bovine serum and then preincubated for 30 min with medium containing TFP. The initial rate of Na^+ influx was measured after 5 min in medium containing 20 μM digitoxin, 10% fetal bovine serum, and TFP at concentrations of 0, 5, 10, 15, and 20 μM. Each point represents the mean ± SEM from four samples.

DISCUSSION

We and others have reported that one of the earliest biochemical events after serum stimulation of cultured fibroblasts is an enhancement of Na^+ influx (5, 6). Although a number of studies have suggested a role for Na^+ influx as a trigger for mitogenesis (5, 9), little is known about the mechanism by which serum stimulates this biochemical event. Some light was shed on this question by the observation that the divalent cation ionophore A23187 mimicked the effects of serum in stimulating Na^+ influx in serum-deprived HSWP cells. The present report demonstrates that the presence of calmodulin antagonists results in inhibition of the serum- or A23187-stimulated net Na^+ influx in human fibroblasts. These results suggest that the mechanism by which serum or A23187 stimulates net Na^+ influx is via an

† Although the absolute K_i values were lower for inhibition of A23187-stimulated relative to serum-stimulated Na^+ influx, the rank order of potency of the six agents was identical.

increase in intracellular Ca^{2+} and a subsequent calmodulin-mediated enzyme process.

The K_i values reported for inhibition of serum-stimulated Na^+ influx by the psychoactive drugs correlate closely with the Ca^{2+}-specific calmodulin binding values reported for these drugs by Levin and Weiss (16). They also correspond closely with the I_{50} for inhibition of phosphodiesterase activity. Although certain of these agents—i.e., the phenothiazines TFP and chlorpromazine—are known to have detergent properties that give them a wide range of membrane effects that are independent of calmodulin (22–24), we can provide two lines of evidence that the inhibition of Na^+ influx is due to specific calmodulin binding. First, we have been able to demonstrate nonspecific effects at concentrations above 50 μM. These agents stimulate Na^+ influx at high concentrations, presumably due to a nonspecific increase in membrane permeability (similar to erythrocyte hemolysis effects). Second, we have demonstrated that these six agents have K_is for inhibition of Na^+ influx that correlate closely with the values for half-saturation of calmodulin binding. Although these compounds are hydrophobic and may interact with other systems, there is a strong correlaton between calmodulin binding and inhibition of Na^+ influx. Thus, in the concentration range we are using, these agents appear to be inhibiting Na^+ influx as a result of their calmodulin antagonism.

The effect of calmodulin on the serum-independent component of Na^+ influx was difficult to evaluate directly due to the low magnitude of the flux (see Fig. 1 *Inset*). However, convincing evidence can be provided, by using fractional inhibition plots as discussed in the text and in ref. 5, that antagonism of calmodulin has little effect on the basal Na^+ influx measured in serum-deprived cells. Thus, the calmodulin-mediated event appears to be involved only in the regulation of Na^+ influx through the serum-dependent amiloride-sensitive Na^+ transport pathway.

There is a precedent for intracellular Ca^{2+} serving as a regulator of Na^+ influx in other cell systems such as toad bladder (25), sea urchin eggs (26), human blood platelets (27), and MDCK cells (cultured dog kidney epithelial cells) (28). These studies and others demonstrate that intracellular Ca^{2+} can be regulated both by alterations in Ca^{2+} permeability (i.e., influx or efflux) and by changes in intracellular Ca^{2+} binding (i.e., redistribution or sequestration). Although the divalent cation ionophore A23187 has been demonstrated to increase intracellular Ca^{2+} both by increasing influx and by inducing redistribution of Ca^{2+} (10), the mechanism by which serum could increase intracellular Ca^{2+} is not clear. In this connection, whereas Tupper *et al.* (4) have shown that serum causes an increase in Ca^{2+} influx, we demonstrated that serum can stimulate net Na^+ influx in the virtual absence of extracellular Ca^{2+} (7). Therefore, it is unlikely that serum stimulates Na^+ influx solely by increasing Ca^{2+} uptake. On the other hand, it is possible that serum may stimulate Na^+ influx by mobilizing Ca^{2+} from intracellular storage sites. Support for this contention is provided by experiments using intracellular Ca^{2+} antagonists such as chlortetracycline (CTC) (29) or trimethoxybenzoate compounds (TMB-8) (30). Both CTC and TMB-8 inhibit serum-stimulated Na^+ influx, presumably by blocking Ca^{2+} mobilization (unpublished data).

One can only speculate at this point as to the intermediate steps between an increase of intracellular Ca^{2+} and the activation of the amiloride-sensitive Na^+ influx pathway. The present data suggest that one step is the formation of a Ca^{2+}–calmodulin complex. Calmodulin has been reported to regulate certain enzymes that could somehow stimulate Na^+ influx—e.g., protein kinases (11, 12). In this connection the existence of a protein phosphorylation system dependent upon both Ca^{2+} and calmodulin has been demonstrated in membrane fractions from a variety of tissues (31). This system is extremely sensitive to low concentrations of free Ca^{2+} and would therefore be responsive to physiological changes in Ca^{2+} concentration. It is possible that such a system is operative in HSWP cells. That is, a Ca^{2+}–calmodulin complex could activate a protein kinase, which would then phosphorylate the Na^+ transport site, resulting in stimulation of net Na^+ influx. An alternative explanation is that the Ca^{2+}–calmodulin complex itself binds to the Na^+ transport site of HSWP cells exclusive of any enzyme involvement.

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