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TUMOR PROMOTER PHORBOL 12-MYRISTATE, 13-ACETATE INHIBITS PHOSPHOINOSITIDE HYDROLYSIS AND CYTOSOLIC Ca^{2+} RISE INDUCED BY THE ACTIVATION OF MUSCARINIC RECEPTORS IN PC12 CELLS

L.M.Vicentini[+], F.Di Virgilio', A.Ambrosini[+]", T.Pozzan' and J.Meldolesi[+]"

Dept. of Pharmacology[+], CNR Center of Cytopharmacology", University of Milan, and Dept. of General Pathology', University of Padova, Italy

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Preincubation of PC12 cells (used both before and after differentiation by NGF) with phorbol myristate acetate (PMA) was without effect on the basal concentration of inositol phosphates (metabolites of phosphoinositide hydrolysis) and of free cytosolic Ca^{2+}, but inhibited considerably the increases induced by the cholinergic agonist carbachol via the activation of the muscarinic receptor. Inasmuch as binding was unaffected, this inhibition might occur at the level of receptor coupling to its transduction mechanism(s). Inhibition appeared within 1 min and was maximal after 3 min. The concentrations of PMA needed (10^{-9}-10^{-8} M) were in the range believed to cause specifically the activation of protein kinase C. The muscarinic receptor, via the hydrolysis of phosphoinositides and the generation of diacylglycerol, participates in the regulation of the latter enzyme. Our data suggest therefore that the receptor operates under stringent feedback control by the metabolites generated as a consequence of its activation.

A great deal of interest has been recently focussed on the role of protein kinase C (PKC, also referred to as the phospholipid-dependent, Ca^{2+}-activated protein kinase) in cell regulation. Present evidence indicated that the enzyme (which is ubiquitous in eukaryotic cells and phosphorylates proteins in serine and threonine residues) is involved in both short-term and long-term processes, such as secretion, smooth muscle contraction and cell growth. Diacylglycerol, a lipid metabolite generated from the hydrolysis of phospholipids (in particular, of the fast turning over phosphoinositides, PI) appears to be responsible for

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+}; CCh, carbachol; DMSO, dimethyl-sulfoxide; KRH, modified Krebs-Ringer medium buffered with Hepes; αLTx, αlatrotoxin; PI, phosphoinositide; PMA, phorbol 12-myristate, 13-acetate; QNB, quinuclidinyl benzylate.

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the activation of PKC in intact cells. Phorbol 12-miristate, 13-acetate (PMA) as well as other tumor promoters,are stable analogs of diacylglycerol. The cellular effects of these molecules are now believed to be mediated by the persistent activation they induce of PKC (1, 2).

During the last several months a number of pharmacological and molecular studies have appeared showing that various receptors and post-receptors events are under the control of PKC activity. Receptors of growth factors (insulin; epidermal growth factor; somatomedin C) are PKC substrates (3-5). Their phosphorylation results in decreased affinity for the ligands, and inhibition of their endogenous tyrosine protein kinase activity (which might be important for transmembrane signalling at these receptors (4, 6, 7). β-adrenergic receptors are also phosphorylated by PKC, with inhibition of their transmembrane coupling to adenylate cyclase (8, 9). In some cultured cells (K562) treatment with PMA induces a large decrease in the number of surface exposed transferrin receptors, with a parallel enlargement of their cytoplasmic pool (10); in other cells (A431; cultured human foreskin fibroblasts) PMA induces blockade of postreceptor events induced by growth factors. The latter effects were observed also at low PMA concentrations, which marginally affected growth factor binding (11; Vicentini L.M. and Villereal M.L., ms. in preparation).

So far, little is known as to whether PKC affects receptors whose activation is transduced intracellularly by rise of free cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$ as well as by PI hydrolysis (2), events which cause the activation of the enzyme (1). The only existing report concerns the inhibition by PMA of glycogenolysis induced by α_1 adrenergic agonists in dissociated rat hepatocytes (12). Here we report that, in a line of neurosecretory cells, pretreatment with PMA inhibits PI hydrolysis and the rise in $[Ca^{2+}]_i$ induced by the cholinergic agonist carbachol (CCh) at the muscarinic receptor (rev. 13). In contrast, the binding properties of the receptor were unaffected by PMA.

METHODS

PC12 cells were cultured as monolayers, with and without nerve growth factor (NGF),as described elsewhere (13, 14). Shortly before the experiments they were detached from the monolayers and dissociated. Cells were used suspended in a modified Krebs Ringer medium (KRH) which contained

in mmol/l : NaCl, 125; $MgSO_4$ and KH_2PO_4: 1.2; KCl, 5; $CaCl_2$, 2; glucose, 6; Hepes-NaOH buffer, pH 7.4, 25. PMA (Sigma) was dissolved in DMSO and applied at the times and concentrations specified in table and figure legends. Controls received the solvent only (0.3-0.5%). CCh (Sigma) was used at 0.5 mM. PI hydrolysis was studied as described previously (14, 15) in cells labeled overnight with [^{3}H]-myo-inositol (Amersham Int.). $[Ca^{2+}]_i$ was measured by the quin2 technique (ref. 16). Binding of the muscarinic antagonist [^{3}H]-quinuclidinyl benzylate [^{3}H]-QNB, Amersham Int.) was measured as described in ref. 17 except that the incubation was for 10 min. Protein was assayed by the procedure of Lowry et al. (18).

RESULTS

Ten-15 day treatment with NGF (50 ng/ml) induces a neuronal like differentiation of PC12 cells (19) which includes a large increase in the number of muscarinic receptors, as measured by [^{3}H]-QNB binding (from 32 to 150 fmoles/mg protein, ref. 17), with no change in receptor affinity. PC12 cells both undifferentiated and differentiated by NGF ($PC12^-$ and $PC12^+$ cells, respectively) were used in the present studies.

Fig. 1 compares the binding of [^{3}H]-QNB to $PC12^+$ cells with and without pretreatment with 100 nM PMA. No difference in the affinity and number of the muscarinic binding sites was observed between the two preparations. In contrast, the post-receptor effects induced by the CCh activation of the receptor, PI hydrolysis and $[Ca^{2+}]_i$ rise (ref.

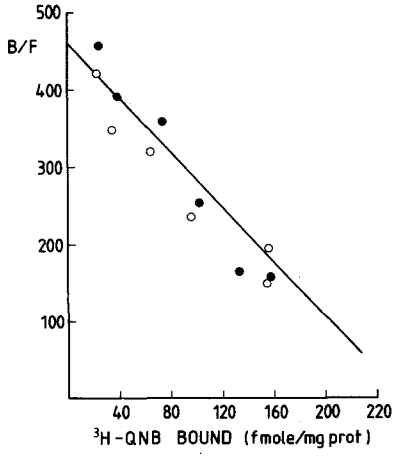

Fig. 1. Scatchard analysis of [^{3}H]-QNB binding. $PC12^+$ cells suspended in KRH at 37° were preincubated for 5 min with PMA (●, 100 nM) in DMSO (0.5%) or with the solvent only (○), and then incubated for 10 min with various concentrations of [^{3}H]-QNB. Data are from one single experiment which was repeated twice. The correlation coefficient was 0.96.

Table I
Effect of PMA on [^{3}H]-inositol phosphate accumulation induced by carbachol in PC12 cells

Additions	[^{3}H]-inositol phosphates (% controls)
- + -	100
- + CCh	185 ± 6
DMSO + CCh	179 ± 5
PMA + CCh	153 ± 5^{++}

PC12$^+$ cells were prelabelled with [^{3}H]-inositol as described in ref. 15. Cells suspensions in plain KRH containing 10 mM LiCl, or this KRH supplemented with either DMSO (0.5%) or PMA (100 nM) in DMSO were preincubated for 5 min and then incubated ± carbachol (CCh, 0.5 mM) for additional 5 min. [^{3}H]-inositol phosphates were extracted as described in ref. 15. Results are averages of 6 separate experiments, expressed as % of basal levels ± S.E.M. Average basal level (cpm/mg pro) was 3,500 ± 600. $^{++}$ indicates significance to the $p<0.01$ level.

13), were considerably inhibited by PMA treatment. Table I summarizes the data on PI hydrolysis in PC12$^+$ cells. In resting cells (no CCh treatment) neither the solvent nor PMA had detectable effect (not shown). In contrast, the CCh-induced PI hydrolytic response was affected. The slight change induced by the solvent remained below statistical significance, whereas PMA (100 nM, 5 min of preincubation) induced a statistically significant inhibition which approached 40% (Table I). Longer preincubations (up to 30 min) induced no further inhibition. The various phosphoinositols generated during a 20 sec exposure of PC12$^+$ cells to CCh were separated (13, 15). Accumulation of inositol trisphosphate was found to be profoundly inhibited (-55%), by PMA pretreatment, whereas inositol bis and monophosphate were inhibited to a lesser extent (data not shown).

Fig. 2 and 3 illustrate the effects of PMA on $[Ca^{2+}]_i$. The tumor promoter had no effect on the resting level, but inhibited substantially the Ca^{2+} rises induced by CCh. Data on PC12$^+$ cells are shown in Fig. 2. When PMA and CCh were applied together, the $[Ca^{2+}]_i$ rise was as large as with CCh alone. A 1 min preincubation with PMA, however, induced a large inhibition (-50%) of the rise , which was further inhibited (-75%)with longer (3-10 min) preincubations. Large inhibitions were observed also in PC12$^-$ cells. In these preparations concentrations of

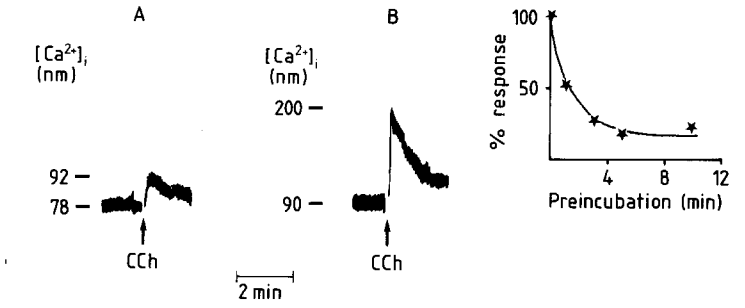

Fig. 2. Effect of the preincubation with PMA on $[Ca^{2+}]_i$ rise induced by carbachol in PC12$^+$ cells.
Suspensions of PC12$^+$ cells were loaded with quin2 (0.34 nmoles/10^6 cells), washed, resuspended in KRH (1.2 x 10^6 cells/ml) and preincubated for 5 min with either PMA (100 nM) in DMSO (0.3%) (A), or with the solvent only (B). Additions of CCh (0.5 mM) are marked by arrows. Calibration of $[Ca^{2+}]_i$ (ref. 16) is indicated to the left of each trace. The inset shows the effect of the length of preincubation with PMA (100 nM) on the maximal rise of $[Ca^{2+}]_i$-induced by CCh (0.5 mM).

PMA in the 10^{-9} range (5 min preincubation) produced already appreciable effects, with maxima (-60%) occurring at 10^{-8}M and above (Fig. 3).

As will be discussed in detail elsewhere, the rise of $[Ca^{2+}]_i$ induced by CCh activation of muscarinic receptors in PC12 cells is the result of two processes: increase influx, due to the opening of a receptor operated channel at the plasmalemma, and redistribution from non mitochondrial particulate store(s) to the cytosol. In order to establish whether the inhibitory effect of PMA was restricted to either one of these processes, suspensions of PC12 cells in a Ca^{2+}-free KR medium

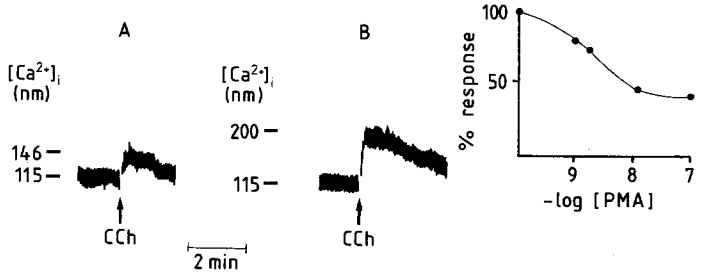

Fig. 3. Effect of the preincubation with PMA on $[Ca^{2+}]_i$ rises induced by carbachol in PC12$^-$ cells.
A and B: Experimental conditions were as in Fig. 2, but cells were PC12$^-$, concentration of quin2 was 0.72 nmoles/10^6 cells, and cell concentration 10^6/ml. Inset: concentration dependence of the inhibitory effect of PMA on the CCh (0.5 mM)-induced maximal $[Ca^{2+}]_i$ rise. Preincubation: 5 min.

containing 1 mM EGTA were investigated. The CCh-induced $[Ca^{2+}]_i$ rise of these cells (due to redistribution only) was inhibited by PMA approximately to the same extent as that observed in the complete medium (increased influx + redistribution)(not shown in figures). We conclude therefore that both these processes are inhibited by PMA.

PI hydrolysis and $[Ca^{2+}]_i$ rise are induced by the activation of not only the muscarinic, but of other receptors as well (2). In PC12 cells these effects are induced by the application of α-latrotoxin (αLTx), a neurotoxin contained in the venom of the black widow spider (15, 16). Contrarywise to the results obtained with CCh, the effects of αLTx were unmodified by pretreatment of $PC12^+$ cells with PMA (100 nM, 5 min, not shown).

DISCUSSION

The inhibition by PMA of PI hydrolysis and $[Ca^{2+}]_i$ rises induced in PC12 cells by CCh activation of the muscarinic receptor occurred at the low concentrations of the phorbol diester which are thought to be specific for the activation of PKC (1), and required 3 min of preincubation to become maximal. These data suggest that phosphorylations by PKC might mediate these effects of PMA. Information on the muscarinic receptor at the molecular level is still very limited, and, to our knowledge, no data have been reported yet as to its regulation by metabolic processes. In our studies no direct attempts have been made to establish whether the effects of PMA occur at the receptor, or at the level of the post receptor processes that we have investigated. A direct effect at the receptor level is however suggested by the following: a) inhibition concerned PI hydrolysis as well as the two components of the Ca^{2+} rise: not only Ca^{2+} redistribution (which may be mediated by PI hydrolysis through the generation of inositol trisphosphate, ref. 2) but also Ca^{2+} influx, which is probably independent of the hydrolytic reaction; and b) the effects of α-LTx, which induce PI hydrolysis and $[Ca^{2+}]_i$ rise through the activation of a receptor different from the muscarinic receptor (15, 16) were not affected by PMA.

Inasmuch as activation of the muscarinic receptor causes PI hydrolysis and generation of diacylglycerol, it is expected to cause activation of PKC in PC12 cells. Our present results, showing that PMA, an exogenous

activator of PKC, causes inhibition of the events triggered by muscarinic receptor activation suggest therefore that the receptor operates under stringent feedback regulation by the metabolites generated as a consequence of its activation. Such a regulation, which seems finalistically oriented to preclude persistent activation of PKC - a potentially dangerous event for the cell (1) - could occur not only at the muscarinic, but also at α_1 adrenergic (12) and,possibly, at other receptors which share the transduction mechanism(s) with the muscarinic and α_1 receptors (2). Another group of receptors which cause activation of PKC, and which are inhibited by PMA and other allosteric activators of the enzyme, are the receptors for growth factors (4, 5, 11, 20). Thus, various receptors, postreceptor events and PKC might be contained within a finely tuned regulatory network, with feedback and feedforward functions in the control of cell activity. Although the experimental evidence supporting this conclusion is at the moment still incomplete, its physiological importance in cell regulation might eventually turn out to be considerable.

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