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Activation of Na^+/H^+ exchange in cultured fibroblasts: Synergism and antagonism between phorbol ester, Ca^{2+} ionophore, and growth factors

(protein kinase C/phorbol 12-myristate 13-acetate/mitogens/vasopressin/bradykinin)

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ABSTRACT **The effects of phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C, on Na^+ influx were investigated in cultured human foreskin fibroblasts (HSWP cells). We report here that in serum-deprived HSWP cells the addition of PMA alone has no significant effect on Na^+ influx. However, the addition of PMA to cells whose Na^+/H^+ exchanger is partially activated with a submaximal dose of the Ca^{2+} ionophore A23187 leads to a larger stimulation than seen with A23187 alone. These data suggest that although stimulation of protein kinase C is not a sufficient signal to activate the Na^+/H^+ exchanger in HSWP cells or in another human foreskin line (Jackson fibroblasts) studied, there are some cooperative effects of protein kinase C activation with a rise in Ca^{2+} to stimulate Na^+/H^+ exchange. In addition, we found that PMA actually inhibits the mitogen-induced stimulation of Na^+ influx in HSWP and Jackson fibroblasts. This observation strengthens the argument that in these cells activation of protein kinase C is not sufficient to activate Na^+/H^+ exchange and suggests that there is a negative feedback control via protein kinase C that inhibits some signal that is necessary for activating Na^+/H^+ exchange. However, in contrast to observations in HSWP cells, we were able to activate the Na^+/H^+ exchanger in mouse 3T3 and human WI-38 cells with PMA alone, suggesting that there is some diversity in the mechanism for activation of Na^+/H^+ exchange in different types of fibroblasts.**

Fibroblasts grown in cell culture can be arrested in the G_0/G_1 phase of the cell cycle by depriving them of growth factors. The re-addition of serum or purified growth factors to the culture medium results in an ordered sequence of biochemical events that lead eventually to an increased cell proliferation. Since activation of Na^+/H^+ exchange is one of the important early responses of cultured fibroblasts to mitogenic stimulation (1–5), it is important to understand the mechanism for this activation. Our previous work suggests that in cultured human fibroblasts (HSWP cells) a rise in intracellular Ca^{2+} activity is a necessary step in this process (2, 6, 7). Early studies from our laboratory, utilizing inhibitors of calmodulin (6, 8), suggested that the activation of Na^+/H^+ exchange in HSWP cells was a calmodulin-dependent event. However, subsequent to that study it became apparent that most of the calmodulin inhibitors used also can inhibit protein kinase C (9). In addition, with the recent discovery that the site of action of tumor promoters is protein kinase C (9), the early observation that the tumor promoter phorbol 12-myristate 13-acetate (PMA) would stimulate Na influx in 3T3 cells (10) suggested that protein kinase C may be involved in the activation of Na^+/H^+ exchange. In fact, a recent report (11) suggested that PMA stimulation of Na^+/H^+ exchange occurs in the absence of a rise in Ca^{2+} activity, which led the authors to suggest that a rise in Ca^{2+} is not a necessary event in fibroblasts for the mitogen activation of Na^+/H^+ exchange. On the basis of these reports, it was important to determine whether the Ca^{2+}-dependent activation seen in HSWP cells might be mediated by protein kinase C rather than by calmodulin. Therefore, we investigated the effects of PMA on Na^+ influx via the Na^+/H^+ exchanger in HSWP cells. Results reported here suggest that, although stimulation of protein kinase C activity may be involved in the activation of Na^+/H^+ exchange in HSWP cells, it is not a sufficient event. In addition, we present evidence which suggests that protein kinase C may mediate a negative feedback signal to turn off the growth factor activation of Na^+/H^+ exchange. More importantly, we present evidence that there may be some diversity among the mechanisms for stimulation of Na^+/H^+ exchange in different types of fibroblasts.

MATERIALS AND METHODS

HSWP cells, a human foreskin fibroblast strain obtained from Jim Regan at Oak Ridge National Laboratory, were grown in Eagle's minimal essential medium plus 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C. WI-38 human embryonic lung cells and Jackson human foreskin fibroblasts were cultured in the same manner as the HSWP cells. Swiss 3T3 mouse cells were grown in Dulbecco's modified minimum essential medium plus 10% fetal bovine serum. Cells subcultured on 60-mm plastic culture dishes were serum deprived in Hepes-buffered, amino acid-free Eagle's medium plus 0.1% fetal bovine serum for 4 hr. The Na^+ influx was initiated by replacement of the serum-deprivation medium with assay medium (Hepes/amino acid-free Eagle's medium/10 μM digitoxin, to inhibit the Na^+/K^+ ATPase) containing the stimulatory or inhibitory agent indicated. For the serum-stimulated cells, serum, dialyzed against a Hanks' buffered salt solution, was added at a concentration of 10% at the time of flux initiation. For the serum-deprived cells, the flux was initiated by addition of serum-free assay medium. To investigate the effect of PMA on Na^+ influx, cells were incubated with the designated dose of PMA for 2 min before addition of assay medium containing the same dose of PMA. Cells were incubated in assay medium for 5 min, freed of assay medium by aspiration, and washed four times in ice-cold isotonic $MgCl_2$ solution to remove extracellular Na^+. Cells were then treated with 5% trichloroacetic acid and the Na^+ content of the extract was determined by atomic

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

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absorption spectrometry. The Na^+ influx, expressed as μmol/min per g of protein, was determined by dividing the Na^+ taken up in 5 min (during which time the Na^+ uptake is linear with time) by the assay time and the protein content of the dish.

RESULTS AND DISCUSSION

The effects of the tumor promoter PMA, which is known to activate protein kinase C *in vitro* (9), on the Na^+ influx in serum-deprived HSWP cells were investigated. For the purposes of this discussion, and on the basis of extensive data in the literature, it is assumed that the actions of PMA at low doses (1–10 ng/ml) are mediated by protein kinase C. However, it should be pointed out that PMA effects at other regulatory steps can never be ruled out entirely. We found that the addition of PMA to HSWP cells in the absence of other agents had no effect on Na^+ influx in either the absence (Fig. 1) or the presence of amiloride (data not shown). Various conditions were tested, including doses of PMA ranging from 1 to 1000 ng/ml, preincubation times ranging from 0 to 30 min, cells at subconfluent and confluent densities, serum deprivation times ranging from 4 hr to 1 week, and serum deprivation in complete CO_2-buffered Eagle's medium versus Hepes-buffered amino acid-free Eagle's medium. Under none of these conditions, or any combination of these conditions, did PMA stimulate Na^+ influx in HSWP cells. In these same experiments the addition of serum caused significant stimulation of Na^+ influx (Fig. 1). As seen in our previous studies (2), all of the serum-stimulated Na^+ influx occurs via an amiloride-sensitive pathway which represents a Na^+/H^+ exchange system (5). The data with PMA alone, and similar data (not shown) in another human foreskin cell strain (Jackson fibroblasts), suggest that if the addition of PMA does stimulate protein kinase C activity in these cells, then this stimulation is not sufficient to activate Na^+/H^+ exchange.

Since there is evidence for synergism between Ca^{2+} ionophores and tumor promoters in the stimulation of enzyme secretion from several secretory cell systems (12, 13), we investigated whether PMA affects Na^+ influx in HSWP cells in the presence of a suboptimal dose of the Ca^{2+} ionophore A23187. As seen in Fig. 2*A*, the addition of A23187 alone will stimulate Na^+ influx in HSWP cells. This agrees with our previous results, which showed that the addition of A23187 to serum-deprived cells stimulates an amiloride-sensitive Na^+ influx (2) and, in contrast to another recent report (4), stimulates a Na^+-dependent amiloride-sensitive rise in intracellular pH (5). Since the A23187-induced alkalinization in HSWP cells (5) and in 3T3 cells (14) is dependent on the presence of extracellular Ca^{2+}, this appears to be a Ca^{2+}-mediated event. The addition of PMA in the presence of submaximal doses of A23187 produces a rise in Na^+ influx above that seen with A23187 alone. The Na^+ flux stimulation by PMA in the presence of A23187 is totally inhibited by amiloride, as judged by an extrapolation of a plot of fractional inhibition versus $[\text{amiloride}]^{-1}$ to infinite amiloride concentrations (data not shown), suggesting that these agents are stimulating Na^+/H^+ exchange.

The PMA dose for the half-maximal synergistic effects with

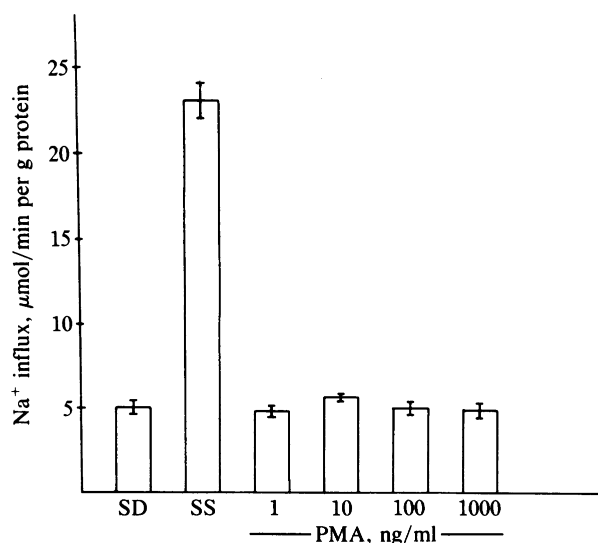

FIG. 1. Effect of addition of PMA alone on Na^+ influx in HSWP cells. Cells were assayed in serum-free medium (SD), medium containing 10% serum (SS), or in medium containing various doses of PMA. Values shown are the mean ± SEM of five replicates from a representative experiment.

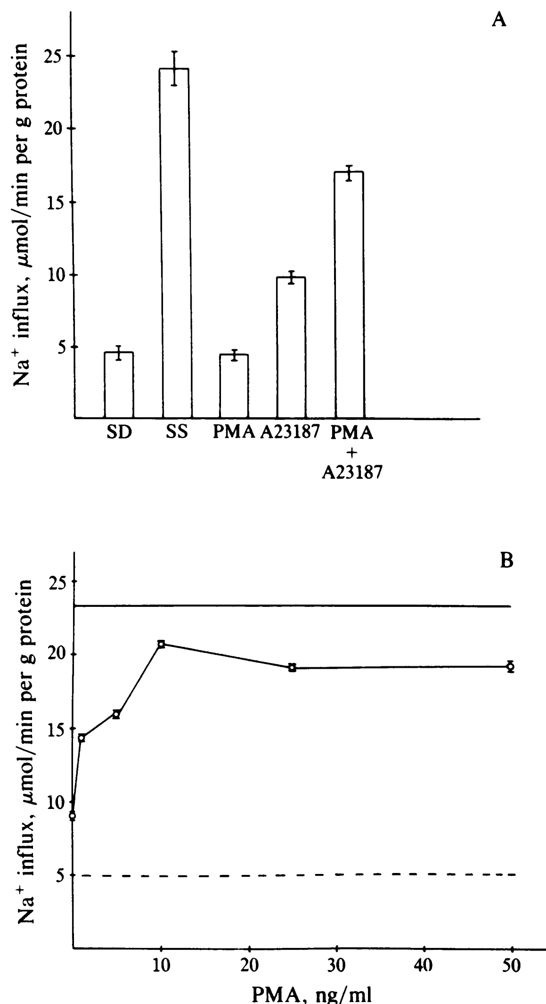

FIG. 2. (*A*) Synergism between PMA and A23187 for stimulation of Na^+ influx in HSWP cells. Cells were assayed in serum-free medium (SD), medium containing 10% serum (SS), medium containing PMA (100 ng/ml), medium containing A23187 (1 μg/ml), or medium containing PMA and A23187. (*B*) Dose–response curve for the PMA effect on Na^+ influx in the presence of A23187. Cells were incubated for 2 min in the designated dose of PMA and then assay medium containing the same dose of PMA and A23187 (1 μg/ml) was added to initiate the net Na^+ influx. The broken line across the bottom of the figure represents the level of basal (serum-free medium) Na^+ influx, while the solid straight line at the top represents the level of serum-stimulated influx (in the absence of PMA) in this experiment. Values shown are means ± SEM from a representative experiment.

A23187 is 1 ng/ml (Fig. 2*B*), which agrees well with the doses of PMA that have been demonstrated to activate protein kinase C *in vitro* (9). The addition of A23187 at 1 μg/ml together with a maximal dose of PMA did not stimulate Na^+ influx to the same degree as seen with serum. However, at 5 μg/ml, A23187 alone stimulates Na^+ influx to approximately 90% of the level seen with serum, and the addition of PMA with this dose of A23187 gives fluxes in excess (approximately 1.3-fold) of those seen with serum (data not shown). In another set of studies, similar synergism between A23187 and PMA was seen in Jackson fibroblasts (data not shown). These data suggest either that a rise in Ca^{2+} is required in these cells to activate protein kinase C (although this is contrary to the current view that PMA can activate protein kinase C in the absence of a rise in Ca^{2+} activity) or that there is synergism between PMA-activated protein kinase C-mediated effects and some Ca^{2+}-dependent event, which could be mediated by calmodulin as we recently suggested (6). Perhaps the Na^+/H^+ exchanger is regulated by multiple phosphorylation events (e.g., mediated by Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C) as is seen in the case of the sarcoplasmic reticulum protein phospholamban (15) and in the case of the myosin light chain (16).

Since PMA shows synergistic effects when added with A23187, which presumably works by elevating intracellular free Ca^{2+} concentration, it was important to determine whether PMA would synergize with mitogens, which also produce a rise in intracellular Ca^{2+} activity (7). HSWP cells were incubated with PMA for 2 min and then assay medium containing growth factors and PMA was added to initiate net Na^+ influx. To our surprise, PMA did not synergize with the added mitogens, but instead it inhibited a significant portion of Na^+ influx induced by the growth factors serum, bradykinin, and vasopressin (Fig. 3*A*). As was seen for the synergism with A23187, the PMA dose for half-maximal inhibition of the serum-stimulated Na^+ flux is 1 ng/ml (Fig. 3*B*). In contrast, the addition of 4α-phorbol 12,13-didecanoate, which is inactive in stimulating protein kinase C, has no effect on the growth factor response when added at 100 nM (data not shown). These results extend recent observations of phorbol ester inhibition of EGF-induced Na^+/H^+ exchange in A431 cells (17) by demonstrating inhibition of bradykinin- and vasopressin-induced Na^+/H^+ exchange. Thus, in addition to inhibiting events mediated by receptors (EGF and insulin) that have tyrosine kinase activity (18, 19), PMA can also inhibit the functional consequences of receptors that appear to be coupled to the release of inositol phosphates (20).

The PMA inhibition results have interesting implications. On the one hand, they demonstrate that the addition of PMA alone during the preincubation period does induce effects in HSWP cells, presumably via activation of protein kinase C. We have recently demonstrated the presence of protein kinase C in HSWP cells and have observed PMA-induced phosphorylation of several proteins in intact cells at a PMA dose of 10 ng/ml (unpublished observation). When taken together these data support the contention that activation of this enzyme alone is not sufficient to stimulate Na^+ influx in HSWP cells. On the other hand, the inhibition of the mitogen effect by PMA suggests the existence of a negative feedback control promoted by the mitogens on their own mechanism of activation. We have previously shown that in HSWP cells serum, vasopressin, and bradykinin stimulate the release of inositol phosphates (20) by a process that presumably also releases diacylglycerol, the endogenous activator of protein kinase C. Elevation of diacylglycerol levels in response to mitogens could produce a negative feedback regulation via protein kinase C activation which could be expressed through the inhibition of some other necessary mitogen-induced signal. This could occur at the receptor level, since recent

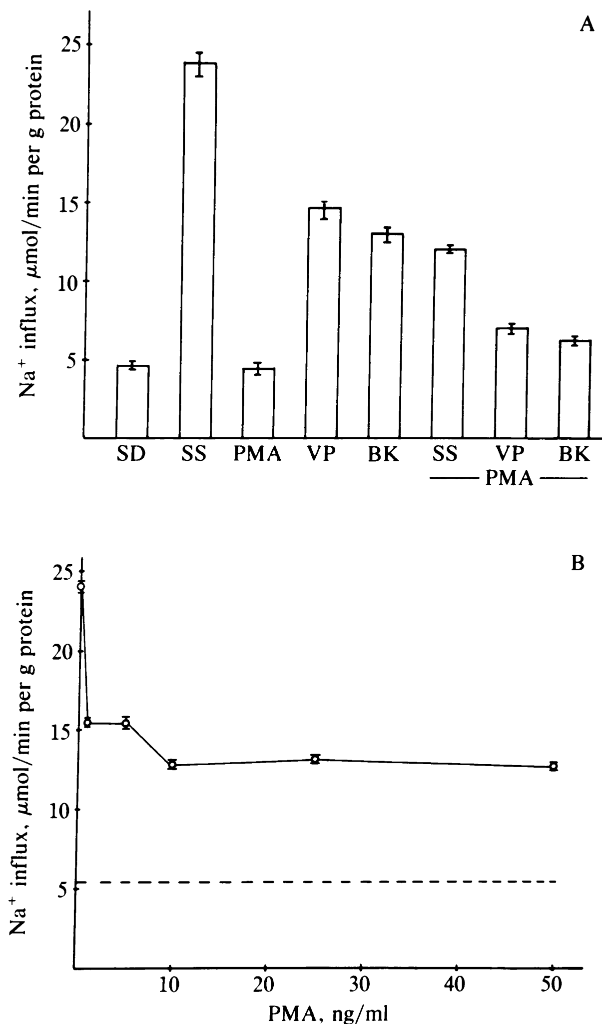

FIG. 3. (*A*) Inhibition of mitogen-stimulated Na^+ influx by PMA in HSWP cells. Cells were incubated with or without PMA (100 ng/ml) for 2 min and then assay medium containing PMA and the noted mitogen was added to initiate net Na^+ influx. Serum (SS) was present at 10%, bradykinin (BK) and vasopressin (VP) were present at 100 ng/ml. The basal flux (SD) was measured in serum-free assay medium. (*B*) Dose–response curve for the PMA inhibition of serum-stimulated Na^+ influx in HSWP cells. Cells were incubated for 2 min in the appropriate dose of PMA and then assay medium containing the same dose of PMA and 10% serum was added to initiate a net Na^+ influx. The broken line across the bottom of the figure represents the level of the basal (SD) Na^+ influx in this experiment. Values are means ± SEM for five replicates in a representative experiment.

studies have demonstrated protein kinase C inhibition of EGF- and insulin-induced tyrosine kinase activity of their respective receptors (18, 19), or it could occur at some postreceptor event such as the phospholipase C-induced release of inositol trisphosphate. The latter possibility is supported by recent observations that the addition of PMA to PC12 cells will inhibit the carbachol-induced release of inositol trisphosphate (21) and that PMA will inhibit the growth factor-induced release of inositol trisphosphate in HSWP cells (unpublished data). Thus, PMA could inhibit the growth factor stimulation of Na^+/H^+ exchange by inhibiting the release of inositol trisphosphate and thereby reducing the rise in Ca^{2+} activity, which appears to be necessary for activation of Na^+/H^+ exchange in HSWP cells. It should be stressed that regardless of whether PMA inhibits Na^+/H^+ exchange by suppressing inositol phosphate release, the PMA inhibition data argue that some signal in addition to the activation of protein kinase C is necessary for the growth

factor stimulation of Na^+/H^+ exchange in HSWP cells. This contention is supported by a recent observation in 3T3 cells, in which epidermal growth factor plus PMA gave a larger activation of the Na^+/H^+ exchanger than the addition of a maximal dose of PMA (14).

In contrast to our studies in two human foreskin fibroblast strains, previous reports indicate that in some cell systems, such as the mouse 3T3 fibroblast (10), addition of PMA alone will stimulate Na^+ influx. It was therefore important to determine whether such a stimulation would occur in our hands. The data in Table 1 indicate that the addition of PMA alone will stimulate Na^+ influx in 3T3 cells. The dose for half-maximal stimulation of Na^+ influx is 10 ng/ml (data not shown). Similar effects were seen with the human lung fibroblast cell line WI-38 (Table 1). The results reported here argue that, in two foreskin fibroblast cell strains, PMA-induced stimulation of protein kinase C activity is not a sufficient stimulus to activate Na^+/H^+ exchange, although activation of protein kinase C does appear to synergize with a Ca^{2+}-mediated activation of this transport system. However, in an immortalized mouse fibroblast line (3T3) and in an embryonal human lung fibroblast cell line (WI-38), activation of protein kinase C does seem to be a sufficient signal to activate Na^+/H^+ exchange, although to a lower level than seen with serum. These data suggest that there is a diversity in the mechanism by which mitogens activate Na^+/H^+ exchange in different cell types and that care should be taken in making generalizations concerning mechanisms for the regulation of Na^+/H^+ exchange based on observations in any one cell system. It remains to be determined whether the diversity in regulation of Na^+ influx between cell types can be explained on the basis of a difference in either their protein kinase C contents or levels of resting Ca^{2+} activity.

Finally, a very important general concept emerges from our data and those recently reported for A431 cells (17). The occupation of mitogen receptors induces hydrolysis of phosphorylated phosphatidylinositols and consequently the generation of diacylglycerol, which causes stimulation of protein kinase C. Our results, showing that PMA activation of protein kinase C inhibits the mitogen-induced activation of Na^+/H^+ exchange, suggest that the production of diacylglycerol may eventually produce a signal that feeds back to inhibit the mitogen-induced response, thereby providing a mechanism for autoregulation that may prevent the cell from overresponding.

Table 1. Effect of PMA on Na^+ influx in 3T3 and WI-38 cells

Cells	Assay conditions	Na^+ influx, μmol/min per g protein
3T3	Serum-free	17.3 ± 0.9
	PMA	32.1 ± 1.6
	Serum	44.3 ± 1.8
WI-38	Serum-free	4.7 ± 0.2
	PMA	16.7 ± 0.8
	Serum	28.7 ± 1.1

Serum was added to cells at the time of initiation of Na^+ influx at a concentration of 10%. PMA (100 ng/ml) was added to the cells 5 min prior to the initiation of the measurement and was also present in the assay medium. Values are means ± SEM for five replicates in a representative experiment.

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