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Ionic responses rapidly elicited by activation of protein kinase C in quiescent Swiss 3T3 cells

(diacylglycerol/phorbol esters/amiloride-sensitive Na^+ influx/Na^+/K^+ pump/intracellular pH)

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ABSTRACT **Diacylglycerol and phorbol esters activate protein kinase C in intact cells. We report here that addition of the synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG) to quiescent cultures of Swiss 3T3 cells caused a marked increase in the rate of ouabain-sensitive $^{86}Rb^+$ uptake, a measure of the activity of the Na^+/K^+ pump. The effect was dose-dependent and could be detected after 1 min of exposure to the diacylglycerol. OAG stimulated Na^+ influx via an amiloride-sensitive pathway and increased intracellular pH by 0.15 pH unit. Phorbol 12,13-dibutyrate (PBt_2) also enhanced ouabain-sensitive $^{86}Rb^+$ uptake and amiloride-sensitive $^{22}Na^+$ influx. Prolonged treatment (40 hr) of 3T3 cells with PBt_2 at a saturating dose, which reduces the number of PBt_2 binding sites and protein kinase C activity, abolished the ionic response of the cells to a subsequent addition of either OAG or PBt_2. Appropriate controls using acid "loads" and the Na^+ ionophore monensin showed that the function of the Na^+/H^+ antiport system and of the Na^+/K^+ pump was not impaired in the PBt_2-desensitized cells. We suggest that activation of protein kinase C elicits, either directly or indirectly, enhanced Na^+/H^+ antiport activity, which, in turn, leads to Na^+ influx, intracellular pH modulation, and stimulation of the Na^+/K^+ pump.**

Quiescent cultures of 3T3 cells can be stimulated to enter DNA synthesis by various mitogenic compounds, including polypeptide growth factors, hormones, tumor promoters, and serum (1). Understanding of the mechanism of action of proliferative stimuli requires the identification of the intracellular signals capable of initiating or modulating the proliferative response. Ion fluxes and redistributions are thought to play a role in mediating the action of mitogenic agents (1–4). Many mitogens stimulate rapid increases in the rate of Na^+, H^+, and K^+ fluxes across the plasma membrane of 3T3 cells (2–4). These early ionic movements can be envisaged as part of a "Na^+ cycle" composed of Na^+ influx (2, 5) via an amiloride-sensitive Na^+/H^+ antiport system, which results in intracellular alkalinization (6–10), and Na^+ efflux via the ouabain-sensitive Na^+/K^+ pump, which leads to K^+ uptake (2, 5, 11) and to the restoration of the electrochemical gradient of Na^+ across the membrane. An early stimulation of Na^+, K^+, and H^+ fluxes also occurs in a variety of other cell types when they are induced to proliferate (12–14), and these increased ion fluxes may contribute to signaling subsequent events leading to cell proliferation. The mechanisms underlying the activation of these ionic events remain poorly understood.

It is increasingly recognized that the Ca^{2+}-sensitive, phospholipid-dependent protein kinase (protein kinase C), which is stimulated by unsaturated diacylglycerol and by the tumor promoters of the phorbol ester family (for review, see ref. 15), may play an important role in signaling a variety of cellular responses including cell growth. It has been hypothesized that the high-affinity specific receptor for phorbol esters, which was detected in a wide variety of cells and tissues (16–20), is a complex formed between protein kinase C, Ca^{2+}, and membrane phospholipids (21, 22) and that unsaturated diacylglycerols generated by phospholipid breakdown (15, 23) may represent the endogenous analogs of phorbol esters. Substantial evidence for this hypothesis has come from recent studies demonstrating that the synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG), which directly competes with [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PBt_2) for binding to specific receptors on intact 3T3 cells and rapidly stimulates protein kinase C in these cells, is a potent mitogen for Swiss 3T3 cells, acting as a phorbol ester agonist (24). In addition, prolonged treatment of Swiss 3T3 cells with phorbol esters, which decreases the number of high-affinity phorbol ester receptors in intact cells (20, 25, 26) and reduces the activity of protein kinase C measured in cell-free preparations (27) or in intact cells (28), blocks the mitogenic response to a subsequent addition of either phorbol esters (20, 26, 28) or OAG (24).

The purpose of the present study was to define whether activation of protein kinase C elicits monovalent ionic fluxes in quiescent Swiss 3T3 cells. Here, we report that addition of OAG to these cells rapidly stimulates ouabain-sensitive $^{86}Rb^+$ uptake and amiloride-sensitive $^{22}Na^+$ influx and increases intracellular pH. Further, prolonged treatment with PBt_2 blocked the ionic responses of the cells to a subsequent addition of either OAG or PBt_2. We conclude that activation of protein kinase C leads either directly or indirectly to enhanced rates of monovalent cation fluxes in quiescent cells.

MATERIALS AND METHODS

Cell Culture. Cultures of Swiss 3T3 cells (29) were grown at 37°C in humidified 10% CO_2/90% air in Dulbecco's modified Eagle's (DME) medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). For experiments, 10^5 cells were subcultured in 33-mm Nunc tissue culture dishes with medium containing 10% fetal bovine serum. Such cultures were used 6–8 days later, when the cells were confluent and quiescent as judged by cytofluorometric and autoradiographic analysis (30).

Measurement of $^{86}Rb^+$ and $^{22}Na^+$ Uptake. Confluent and quiescent cultures of Swiss 3T3 cells were washed twice with 2 ml of a modified DME medium (ESB) in which the NaCl (110 mM) was replaced with 220 mM sucrose and which was supplemented with bovine serum albumin (fatty acid- and globulin-free) at 1 mg/ml and pre-equilibrated at 37°C. Then, the cultures were incubated with ESB at 37°C in the presence of tracer amounts of $^{22}Na^+$ (5 μCi/ml; 1 Ci = 37

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Abbreviations: OAG, 1-oleoyl-2-acetylglycerol; PBt_2, phorbol 12,13-dibutyrate; DMO, 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione.

GBq) or $^{86}Rb^+$ (2.5 μCi/ml) and other additions as indicated for individual experiments. The replacement of NaCl with sucrose, done to increase the specific activity of ^{22}Na, reduced the concentration of Na^+ in ESB to 50 mM. The concentration of K^+ was 5.1 mM. Thus, the uptakes of $^{86}Rb^+$ and $^{22}Na^+$ were carried out in the same medium (ESB) throughout the studies.

The uptake of $^{86}Rb^+$ was measured in the absence or presence of 2 mM ouabain. The cultures were incubated with $^{86}Rb^+$ for 10 min (unless otherwise indicated), during which time its uptake by 3T3 cells is linear. The radioactivity taken up by cultures treated with ouabain was subtracted from the total $^{86}Rb^+$ uptake (measured in the absence of ouabain) to determine the rate of ouabain-sensitive $^{86}Rb^+$ uptake.

The uptake of $^{22}Na^+$ was carried out in the presence of 2 mM ouabain to prevent the isotope from exiting the cell via the Na^+/K^+ pump. The cultures were incubated with ^{22}Na for 3 min, during which time its uptake by 3T3 cells was linear.

At the end of the incubation period, the uptakes of $^{86}Rb^+$ and of $^{22}Na^+$ were stopped by washing each dish rapidly (10 sec) six times with 5 ml of 0.1 M $MgCl_2$ at 4°C, and the radioactivity was extracted and measured as described (31).

Intracellular pH. Intracellular pH was measured with the weak acid [^{14}C]DMO (5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione) by using a modification of a previously described method (6, 7, 9). Briefly, cultures were washed twice with 2 ml of electrolyte solution [50 mM NaCl/5 mM KCl/0.9 mM $MgCl_2$/1.8 mM $CaCl_2$/25 mM glucose/220 mM sucrose/bovine serum albumin (fatty acid- and globulin-free; 1 mg/ml)/16 mM Hepes/7 mM Tris, pH 6.94. The dishes were incubated at 37°C for 1 hr with 1925 μl of the electrolyte solution in the presence of different factors. After this incubation, 75 μl of [^{14}C]DMO was added (final concentration 150 μM, 3–4 $\times$ 10^6 cpm per dish) to the cultures. After 15 min of incubation in the same conditions, the medium was aspirated and the cultures were washed as described (9).

Intracellular pH was calculated from extracellular pH, [^{14}C]DMO in washed monolayers and media, protein, and intracellular water by using the formula of Waddell and Butler (32). Correction for DMO trapped in extracellular water was made by subtracting the radioactivity remaining in precooled cultures washed immediately after addition of [^{14}C]DMO. Four replicates were determined for each experimental value. A pK_a of 6.3 was used for calculations. Intracellular water was determined by using [^{14}C]urea as previously described (6, 7, 9).

Materials. Bovine albumin (essentially fatty acid- and globulin-free), ouabain, and PBt_2 were obtained from Sigma. Fetal bovine serum was purchased from GIBCO Europe (U.K.). $^{22}Na^+$ and $^{86}Rb^+$ were obtained from the Radiochemical Centre (Amersham, U.K.). [^{14}C]urea and [2-^{14}C]DMO were from New England Nuclear. Amiloride (*N*-amidino-3,5-diamino-6-chlorpyrazine carboxamide) was a gift from Merck Sharp & Dohme (Hertfordshire, U.K.). OAG was prepared (24) and kindly provided by M. M. Coombs (Chemistry Laboratory, Imperial Cancer Research Fund). All other materials were reagent grade.

RESULTS

OAG Rapidly Stimulates Ouabain-Sensitive $^{86}Rb^+$ Uptake. Addition of OAG to quiescent monolayers of Swiss 3T3 cells increases the rate of ouabain-sensitive $^{86}Rb^+$ uptake as much as 3-fold, in a dose-dependent manner (Fig. 1). Maximum stimulation of cation influx was observed at a concentration of 20 μg/ml. There is little or no effect on the ouabain-insensitive component of $^{86}Rb^+$ uptake, which comprises 30–50% of total uptake, suggesting that the enhanced uptake of this cation is mediated by the Na^+/K^+ pump.

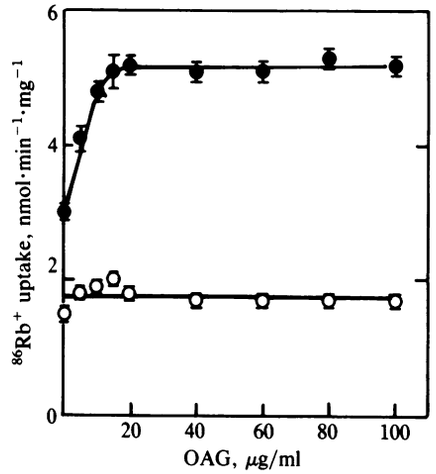

FIG. 1. Dose–response for the effect of OAG on $^{86}Rb^+$ uptake. Confluent, quiescent cultures of Swiss 3T3 cells were washed twice with ESB medium (see *Materials and Methods*) at 37°C. The cultures then were incubated at 37°C with the same medium and various concentrations of OAG in the absence (●) or presence (○) of 2 mM ouabain. After 15 min, $^{86}Rb^+$ was added and the cultures were incubated for an additional 10 min. Shown are the mean ± SEM of two independent experiments (n = 9).

To determine the time-course of the stimulation of the Na^+/K^+ pump by OAG, quiescent cultures of 3T3 cells were exposed to the diacylglycerol for various times prior to the addition of $^{86}Rb^+$. The uptake reaction was terminated after 1 min of further incubation. Maximum stimulation of ouabain-sensitive $^{86}Rb^+$ uptake was achieved after 2 min of incubation (Fig. 2); diacylglycerol enhanced cation uptake as early as 1 min after adding OAG and $^{86}Rb^+$ simultaneously. Thus, OAG, like phorbol esters (33, 34), rapidly stimulates ouabain-sensitive $^{86}Rb^+$ uptake in quiescent cultures of Swiss 3T3 cells.

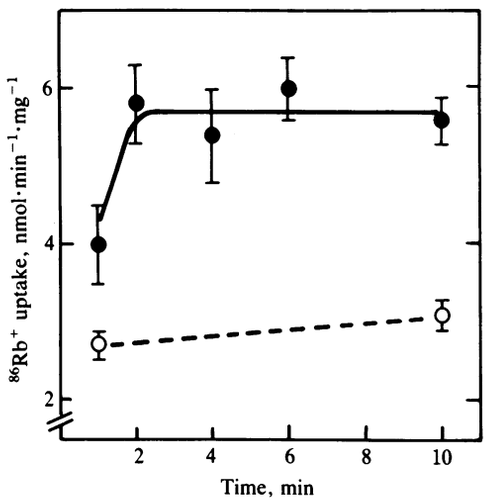

FIG. 2. Time course of stimulation of ouabain-sensitive $^{86}Rb^+$ uptake by OAG. Confluent, quiescent cultures were washed twice with ESB medium and then incubated at 37°C for 15 min with 2 ml of the same medium. At time zero, the cultures were transferred to ESB medium with (●) or without (○) OAG (100 μg/ml). At various times, $^{86}Rb^+$ (12.5 μCi/ml) was added and the uptake after 60 sec at 37°C was measured. Parallel cultures were treated identically but in the presence of 2 mM ouabain, and the radioactivity taken up by these cultures was subtracted from the total $^{86}Rb^+$ uptake measured in cultures without ouabain to determine the rate of ouabain-sensitive $^{86}Rb^+$ uptake. The data represent the mean ± SEM of six independent experiments (n = 18).

The Effect of Prolonged Incubation with PBt_2 on the Responsiveness of the Cells to OAG and PBt_2. Prolonged exposure of Swiss 3T3 cells to PBt_2 leads to a progressive decline in high-affinity phorbol ester binding sites (20, 25, 26) and in the specific activity of protein kinase C measured in cell-free preparations (27) and to desensitization to further biological effects of phorbol esters (26, 28). If the action of OAG and PBt_2 on cation uptake is mediated through activation of protein kinase C, prolonged exposure to PBt_2 should block the stimulation of $^{86}Rb^+$ uptake caused by a subsequent challenge with either PBt_2 or OAG. To test this possibility, cultures of 3T3 cells were treated with PBt_2 at a saturating concentration (400 ng/ml). After 40 hr, control and treated cultures were washed extensively to remove residual PBt_2 and then transferred to medium containing PBt_2 or OAG to ascertain the efficacy of these agents in stimulating $^{86}Rb^+$ uptake. As shown in Fig. 3*A*, addition of PBt_2 or OAG to control 3T3 cultures stimulated ouabain-sensitive $^{86}Rb^+$ uptake by 2.2- and 1.85-fold, respectively. In sharp contrast, neither PBt_2 nor OAG caused a significant stimulation of cation uptake in the cultures pretreated with PBt_2 (Fig. 3*B*).

The activity of the Na^+/K^+ pump is limited and regulated by the availability of cytosolic Na^+ (2, 5, 31). In accord with this, the rate of $^{86}Rb^+$ uptake by intact cells is markedly increased by the addition of the monocarboxylic acid ionophore monensin, which carries Na^+ into the cell (2, 31). To eliminate the possibility that a prolonged incubation with PBt_2 impairs the function of the Na^+/K^+ pump itself rather than its responsiveness to a subsequent exposure to PBt_2 or OAG, we verified that monensin, added at various concentrations, was as effective in control as in PBt_2-treated cells in stimulating $^{86}Rb^+$ uptake (Fig. 4). At 1.2 μg/ml, monensin caused an 8-fold increase in cation influx in both control and PBt_2-treated cells (Fig. 4).

Effect of OAG and PBt_2 on Amiloride-Sensitive $^{22}Na^+$ Influx. To test whether the rapid increase in Na^+/K^+ pump activity by OAG or PBt_2 results from increased Na^+ entry via the Na^+/H^+ antiport system, quiescent cultures were incubated in the absence or presence of these ligands and

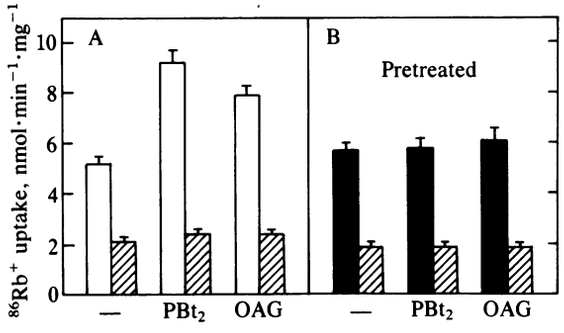

FIG. 3. Effect of prolonged pretreatment with PBt_2 on the stimulation of $^{86}Rb^+$ uptake by a subsequent addition of PBt_2 or OAG. Cells were plated in DME medium containing 10% fetal bovine serum and incubated at 37°C in a humidified 90% air/10% CO_2 atmosphere for 7 days, at which time the cultures were confluent and quiescent. The cells then were incubated for 40 hr in a mixture of their own conditioned medium, fresh DME, and Waymouth medium (1:1.5:1.5, vol/vol) either in the absence (*A*) or in the presence (*B*) of PBt_2 (400 ng/ml). At this time, the cells were washed three times in ESB and incubated in this medium for 20 min at 37°C before two final washes in the same medium. This procedure was shown previously to remove residual PBt_2 (26). The cultures then were incubated with 1.9 ml of ESB at 37°C, either in the absence (—) or presence of either PBt_2 at 200 ng/ml or OAG at 100 μg/ml and with (hatched bars) or without 2 mM ouabain. After 15 min of incubation, $^{86}Rb^+$ was added and the cultures were incubated for an additional 10 min. Shown are the mean + SEM (n = 8) of three independent experiments.

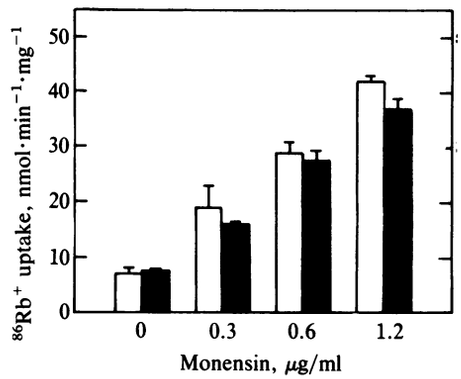

FIG. 4. Stimulation of $^{86}Rb^+$ uptake by various concentrations of monensin in control or PBt_2-treated cultures. Confluent cells were incubated for 40 hr either in the absence (control; open bars) or presence (shaded bars) of PBt_2 at 400 ng/ml. These incubations and subsequent washes were exactly as described in the legend to Fig. 3. Then, the cultures were incubated in ESB with various concentrations of monensin. After 15 min, $^{86}Rb^+$ uptake was measured; shown are mean + SEM, n = 4.

with or without amiloride, an inhibitor of Na^+/H^+ exchange in 3T3 cells and other cell types (6, 35–40). Fig. 5*A* shows that PBt_2 or OAG caused a marked increase in the rate of $^{22}Na^+$ influx, an effect prevented by the presence of amiloride. In view of the results shown in Fig. 3, it was of interest to determine whether prolonged exposure to PBt_2 prevents subsequent addition of PBt_2 or OAG from increasing amiloride-sensitive Na^+ influx into 3T3 cells. The results shown in Fig. 5*B* demonstrate that pretreatment of 3T3 cells with PBt_2 abolished the stimulation of Na^+ influx promoted by subsequent addition of PBt_2 or OAG.

An increase in the intracellular concentration of H^+ (i.e., an acid "load") activates the Na^+/H^+ antiport in intact 3T3 cells (7). If prolonged pretreatment with PBt_2 prevents the stimulation of the Na^+/H^+ antiport system by OAG or PBt_2 but does not affect the activity of the antiport system itself, the increase in Na^+ influx in control and PBt_2-treated cells in response to an acid load should be similar. To test this, control and PBt_2-treated cultures were preincubated in the absence or presence of NH_4Cl for 13 min, washed, and transferred to NH_4Cl-free medium; $^{22}Na^+$ uptake was then measured with or without amiloride. Removal of NH_4Cl

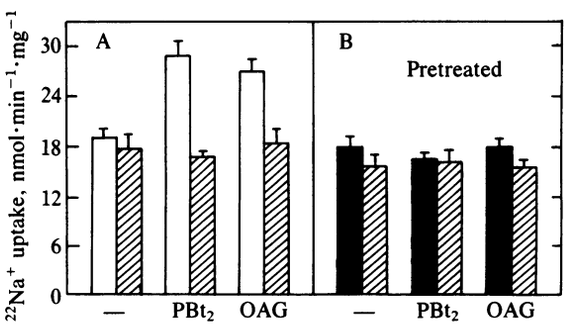

FIG. 5. Effect of prolonged pretreatment with PBt_2 on the stimulation of $^{22}Na^+$ uptake by a subsequent addition of PBt_2 or OAG. Confluent cells were incubated for 40 hr either in the absence (*A*) or presence (*B*) of PBt_2 (400 ng/ml). These incubations and subsequent washes were exactly as described in the legend to Fig. 3. Then, the cultures were incubated with ESB containing 2 mM ouabain either in the absence (—) or presence of either PBt_2 at 200 ng/ml or OAG at 100 μg/ml and with (hatched bars) or without 2 mM amiloride. After 15 min of incubation, $^{22}Na^+$ uptake was measured; shown are the mean + SEM of two independent experiments (n = 6).

from the medium results in cellular acidification (i.e., acid load) as the cellular NH_4^+ accumulated during the preincubation period gives up H^+ and rapidly exits the cell as NH_3 (7). As shown in Fig. 6, the NH_4Cl "pulse" caused a striking increase in $^{22}Na^+$ influx, in both control and PBt_2-treated cells. These results indicate that a functional Na^+/H^+ antiport system is present in PBt_2-desensitized cells but is no longer responsive to either PBt_2 or OAG.

Effect of OAG on Intracellular pH. Since OAG and PBt_2 stimulate Na^+ influx into 3T3 cells through an amiloride-sensitive Na^+/H^+ antiport system, a rise in intracellular pH should occur simultaneously with the increase in Na^+ entry. As shown in Table 1, OAG enhanced the uptake of [^{14}C]DMO into 3T3 cells. In contrast, OAG had very little effect on intracellular water content, as measured by the uptake of [^{14}C]urea. The stimulation by OAG of [^{14}C]DMO uptake indicates a net increase (0.15 pH units) in the intracellular pH of Swiss 3T3 cells.

DISCUSSION

The synthetic diacylglycerol OAG competes with [^{3}H]PBt_2 for binding sites in monolayer cultures of 3T3 cells and rapidly activates protein kinase C in intact cells (24). OAG, like phorbol esters (20, 25, 26, 30, 41), stimulates reinitiation of DNA synthesis and cell division in 3T3 cells (24). The purpose of the present study was to determine the role of protein kinase C in the stimulation of monovalent ion fluxes in quiescent cells. The results presented here show that OAG rapidly stimulates ouabain-sensitive $^{86}Rb^+$ uptake, a measurement of the activity of the Na^+/K^+ pump in intact cells. The effect is dose-dependent and can be detected as early as 1 min after the addition of OAG. Further, OAG or PBt_2 increases $^{22}Na^+$ entry into 3T3 cells, an effect prevented by amiloride. These findings strongly suggest that OAG and PBt_2 enhance amiloride-sensitive Na^+/H^+ antiport activity leading to stimulation of the Na^+/K^+ pump, which is highly sensitive to small increases in cellular Na^+. The data are consistent with previous reports from this laboratory showing that the biologically active phorbol esters PBt_2 and phorbol 12-myristate 13-acetate stimulated Na^+ entry and the Na^+/K^+ pump in 3T3 cells (34); the half-maximal concentration of PBt_2 to elicit this effect was virtually identical to the K_d of [^{3}H]PBt_2 for its high-affinity receptor in these cells (20).

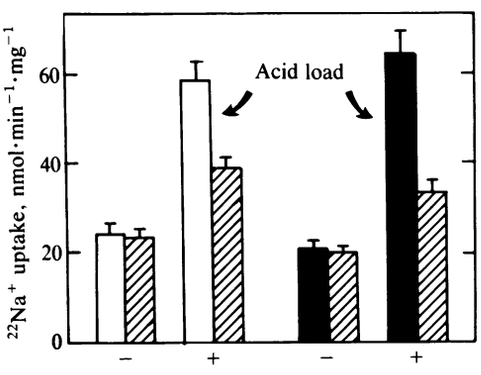

FIG. 6. Stimulation of $^{22}Na^+$ uptake by an acid load generated by a transient exposure to NH_4Cl in control and PBt_2-treated cultures of Swiss 3T3 cells. Confluent cells were incubated for 40 hr either in the absence (control; open bars) or presence of PBt_2 (400 ng/ml; shaded bars). These incubations and subsequent washes were exactly as described in the legend to Fig. 3. Then, the cultures were incubated in a modified ESB medium containing 110 mM sucrose and either 50 mM choline Cl (−) or 50 mM NH_4Cl (+). After 13 min, the media were rapidly removed and replaced by ESB containing 2 mM ouabain and $^{22}Na^+$ and with (hatched bars) or without 2 mM amiloride. Shown are the mean + SEM; $n = 4$.

Table 1. Addition of OAG enhances intracellular pH in Swiss 3T3 cells

	Intracellular water, μl/mg of protein	[^{14}C]DMO uptake, cpm/μg of protein	pH_i	ΔpH_i
Control	5.13 ± 0.10 (n = 9)	9.76 ± 0.14 (n = 15)	6.98 ± 0.012	—
OAG (100 μg/ml)	5.22 ± 0.06 (n = 10)	12.44 ± 0.34 (n = 20)	7.13 ± 0.017	0.15

The measurements of the steady-state concentration of [^{14}C]DMO and of the intracellular water content as well as the calculation of intracellular pH (pH_i) were carried out as described in *Materials and Methods*.

The stimulation of Na^+ influx via the Na^+/H^+ antiport could reflect either an increase in Na^+ for H^+ exchange, leading to cytoplasmic alkalinization, or, alternatively, a compensatory mechanism for the extrusion of protons generated by cellular metabolism in PBt_2 or OAG-stimulated cells. To distinguish between these possibilities, we measured the uptake of [^{14}C]DMO as a measure of intracellular pH. We found that OAG caused a significant increase in the steady-state concentration of labeled DMO; the intracellular pH was increased by 0.15 pH units (Table 1). As previously reported by Burns and Rozengurt (7) and confirmed under our experimental conditions, PBt_2 also causes cytoplasmic alkalinization in Swiss 3T3 cells. These findings are in good agreement with recent reports that phorbol esters enhance amiloride-sensitive Na^+/H^+ exchange in a murine pre-B-lymphocyte cell line (42) and a human leukemic cell line (43).

Prolonged pretreatment with PBt_2 markedly reduces the number of high-affinity phorbol ester binding sites (20, 25, 26) and the specific activity of protein kinase C in cell-free preparations (27) and desensitizes the cell to further biological effects of phorbol esters (26, 28). It is noteworthy that both the magnitude of stimulation of the Na^+/H^+ antiport system by an acid load achieved by a transient exposure to NH_4Cl and the enhancement of Na^+/K^+ pump activity induced by the Na^+ ionophore monensin (31) were virtually identical in control and PBt_2-treated cells. These important controls indicate that the Na^+/H^+ antiport system and the Na^+/K^+ pump are present and functional in PBt_2-desensitized cells.

A salient feature of the results presented in this paper is that prolonged pretreatment of the cells with PBt_2 abolished the stimulation of $^{86}Rb^+$ or $^{22}Na^+$ uptake by a subsequent addition of either PBt_2 or OAG. This striking loss of ionic responses to OAG and PBt_2 seen in 3T3 cells with a greatly reduced number of high-affinity phorbol ester receptors (20, 25, 26) and activity of protein kinase C (27, 28) strongly implicates this phosphotransferase system in the stimulation of monovalent cation fluxes. We conclude that activation of protein kinase C leads either directly or indirectly to increased activity of the Na^+/H^+ antiport system, which in turn promotes Na^+ influx, enhances intracellular pH, and stimulates the Na^+/K^+ pump activity.

Addition of a variety of mitogens, including serum, platelet-derived growth factor, fibroblast-derived growth factor, and the peptides vasopressin and bombesin, to quiescent 3T3 cells rapidly activates protein kinase C (refs. 28 and 44; unpublished results) and increases the rate of monovalent cation fluxes (2, 5–10). In view of our findings, it is likely that activation of protein kinase C mediates the early ionic response elicited by these growth-promoting factors. The use of PBt_2-desensitized 3T3 cells, as shown

here, provides an experimental approach for evaluating the contribution of protein kinase C in mediating the ionic responses elicited by growth factors and mitogenic hormones.

Note Added in Proof. Moolenaar *et al.* (45) recently reported that phorbol esters and OAG also increase intracellular pH in human fibroblasts.

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