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PHORBOL ESTER STIMULATION OF Na INFLUX AND Na-K PUMP ACTIVITY IN SWISS 3T3 CELLS

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SUMMARY

Tumor promoting phorbol esters, at nM concentrations, rapidly stimulate Na^+ entry into intact 3T3 cells incubated in the presence of ouabain. The effect is specific and time and dose-dependant. In the absence of ouabain phorbol esters increase Na-K pump activity. Since stimulation of Na-K pump activity by TPA is i) dependant on the Na^+ concentration in the medium ii) abolished by the sodium ionophore monensin, we suggest that the phorbol esters stimulate Na-K pump activity by increasing Na^+ influx into 3T3 cells.

Recent studies on the mitogenic actions of vasopressin (1) and of 12-0-tetradecanoyl-phorbol-13-acetate (TPA)*, the most potent tumor promoting agent of the phorbol ester family (2), led to the proposal that these chemically diverse molecules modulate 3T3 cell function through a common mechanism (3). Thus, vasopressin or TPA synergistically stimulate DNA synthesis to an identical level when added in the presence of other growth factors, but show neither synergistic nor additive effects with each other (3). TPA and vasopressin also have equivalent non-synergistic, effects in inducing 2-deoxyglucose uptake and ornithine decarboxylase activity many hours prior to the

* ABBREVIATIONS: DMEM: Dulbecco's modified Eagle's medium. Me-TPA: 4-0-Methyl-12-0-tetradecanoyl-phorbol-13-acetate. TPA: 12-0-tetradecanoyl-phorbol-13-acetate. PDB: Phorbol-12-13-dibutyrate.

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stimulation of DNA synthesis (3). Furthermore, within minutes after their addition to cells, both agents inhibit the binding of radiolabelled epidermal growth factor to specific surface receptors (4,5,6,7). These findings suggest that the action of phorbol esters and vasopressin rapidly converges into a common mechanism, probably at the level of the plasma membrane (3).

A striking functional change occurring in the plasma membrane is the activation of the Na-K pump within minutes of the addition of a variety of mitogenic molecules (8,9), including vasopressin (10) and TPA (11), to quiescent 3T3 cells. Recent evidence indicates that the activity of the Na-K pump in intact cells is limited and regulated by the internal Na^+ content and that vasopressin and other polypeptide growth factors stimulate the Na-K pump in quiescent 3T3 cells by increasing Na^+ entry and availability to the intracellular Na^+ transport site of the pump (10,12-15). The mechanism by which TPA increases the activity of the pump remains unknown. The results presented here suggest that TPA, like vasopressin and other growth-promoting factors, stimulates the activity of the Na-K pump by increasing Na^+ entry into the cell.

MATERIALS AND METHODS

Cell Culture: Swiss 3T3 cells (16) were maintained as previously described (1). The cells were subcultured to 30 mm Nunc Petri dishes with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The medium was changed 2 days after plating and studies were performed on quiescent, confluent cultures 4-7 days later. Cultures were shown to be quiescent by autoradiography (labelling index 0.5%) after 40 hrs with (^{3}H)-thymidine as previously described (8).

Ion fluxes and content: Intracellular electrolyte content and $^{86}Rb^+$ and $^{22}Na^+$ uptake was measured as previously described (12). All values are the means of duplicate (Rb^+ uptake) quadruplicate (Na^+ uptake and ion measurements) dishes which did not vary by more than 10% from the mean.

Materials: Bovine insulin (26 international units/mg) and (Arg) vasopressin were obtained from Sigma. Phorbol esters were obtained from Consolidated Midland Corporation (Brewster, New York). The serum used was fetal bovine (Flow Laboratories, Rockville, Md.) which had been exhaustively dialysed against 0.14M choline chloride. All other material used were of reagent grade.

RESULTS AND DISCUSSION

Incubation of quiescent Swiss 3T3 cells with 100 ng/ml TPA, a saturating concentration for induction of mitogenesis (3) increases the initial rate of $^{86}Rb^{+}$ uptake (Fig. 1). This stimulation is almost exclusively on the ouabain-sensitive component of $^{86}Rb^{+}$ influx, which comprises 50 to 80% of total influx. The stimulatory effect of phorbol esters on the Na-K pump displays remarkable specificity. Only those phorbol esters which stimulate mitogenesis in 3T3 cells, e.g. TPA and phorbol-12-13-dibutyrate (PDB) but not 4-0-methyl-12-0-tetradecanoyl-phorbol-13-acetate (MeTPA), the inactive analogue of TPA (2,17), enhance ouabain sensitive $^{86}Rb^{+}$ influx (Fig. 1). The stimulation of $^{86}Rb^{+}$ uptake by TPA was generally less than that induced by 10% fetal bovine serum but comparable to that elicited by vasopressin (Fig. 1).

As reported previously, $^{86}Rb^{+}$ uptake depends on the concentration of K^{+} in the medium (8,10). TPA, like serum and vasopressin (8,10) does not change the apparent affinity of the Na-K pump for extracellular K^{+} but increases the V max. for K^{+} transport. As shown in Fig. 1, (right panel) the apparent K_M for K^{+} uptake was 1.7 mM either in the presence or absence of TPA, whereas the tumor promoter increased the V max of uptake from 6.1 to 9.0 n moles/mg protein/min.

The activity of the Na-K pump in quiescent Swiss 3T3 cells is highly sensitive to changes in intracellular Na^{+} concentration

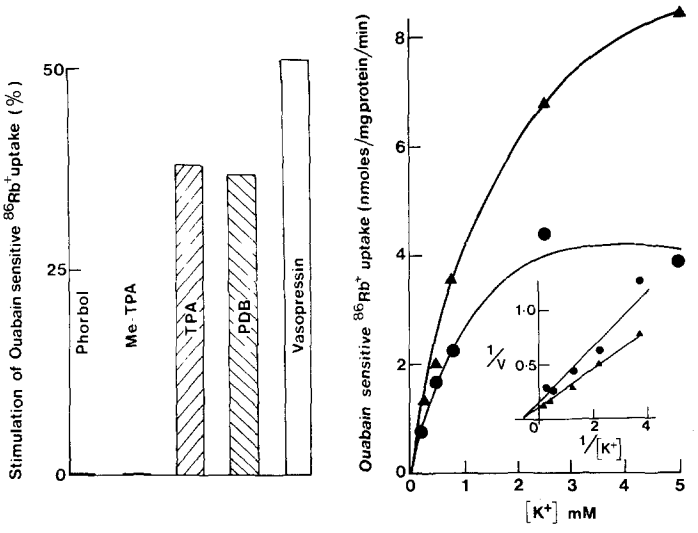

Fig. 1. Stimulation of ouabain sensitive $^{86}Rb^+$ uptake in Swiss 3T3 cells by phorbol esters. Left panel. Quiescent cultures were incubated 45 min in DMEM plus the additions as indicated in the presence or absence of 2mM ouabain. ^{86}Rb (1.5×10^6 cpm) was added and after an additional 15 min (8,14) the uptake of $^{86}Rb^+$ was measured as in Materials and Methods. All phorbol esters had a final concentration of 200 ng/ml, and vasopressin was present at 50 ng/ml. 10% (vol/vol) serum induced an 82% increase in ouabain sensitive Rb^+ uptake in this experiment. Right panel. Rate of ouabain-sensitive $^{86}Rb^+$ uptake as a function of K^+ concentration in the absence (●), or presence of 200 ng/ml TPA (▲). Quiescent cultures of Swiss 3T3 cells were rapidly washed three times with KCl-free medium at 37°C and then incubated with medium containing different concentrations of K^+ (checked by flame photometry) in the absence or presence of 2mM ouabain and, in each case, with or without 200 ng/ml TPA. After 20 min, the cultures were labelled with $^{86}Rb^+$ and incubated for an additional 10 min, during which period uptake was linear with time. All other experimental details were as described under Materials and Methods.

(12) and both serum and vasopressin stimulate the activity of the pump by increasing Na^+ entry into the cell (10,12,15). Several lines of evidence discussed below suggest that TPA stimulates pump activity in a similar fashion.

The rate of $^{86}Rb^+$ influx decreases when the sodium concentration of the medium is lowered and sodium replaced by choline. When Na^+ is present in the media at low concentrations (40 mM), TPA causes a 100% increase in ouabain sensitive $^{86}Rb^+$ influx while at high (140 mM) Na^+ concentrations the phorbol

TABLE I : Stimulation of ouabain sensitive $^{86}Rb^+$ influx in 3T3 cells by TPA or serum in the presence of 40 or 140 mM Na^+ in the medium

Addition	Ouabain sensitive $^{86}Rb^+$ influx (nmoles/mg protein/min)			
	40 mM Na^+	% Increase	140 mM Na^+	% increase
None	1.1	-	2.0	-
TPA	2.2	100	2.8	40
Fetal bovine serum	5.0	354	6.0	200

Quiescent 3T3 cultures were preincubated for 40 min in DMEM containing additions as shown. Then, the cultures were washed three times in serum-free DMEM lacking NaCl (but containing 40 mM Na HCO_3), which was supplemented with NaCl or choline chloride to a final Na^+ concentration of 40 or 140 mM. Cultures were then incubated for 15 min with 1 ml of the same medium containing the additions as indicated, in the presence or absence of 2 mM ouabain. $^{86}Rb^+$ (2.5×10^6 cpm/dish) was added, then the cells were incubated for an additional 10 min and ouabain-sensitive $^{86}Rb^+$ uptake determined as in Materials and Methods. The concentrations of TPA and fetal bovine serum were 200 ng/ml and 10% vol/vol respectively.

ester increases the activity of the pump by 40% (Table I). Similarly, serum (Table I) or vasopressin (10) induce a more pronounced stimulation of $^{86}Rb^+$ influx at low Na^+ concentrations.

In order to test directly if biologically active phorbol esters stimulate Na^+ entry into the cells we measured total intracellular Na^+ content in 3T3 cells incubated in the presence of ouabain (to prevent the exit of sodium from cells via the Na-K pump) with or without various phorbol esters. Table II shows that in cultures incubated with TPA and ouabain, the intracellular Na^+ content increases 60% more than in cultures exposed to ouabain in the absence of TPA. This effect was specific since Me-TPA did not augment Na^+ content, whereas PDB enhanced the accumulation of intracellular Na^+ as effectively as TPA. The stimulation of Na^+ entry by the tumor promoters was similar in magnitude to that caused by vasopressin, but less than that induced by serum. Thus, the relative effects of these

TABLE II : Effect of phorbol esters, vasopressin and serum on Na^+ accumulation into 3T3 cells incubated in the presence of ouabain

Addition	Increase in intracellular Na^+ (μ moles/mg protein)
None	0.34
TPA	0.56
PDB	0.54
Me-TPA	0.40
Vasopressin	0.57
Serum	0.69

The conditions were as in Fig. 2 (left panel) except that the additions were as indicated above. All the phorbol esters were present at 200 ng/ml, serum at 10% vol/vol and vasopressin at 50 ng/ml.

diverse agents on Na^+ accumulation (Table II) and on the activity of the Na-K pump (Fig. 1) are identical. The results shown in Table III demonstrate that TPA also increases the rate of influx of $^{22}Na^+$ into 3T3 cells.

The ability of TPA to accelerate the accumulation of Na^+ in cells incubated with ouabain is further characterized in Fig. 2. The left panel shows that TPA induces this effect in a concentration-dependent fashion; a detectable effect is seen at concentrations as low as 3 ng/ml. The shape of this dose-response is similar to that with which TPA acts synergistically with polypeptide growth factors to stimulate DNA synthesis in 3T3 cells (3). Fig. 2 (right panel) illustrates the time-course of the enhanced accumulation of Na^+ caused by TPA. These findings raise the possibility that TPA stimulates the Na-K pump primarily by increasing the entry of Na^+ into the cells.

Monensin, a monovalent ion ionophore with an affinity for Na^+ 10-fold higher than for K^+ (18), has been shown to carry Na^+

TABLE III : Effect of phorbol esters and serum on $^{22}Na^+$ influx into 3T3 cells incubated in the presence of ouabain

Addition	Rate of $^{22}Na^+$ uptake (nmoles/mg protein min)
Control	3.42 ± 0.25
TPA	4.73 ± 0.36 *
Serum	7.54 ± 0.45 *

Quiescent cultures were incubated for 45 min in DMEM plus the additions as indicated and then washed twice in DMEM without NaCl (but with 40 mM $NaHCO_3$) and supplemented with 100 mM choline chloride. Cultures were then incubated in 0.5 ml of the latter medium containing 2 mM ouabain plus $^{22}Na^+$ (5 μCi/ml) and the additions as indicated. After 3 mins (10,12) $^{22}Na^+$ uptake into cultures was determined as in Materials and Methods. The data represent the means ± SD from quadruplicate dishes. *Indicates $p < 0.001$ when compared with control cultures.

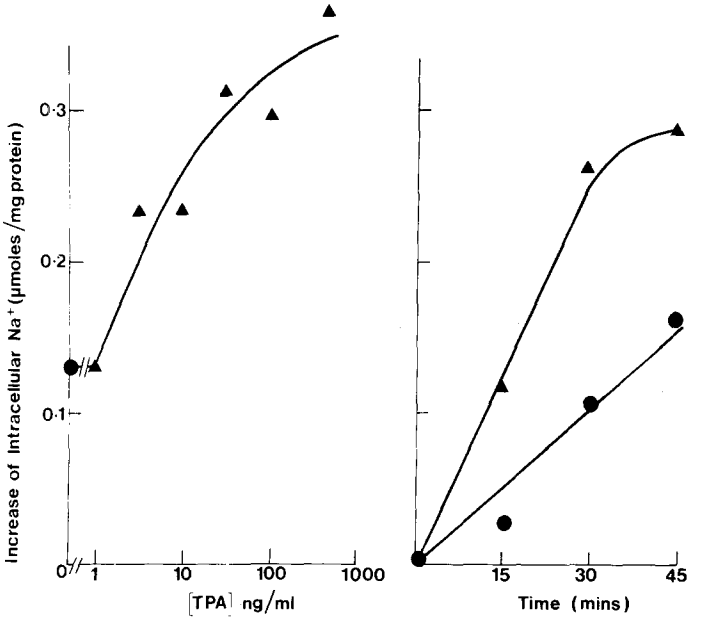

Fig. 2. Effect of TPA on the increase in Na^+ content of 3T3 cells incubated in the presence of ouabain as a function of TPA concentration (left panel) or time (right panel). Left panel. Cultures were incubated in DMEM containing the indicated concentrations of TPA for 45 min. Then, 2mM ouabain was added and after an additional 45 min total intracellular Na^+ measured as in Materials and Methods. The initial intracellular Na^+ content was 0.15 μmoles/mg protein. Right panel. Cultures were incubated in DMEM without (●) or with (▲) 200 ng/ml TPA. After 45 min 2 mM ouabain was added and after the times indicated on the abscissa total intracellular Na^+ measured as in Materials and Methods.

TABLE IV : Stimulation of ouabain sensitive $^{86}Rb^{+}$ uptake in Swiss 3T3 cells by TPA and serum in the absence or presence of monensin

Addition	Ouabain sensitive $^{86}Rb^{+}$ uptake (n moles/mg protein/min)	
	No other addition	Monensin
None	1.6	8.3
TPA	2.5	8.3
Serum	3.3	6.7

Conditions were as in Fig. 1 (left panel) except additions were as indicated above. The concentrations of TPA, serum, and monensin were 100 ng/ml, 10% vol/vol and 5 µg/ml respectively.

into 3T3 cells (12). Table IV shows that addition of 5 µg/ml monensin caused a 5-fold stimulation in ouabain-sensitive $^{86}Rb^{+}$ influx. When TPA or serum were added in the presence of monensin they caused no further increase. Thus, under conditions in which the intracellular availability of Na^{+} is no longer rate limiting for the activity of the pump, TPA does not stimulate ouabain-sensitive $^{86}Rb^{+}$ uptake. Taken together, our findings strongly suggest that phorbol esters enhance Na-K pump activity by increasing the influx of Na^{+} into the cells.

We have previously show that vasopressin and TPA stimulate DNA synthesis in 3T3 cells by pathways which rapidly converge. The cellular receptors for vasopressin appear to be different from those to which phorbol esters bind (3,19). It is plausible that vasopressin and TPA bind to specific and different receptor sites on 3T3 cells, to initiate an identical stimulation of Na^{+} influx, which in turn causes at least one other biological effect, the stimulation of the Na-K pump. In addition, changes in Na^{+} fluxes may play a role in the regulation of Ca^{++} movements and redistributions in the cell. A Na^{+}-Ca^{++} exchange system is present in the mitochondrial membrane of a variety of cell types

(20-22). Increased Na^+ entry stimulated by TPA or vasopressin could induce Ca^{++} mobilization from the mitochondrial pool, thereby increasing the cytosolic Ca^{++} concentration. The possibility that changes in monovalent and divalent cation movements mediate the complex set of cellular functions modulated by phorbol esters in responsive cells warrants further investigation.

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