Tumor Promoter Phorbol 12-Myristate 13-Acetate Inhibits Mitogen-stimulated Na^+/H^+ Exchange in Human Epidermoid Carcinoma A431 Cells

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ABSTRACT Addition of polypeptide growth factors to cultured cells results in a rapid stimulation of Na^+/H^+ exchange, which leads to cytoplasmic alkalinization. We studied the effects of the potent tumor promoter phorbol 12-myristate 13-acetate (PMA) on the Na^+/H^+ exchange system of A431 cells. Stimulation of Na^+/H^+ exchange by epidermal growth factor (EGF) and serum as well as by vanadate ions is strongly inhibited after treatment of cells with nanomolar concentrations of PMA. Phorbol esters that have no activity as tumor promotors also do not modulate the activation of Na^+/H^+ exchange. By contrast, the stimulation of Na^+/H^+ exchange that is produced upon exposure of cells to hypertonic solution is only slightly inhibited by PMA treatment, indicating that PMA treatment does not directly block the activity of the Na^+/H^+ antiporter. Furthermore, incubation of cells with PMA causes a weak stimulation of Na^+/H^+ exchange, although this effect is mostly observed at relatively high PMA concentrations and appears to require external Ca^{2+}. The inhibition BY PMA of EGF-promoted Na^+/H^+ exchange is not due to inhibition of EGF-binding to the EGF receptor. Since PMA activates protein kinase C, our observations are consistent with the hypothesis that protein kinase C functions to attenuate the stimulation of Na^+/H^+ exchange by polypeptide growth factors.

Stimulation of an amiloride-sensitive Na^+ uptake is a ubiquitous consequence of the addition of polypeptide growth factors (mitogens) to cells. This increased Na^+ uptake is primarily due to activation of Na^+/H^+ exchange (1–7). Recent studies have demonstrated that stimulation of Na^+/H^+ exchange by mitogens leads to an increase in cytoplasmic pH (8–11). This cytoplasmic alkalinization is likely to have a permissive role in the mitogenic response of cells to growth factors (12).

Phorbol diester derivatives that are potent tumor promoters in the mouse skin model system have recently been found to exert a wide variety of biological responses in cells. Phorbol 12-myristate 13-acetate (PMA),[1] the most potent compound from this series, is mitogenic for a number of fibroblasts, acting synergistically with growth factors (see reference 13 and references therein), while in other cells PMA induces differentiation (for a recent review, see reference 14). Biochemical investigations suggest that the Ca^{2+} and phospholipid-dependent protein kinase (protein kinase C) is the major target for PMA action. PMA is a potent protein kinase C activator in vitro, apparently substituting for the natural activator diacylglycerol, which is produced upon hormonal stimulation of phosphatidylinositol turnover (reviewed in reference 15). Binding studies (16–18) suggest that protein kinase C is also the cellular phorbol diester "receptor." One of the substrates of activated protein kinase C is the epidermal growth factor (EGF) receptor. Activated protein kinase C catalyzes the phosphorylation of serine and threonine residues on the EGF receptor, resulting in a decrease in the tyrosine-specific protein kinase activity of this mitogen receptor (19, 20). These observations suggest a regulatory interrelationship between protein kinase C and growth factor receptors. That such regulation may indeed operate in cells is suggested by the findings that addition of EGF to A431 cells (21, 22) or platelet-derived growth factor to 3T3 cells (23) stimulates phosphatidylinositol turnover which is likely to result in protein kinase C activation.

In this study, we investigated the functional consequences of treatment of cells with PMA upon an early growth factor-mediated response, that is the stimulation of Na^+/H^+ exchange. We show that PMA treatment strongly inhibits this

[1] *Abbreviations used in this paper*: EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate.

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reaction and suggest that this PMA effect is due to the activation of protein kinase C.

MATERIALS AND METHODS

Phorbol derivatives were purchased from Sigma Chemical Co. (St. Louis, MO) and $^{22}Na^+$ was from New England Nuclear (Boston, MA). Sources of other reagent and cell culture methods have been described (10). Amiloride and 5-alkylamino substituted amiloride analogs were provided by Dr. E. J. Cragoe, Jr. (Merck Sharp and Dohme Research Laboratories). [^{125}I]EGF was prepared as described by Lieberman et al. (24).

Physiological Salt Solution (Solution A): 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Pi, 25 mM glucose, 25 mM morpholinopropane sulfonic acid-NaOH, pH 6.8 or 7.2.

Fluorimetric Measurements of Intracellular pH: A431 cells grown on glass slides that have just reached confluency were loaded with the fluorescent pH indicator 4',5'-dimethylfluorescein coupled to dextran as previously described (10), except that the concentration of sucrose in the hypertonic loading solution was increased to 1.0 M. This modification of the loading solution was found to increase the amount of dye in the cell cytoplasm three to five times, which allowed a reduction of the 4',5'-dimethylfluorescein-dextran concentration in the solution to 10%. For measurement of pHi, cells were incubated in solution A, pH 6.8, at 37°C in a thermostated fluorimeter as described previously (10). Calibration of pHi vs. fluorescence was obtained following the equilibration of pHi with extracellular pH in the presence of ouabain (10).

RESULTS

Effect of PMA on the Intracellular pH

Addition of PMA to A431 cells (Fig. 1) results in a small increase in pHi following a brief lag period. Relatively high PMA concentrations (~0.1 μM) are required to rapidly promote pHi elevation, while 10 nM PMA has little effect during the first 15 min (Fig. 1) or even after 1 h (ΔpHi ~0.05, data not shown). Similarly, Burns and Rozengurt (25) have shown that 100 nM phorbol 12,13-dibutyrate induces a pHi increase of 0.08 U in Swiss 3T3 cells. pHi elevation by PMA in A431

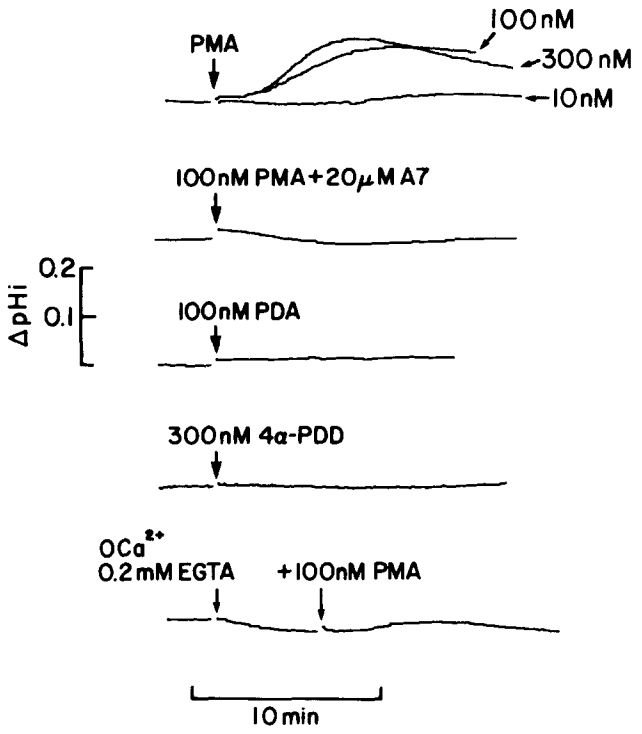

FIGURE 1 Effect of PMA on pHi in A431 cells. pHi was monitored as described in Materials and Methods. In the second experiment the cells were preincubated for 10 min with 20 μM 5-*N*(ethyl isopropyl)amiloride (A7) (27) before addition of PMA.

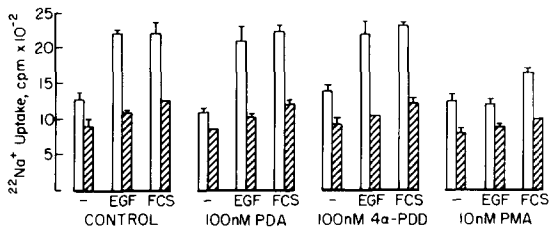

FIGURE 2 Effect of PMA treatment on mitogen-stimulated $^{22}Na^+$ uptake. Cells in 35-mm dishes (1.2 × 10^6 cells/dish) were preincubated for 1 h in solution A, pH 7.2 in the presence or absence of phorbol diesters. $^{22}Na^+$ uptake was subsequently assayed by switching the cells to 1 ml solution A, pH 7.2, containing 0.5 mM ouabain and 2.5 × 10^6 cpm/ml [$^{22}Na^+$] with or without EGF (300 ng/ml) or dialyzed FCS (5% vol/vol). Hatched bars indicate the addition of 50 μM 5-*N*,*N*-dimethylamiloride. Mitogens increase the rate of amiloride-sensitive Na^+ uptake, i.e., the difference in the rate shown by the open bars and cross-hatched bars. After a 12-min uptake period at 37°C cellular $^{22}Na^+$ content was assayed as described (7). The same results, as shown in this figure, have been obtained in at least three independent experiments. Note that while PMA blocks the mitogen-induced amiloride-sensitive Na^+ uptake, phorbol esters which are inactive as tumor promoters and do not bind to protein kinase C, i.e., 4α-phorbol 12,13-didecanoate and phorbol 12,13-diacetate, do not modulate the mitogen-induced Na^+ uptake.

cells depends on the presence of Na^+ in the incubation solution (not shown) and is eliminated in the presence of the potent Na^+/H^+ exchange inhibitor 5-*N*-(ethyl isopropyl)-amiloride (A7) (see references 12, 26, 27). These findings suggest that PMA promotes cytoplasmic alkalinization by weakly stimulating Na^+/H^+ exchange activity. Unlike PMA, the inactive phorbol analogues phorbol 12,13-diacetate and 4α-phorbol 12,13-didecanoate did not cause cytoplasmic alkalinization. Thus, the capacity of phorbol diesters to cause pHi elevation shows similar structural requirements as those previously reported for the activity of these compounds as tumor promoters (for a review, see reference 28) and as activators of protein kinase C (29).

Previous studies (7, 10) have shown that the stimulation of Na^+/H^+ exchange by mitogens is independent of external calcium. By contrast, pHi elevation by PMA is essentially abolished in Ca^{2+}-free solution (Fig. 1).

Effect of PMA on the Stimulation of Na^+/H^+ Exchange by Mitogens

We have reported previously that EGF and serum stimulate Na^+/H^+ exchange in A431 cells resulting in the stimulation of an amiloride-sensitive $^{22}Na^+$ uptake and pHi elevation (7, 10). In Fig. 2, we tested the effect of phorbol diesters on mitogen-stimulated $^{22}Na^+$ uptake. It can be seen that treatment of cells with 10 nM PMA strongly inhibits EGF and serum-stimulated $^{22}Na^+$ uptake while only slightly affecting the basal Na^+ uptake determined in the presence of 5-*N*,*N*-dimethylamiloride. The inactivate phorbol diesters phorbol 12,13-diacetate and 4α-phorbol 12,13-didecanoate at a concentration 10 times higher than that of PMA have no effect on $^{22}Na^+$ uptake either in the presence or absence of mitogens.

In agreement with the $^{22}Na^+$ uptake data, treatment of cells with 10 nM PMA for 1 h, strongly inhibits pHi elevation induced by EGF and serum (Fig. 3, *A* and *B*) while the inactive analogues phorbol 12,13-diacetate and 4α-phorbol 12,13-didecanoate have no effect (not shown). We previously found (30) that addition of vanadate to A431 cells also results

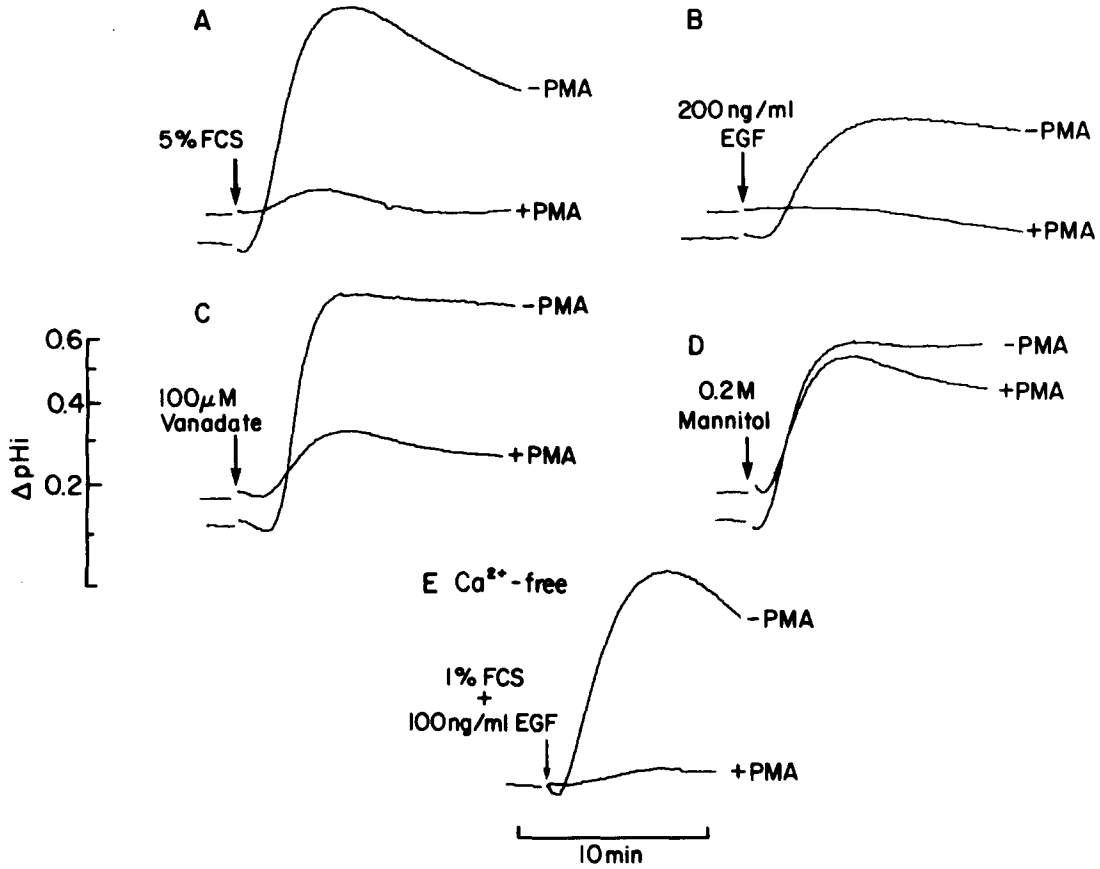

FIGURE 3 Effect of PMA treatment on pHi elevation by various activators of Na^+/H^+ exchange. (*A–D*) Cells were preincubated for 1 h with or without 10 nM PMA, followed by the addition of Na^+/H^+ exchange activators as indicated. Baselines indicate the fact that pHi increases by ~0.05 U during the PMA treatment (see Fig. 1). (*E*) Cells were preincubated for 15 min in Ca^{2+}-free solution A containing 0.2 mM EGTA in the presence or absence of 100 nM PMA before the addition of EGF and serum.

in a stimulation of Na^+/H^+ exchange and cytoplasmic alkalinization. As shown in Fig. 3*C*, pHi elevation by vanadate is inhibited by PMA treatment by ~75%. Na^+/H^+ exchange can also be activated in A431 cells by exposing the cells to hypertonic medium (D. Cassel and B. Whiteley, unpublished observations). The data in Fig. 3*D* show that the activation of Na^+/H^+ exchange upon exposure to hypertonic medium is only slightly inhibited by PMA. Activation of Na^+/H^+ exchange by loading cells with Na^+ (7, 10) is also not affected by PMA (data not shown). This indicates that treatment of cells with PMA does not impair the functionality of the Na^+/H^+ antiporter, but rather impairs the mechanism by which growth factors and vanadate activate this antiporter.

Since we found that PMA fails to elevate pHi in Ca^{2+}-free solution (Fig. 1), it was interesting to determine whether PMA treatment can inhibit mitogen-dependent pHi elevation under these conditions. The results (Fig. 3*E*) show that treatment of cells with PMA in Ca^{2+}-free solution results in an effective inhibition of mitogen-induced pHi elevation similar to that observed in normal physiological solution containing 1.8 mM Ca^{2+}. Note that the experiment in Fig. 3*E* was carried out with a rather high PMA concentration of 100 mM to minimize the time of exposure of cells to Ca^{2+}-free medium (the inverse relationship between PMA concentration and the time required for inhibition of Na^+/H^+ exchange is described below).

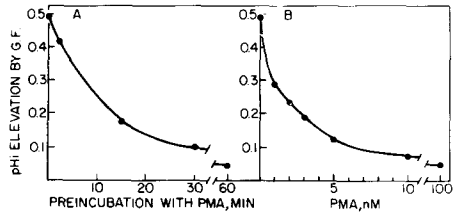

FIGURE 4 Kinetics of the inhibition by PMA of mitogen-promoted pHi elevation. (*A*) Time course. Following preincubation with 10 nM PMA for the indicated periods, EGF (100 ng/ml) + fetal calf serum (1% vol/vol) were added and the increase in pHi was determined. Zero time point indicates pHi elevation in control system that did not receive PMA. (*B*) Dose response. Cells were treated with the indicated PMA concentrations for 1 h. Subsequently, EGF (100 ng/ml) + fetal calf serum (1%) were added and the increase in pHi was determined.

Kinetics of PMA Effect on Mitogen-induced pHi Elevation

As shown in Fig. 4*A*, inhibition of mitogen-induced pHi elevation, assayed in this experiment using the potent combination of EGF and serum, depends on preincubation of the cells with PMA. While treatment of cells with 10 nM PMA for 2 min has little effect, treatment for longer periods leads to progressive decrease in mitogen-dependent pHi elevation.

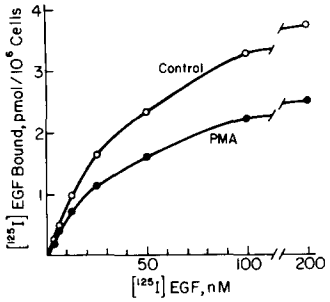

FIGURE 5 [^{125}I]EGF-binding in control and PMA-treated A431 cells. Cells in 35-mm dishes (1.2×10^6 cells/dish) were preincubated for 1 h at 37°C in solution A, pH 7.2, with or without 10 nM PMA. Subsequently, the medium was removed and replaced with 0.9 ml cold solution A, pH 7.2, containing 0.2 ng/ml bovine serum albumin and the indicated [^{125}I]EGF concentrations. After incubation at 0°C for 15 min, the cells were washed three times with cold buffer. [^{125}I]EGF was extracted with 0.3 M acetic acid and radioactivity was assayed in a γ counter. Nonspecific binding at the lower [^{125}I]EGF concentrations, determined by the addition of 250-fold excess of unlabeled EGF, was only 2–4% of the total binding and was not significantly affected by PMA treatment.

The time required for inhibition of the mitogen-induced pHi elevation decreases with increasing the PMA concentration (data not shown). For example, at 100 mM PMA, full inhibition is observed after 15 min incubation, while 1 h is required at 10 mM PMA, as shown in Fig. 4*A*. A dose response for the inhibition of mitogen-dependent pHi elevation upon treatment of cells with PMA for 1 h is shown in Fig. 4*B*. The effect of PMA is half maximal at 1–2 nM and becomes maximal at ~10 nM. Similar PMA concentrations were reported to be required for inhibition of [^{3}H]phorbol 12,13-dibutyrate binding to the phorbol diester receptor (16, 31) and for the activation of protein kinase C by PMA (29).

Effect of PMA on [^{125}I]EGF Binding

To investigate whether the inhibition by PMA of EGF-stimulated Na^+/H^+ exchange is due to a decrease in the number of EGF receptors in the cell membrane, we tested the effect of PMA treatment on [^{125}I]EGF binding to A431 cells. Cells were preincubated for 1 h at 37°C in the presence or absence of 10 nM PMA, and [^{125}I]EGF binding was subsequently assayed at 0°C to eliminate ligand internalization and degradation. As shown in Fig. 5, PMA treatment causes a uniform decrease in [^{125}I]EGF binding by ~35% over the entire range of [^{125}I]EGF concentrations tested (4–200 nM). A similar decrease in [^{125}I]EGF binding was also observed at still lower EGF concentrations of 10–1,000 pM (not shown). Thus, unlike in some other cells (32), PMA treatment of A431 cells appears to reduce the number of EGF receptors, rather than reduce receptor affinity. Smith et al. (22) have reported that PMA treatment has no effect on the binding of [^{125}I]EGF (1 ng/ml) at 37°C to A431 cells. We have confirmed these results but found that PMA treatment does inhibit [^{125}I]EGF binding at 37°C by 20–25% when higher [^{125}I]EGF concentrations (25–200 nM) are employed (data not shown). In any event, the observed decrease in the number of EGF receptors as measured either at 0°C or at 37°C cannot account for the complete inhibition by PMA of the stimulation of Na^+/H^+ exchange by EGF, suggesting that this inhibition is largely due to some additional PMA-induced modification of the EGF receptor or other cellular targets.

DISCUSSION

We show in this study that treatment of A431 cells with PMA inhibits the stimulation of Na^+/H^+ exchange by mitogens. The dose response for PMA (IC_{50} = 1–2 nM) and the specific structural requirements of phorbol esters mediating this effect are consistent with the hypothesis that PMA inhibits mitogen-promoted Na^+/H^+ exchange by activating the phospholipid and Ca^{2+}-dependent protein kinase (protein kinase C). Recent work has demonstrated that protein kinase C phosphorylates serine and threonine residues on the EGF receptor in A431 membrane preparations and that this modification results in a decrease in receptor autophosphorylation on tyrosine residues (19, 20). A similar modification of the EGF receptor was detected upon incubation of intact A431 cells with PMA, which also resulted in a decrease in EGF-promoted phosphorylation of tyrosine residues on cellular proteins (19). This suggests that the inhibition by PMA of mitogen-promoted Na^+/H^+ exchange could be due to a decrease in the protein kinase activity that is associated with growth factor receptors. Consistent with the hypothesis that phosphorylation may be involved in the activation of Na^+/H^+ exchange is the finding that vanadate, a known inhibitor of phosphotyrosine phosphatase in vitro (33), stimulates Na^+/H^+ exchange in A431 cells (30) and that this stimulation is inhibited upon PMA treatment (present results). On the other hand, PMA causes only a small inhibition of the stimulation of Na^+/H^+ exchange that is produced upon exposure of cells to hypertonic solutions. This suggests that the osmotic mechanism for the activation of the Na^+/H^+ antiporter bypasses certain stages in the pathway of the growth factor-mediated activation that are sensitive to PMA action.

Phorbol esters that can block the mitogen-dependent activation of Na^+/H^+ exchange can by themselves also induce a small elevation of pHi, apparently due to activation of Na^+/H^+ exchange. Phorbol esters that do not activate protein kinase C are inactive in either system. The two effects of phorbol esters differ, however, in that activation of Na^+/H^+ exchange by phorbol esters requires external Ca^{2+}, while the inhibition of the mitogen-induced activation of Na^+/H^+ exchange can take place in the absence of external Ca^{2+}. The difference in Ca^{2+} requirements implies that the two PMA effects are mediated at least in part by different pathways, even though both may require interaction of PMA with protein kinase C. Since the PMA-promoted elevation of pHi is small compared to that produced by mitogens, it seems unlikely that the mitogen-induced elevation of pHi occurs by a protein kinase C dependent pathway.

The control of many metabolic pathways is often affected by the interplay of positive and negative effectors. The results presented here, together with previous observations on tyrosine phosphorylation (19, 20), suggest that some of the mitogen-induced effects are attenuated by activation of protein kinase C. Several reports have documented activation of phosphatidylinositol turnover by mitogens (15, 21–23). This increased turnover is likely to result in an activation of protein kinase C, and thereby diminish the mitogen-induced activation of Na^+/H^+ exchange and possibly other mitogen-induced events.

In several cells, the long-term effects of PMA and mitogens are synergistic rather than antagonistic. For example, PMA and EGF synergistically inhibit the growth of A431 cells after several generations (22), whereas in 3T3 cells PMA and a variety of growth factors synergistically enhance cell growth (13). The implication of the apparent paradox, whereby PMA antagonizes some of the early events following mitogen ad-

dition to cells and yet acts synergistically with mitogens to affect cell growth, remains to be explored. We have recently shown (unpublished observations) that mitogen-induced changes in pHi human foreskin fibroblasts, i.e., normal cells, is also modulated by PMA in the same way as described here for A431 cells.

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