Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 261, No. 1, Issue of January 5, pp. 32–35, 1986
© 1986 by The American Society of Biological Chemists, Inc.
Printed in U.S.A.

Tumor Promoter Phorbol Myristate Acetate Inhibits Ca^{2+} Influx Through Voltage-gated Ca^{2+} Channels in Two Secretory Cell Lines, PC12 and RINm5F*

(Received for publication, July 18, 1985)

Francesco Di Virgilio‡, Tullio Pozzan‡§¶, Claes B. Wollheim‖, Lucia M. Vicentini‡‡, and Jacopo Meldolesi§‡‡**

From the ‡Institute of General Pathology and Consiglio Nazionale delle Ricerche Center for the Study of the Physiology of Mitochondria, via Loredan 16, 35131 Padova, University of Padova, Italy, the ‖Institute of Clinical Biochemistry, University Medical Center, 1211 Geneva 4, University of Geneva, Switzerland, and the ‡‡Department of Medical Pharmacology and Consiglio Nazionale delle Ricerche Center of Cytopharmacology, via Vanvitelli 32, 20129 Milano, University of Milano, Italy

Protein kinase C is known to be involved both in initiation and termination of cellular responses due to phosphoinositide breakdown. Here we report that in PC12 cells (a line of neurosecretory cells derived from a rat pheochromocytoma), pretreatment with nanomolar concentrations of phorbol myristate acetate, PMA, which is believed to specifically activate protein kinase C, inhibits the cytosolic-free Ca^{2+} concentration rise induced by depolarizing agents. In contrast, plasma membrane potential and ^{45}Ca efflux from preloaded cells were unaffected by PMA pretreatment. Inhibition by PMA and diacylglycerol of the cytosolic-free Ca^{2+} concentration rise induced by depolarization was observed also in another cell line, the insulin secreting line RINm5F. These results raise the possibility that the voltage-gated Ca^{2+} channel is under inhibitory control by protein kinase C.

Changes in cytosolic-free Ca^{2+} concentration, $[Ca^{2+}]_i$,[1] can be elicited physiologically either by mobilizing Ca^{2+} from intracellular stores and/or by increasing the Ca^{2+} permeability of the plasma membrane. The best known example of the latter mechanism is the opening of voltage-gated Ca^{2+} channels in excitable tissues. The opening probability of these channels is increased by cyclic AMP-dependent phosphorylation, studied in detail in the heart (1–4). Little is known, however, about the effect of other second messengers on the function of these channels. The mechanism of Ca^{2+} mobilization, in nonmuscle cells, on the other hand, has been clarified only recently (5, 6) and the understanding of the mode of action of Ca^{2+}-mobilizing agonists has been one of the major recent breakthroughs in cell biology. Following the hydrolysis of phosphatidylinositolbisphosphate by a receptor-modulated phospholipase C, two second messengers are generated: (*a*) inositoltrisphosphate, which releases Ca^{2+} from endoplasmic reticulum (5, 6) and (*b*) diacylglycerol which activates the ubiquitous phospholipid-dependent, Ca^{2+} activated, enzyme protein kinase C (7). The action of endogenously produced diacylglycerol can be mimicked by active tumor promoters, the most widely used being PMA. Initially attention has been focused on the synergistic interaction between Ca^{2+} and protein kinase C (6–9), while recently a role of protein kinase C as a negative feedback regulator of cell functions has also been proposed (10–15). However, so far, most reports have emphasized the negative feedback regulation by hyperstimulation of protein kinase C of phosphoinositide breakdown, and of related processes. An unexpected negative modulation by PMA of voltage-gated Ca^{2+} channel function forms the basis of this report.

MATERIALS AND METHODS

Preparation of PC12 and of RINm5F cell suspensions, quin2 loading, and calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ were carried out as described in Refs. 16 and 17. Before each experiment, unless otherwise specified, an aliquot of the cell suspension was centrifuged (100 × *g*) for 5 min and resuspended in a modified Krebs-Ringer bicarbonate medium containing: 125 mmol/liter NaCl, 5 mmol/liter KCl, 1.2 mmol/liter $MgSO_4$ and KH_2PO_4, 1 mmol/liter $CaCl_2$, 6 mmol/liter glucose, 25 mmol/liter Hepes/NaOH buffer, pH 7.4.

^{45}Ca experiments were performed essentially as described previously for synaptosomes (18). Briefly, for the influx experiments, cells were incubated with ^{45}Ca (5 μCi/ml), treated with the stimulants, and at the indicated time points, aliquots of the cell suspension were withdrawn and rapidly mixed with EGTA and ruthenium red (500 and 5 μM final free concentration, respectively) to remove superficially absorbed Ca^{2+}. Within 5–10 s the samples were then centrifuged through layers of silicon oil to effectively separate cells from the medium. The supernatant and the oil were carefully removed and the radioactivity in the pellet measured by liquid scintillation counting. The contribution of contaminating extracellular ^{45}Ca trapped in the pellet was substracted by including contemporarily [^{3}H]sucrose as a marker of extracellular space. Efflux of ^{45}Ca was performed essentially as described above, but the cells were preloaded for 30 min with 10 μCi/ml ^{45}Ca, and then washed in ^{45}Ca-free medium, before initiating the experiments. All experiments were performed at 37 °C. PMA was purchased from Sigma, quin2/AM from Calbiochem-Behring, and all other compounds were of analytical grade.

RESULTS AND DISCUSSION

Figs. 1 and 2 summarize results on $[Ca^{2+}]_i$ and membrane potential in PC12 cells. In agreement with our previous results (16) we found that raising the concentration of KCl in the incubation medium induces an immediate, marked rise of intracellular quin2 fluorescence, reflecting an increase of $[Ca^{2+}]_i$. Thereafter, the fluorescent signal remained elevated for several minutes at a slowly declining plateau level (Fig. 1*A*). The size of the measured $[Ca^{2+}]_i$ rise depended on the concentration of KCl. A clear effect was measured at 18 mM, while maximum (3-fold the resting level) was reached above

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by grants from the Italian National Research Council, Special projects "Oncology."

¶ Also supported by a grant from the ZYMA Foundation (Nyon, Switzerland) during part of this work.

** Supported by Grant 3.246:0.82 from the Swiss National Science Foundation.

[1] The abbreviations used are: $[Ca^{2+}]_i$, cytosolic-free Ca^{2+} concentration; PMA, phorbol myristate acetate; Hepes; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

This is an Open Access article under the CC BY license.

50 mM (Fig. 2*A*). The rise of $[Ca^{2+}]_i$ is due to opening of voltage-gated Ca^{2+} channels, because (*a*) it depends on the presence of Ca^{2+} in the extracellular medium (16); (*b*) it is inhibited by verapamil, D600, and nifedipine at concentrations specific for blocking voltage-gated Ca^{2+} channels (16, 19); (*c*) it can be elicited by other depolarizing agents (20).

When cells were treated with PMA, the resting $[Ca^{2+}]_i$, was practically unchanged. However, the K^+ induced $[Ca^{2+}]_i$ rise, although still visible, was greatly attenuated by PMA pretreatment at all KCl concentrations (Figs. 1*B* and 2*A*). Moreover PMA added to cells already depolarized by K^+ induced within 1–2 min a decline of $[Ca^{2+}]_i$ approximately to the levels reached in cells depolarized after PMA pretreatment (Fig. 1*A*). When cells were depolarized by high K^+ in Ca^{2+}-free medium, $[Ca^{2+}]_i$ only rose upon readdition of Ca^{2+} to the medium. Even in this latter case the $[Ca^{2+}]_i$ rise was greatly reduced by PMA, added to the cells together with K^+ (Fig. 1, *C* and *D*).

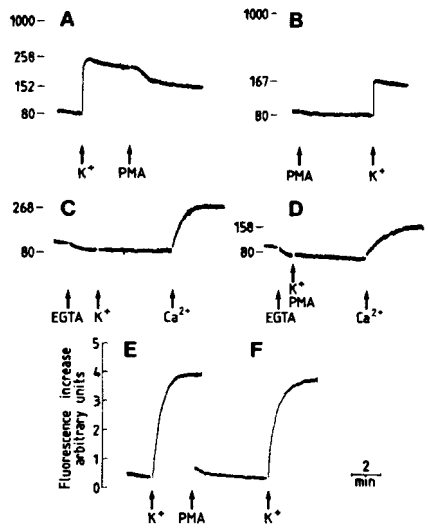

FIG. 1. **Effect of K^+ and PMA on $[Ca^{2+}]_i$ (*panels A–D*) and membrane potential (*panels E and F*) in PC12 cells.** In *panels A–D* the number of cells was 6×10^5/ml and the intracellular quin2 concentration 1 nmol/10^6 cells. Where indicated KCl and PMA were added to the final concentration of 50 mM and 30 nM, respectively; EGTA and Ca^{2+} to the final free concentration of 1 and 1.5 mM, respectively. Cells not treated with PMA received the solvent alone (0.1% dimethyl sulfoxide). The *number* on the *left* of each trace represents the $[Ca^{2+}]_i$ calibration scale (nM). In *panels E* and *F* the number of cells was 10^5/ml and bisoxonol was 100 nM. An increase of fluorescence indicates depolarization (16, 17). Additions were as *panels A–D*. The exitation and emission wavelengths for bisoxonol were 540 and 580 nm, respectively. These and the following experiments are representative examples of experiments performed with very similar results in at least three different batches of cells.

The PMA inhibition of the K^+-induced $[Ca^{2+}]_i$ rise was concentration dependent. A clear effect was observed already with 2 nM PMA, and a large inhibition (60–70%) with 30 nM or more (Fig. 2*B*). As far as the time dependence of the inhibition, a considerable effect was observed already after 1 min of preincubation, and an almost maximal effect after 2 min (Fig. 2*C*). In a parallel series of experiments we investigated the effect of PMA pretreatment on $[Ca^{2+}]_i$ rises induced by the application of another depolarizing agent, the pore forming ionophore gramicidin. Gramicidin causes depolarization by equilibrating Na^+ and K^+ gradients across the plasma membrane. In this case too, a substantial inhibition by PMA was observed (not shown).

The inhibition by PMA pretreatment of the $[Ca^{2+}]_i$ rise induced by depolarizing agents could be due to different processes. An effect of PMA on plasma membrane potential was excluded by the experiments illustrated in Fig. 1, *E* and *F*. In these experiments the plasma membrane potential was measured with the fluorescent probe bisoxonol (16, 17). PMA was without effect on both the resting membrane potential of PC12 cells, and on the depolarization induced by high K^+. Likewise, the effect of PMA cannot be due to stimulation of Ca^{2+} efflux, because cells preloaded with ^{45}Ca for 30 min, and then incubated with or without PMA (30 nM), released the tracer at the same rate (Fig. 3*B*). This result is consistent with our previous finding that PMA is without effect on $[Ca^{2+}]_i$ rises induced by Ca^{2+} ionophores, such as ionomycin (16). In contrast, the depolarization-induced increase of ^{45}Ca influx was severely curtailed by PMA. As can be seen in Fig. 3*A*, a 6-min preincubation with PMA was without effect on the basal ^{45}Ca accumulation, but blocked almost completely the influx stimulated by 50 mM KCl.

Additional experiments were carried out on RINm5F cells, a rat insulin-secreting cell line that also possesses voltage-gated Ca^{2+} channels (17). The quin2 results obtained with this line (Fig. 4) were almost identical to those obtained with PC12 cells. Thus, PMA pretreatment of the RINm5F cells had no effect on the resting $[Ca^{2+}]_i$, but greatly reduced the rise induced by K^+ depolarization (Fig. 4, *A* and *B*). Moreover

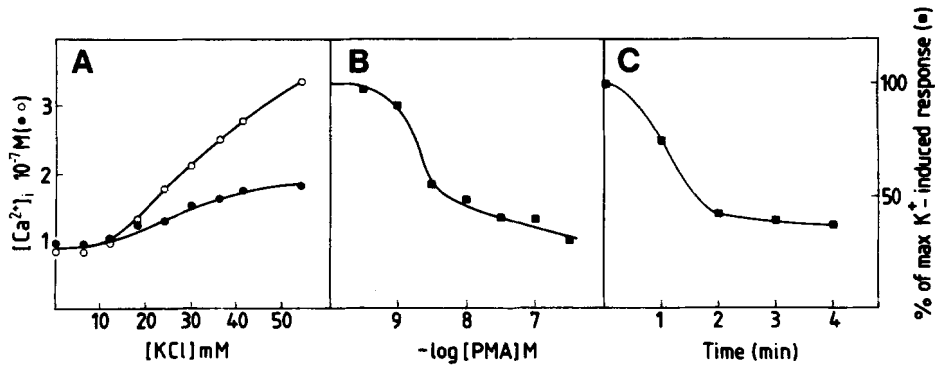

FIG. 2. **Inhibition by PMA of the high K^+-induced $[Ca^{2+}]_i$ rise in PC12 cells.** Dependence on the concentration of K^+ (*A*), of PMA (*B*), and on the length of PMA pretreatment (*C*). General experimental conditions were as described in the legend to Fig. 1, but the number of cells was 10^6/ml and the intracellular quin2 concentration 0.75 nmol/10^6 cells. Values plotted refer to the peaks of $[Ca^{2+}]_i$ observed after the various treatments. In *panels A* and *C* the concentration of PMA was 30 nM, while the concentration of the solvent (dimethyl sulfoxide) was always 0.1%. In *panels A* and *B* the time of PMA preincubation was 5 min. In *panels B* and *C* the concentration of KCl was 30 mM. ○, controls; ●, +PMA.

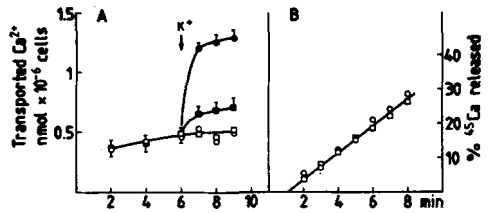

FIG. 3. **Effect of K^+ and PMA on ^{45}Ca transport in PC12 cells.** Influx of ^{45}Ca (*A*) was measured in cell suspensions depolarized with 50 mM KCl during incubation with the tracer. ^{45}Ca efflux (*B*) was measured in cells preloaded with ^{45}Ca for 30 min and then washed (see above). In either case PMA (30 nM, ■ and □) or solvent (0.1% dimethyl sulfoxide, ● and ○) were added at zero time. *Solid symbols*: K^+-treated samples. The results shown are averages of two–six experiments ± S.E. The number of cells ranged between 2.5 and 3.5 × 10^6/ml. In the efflux experiment, 100% radioactivity was between 8.5 and 10 × 10^4 dpm.

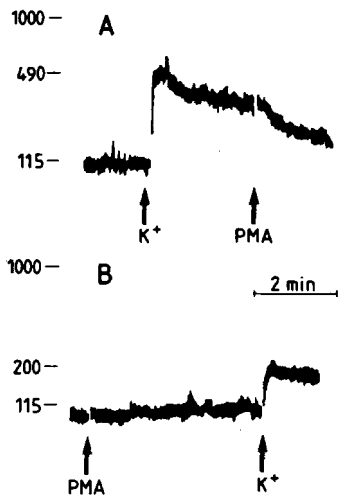

FIG. 4. **Effect of K^+ and PMA on $[Ca^{2+}]_i$ in RINm5F cells.** Cell number was 1.25 × 10^6/ml and intracellular concentration of quin2 was 0.8 nmol/10^6 cells. Experimental conditions and presentation of the data were as described in the legend to Fig. 1, except that the concentration of PMA was 50 nM.

PMA added after K^+ accelerated the return of $[Ca^{2+}]_i$ towards basal (Fig. 4*A*). 50 μM 1-oleoyl-2-acetylglycerol, a membrane permeant diacylglycerol that is also able to activate protein kinase C (7), inhibited the K^+-induced $[Ca^{2+}]_i$ rise in RINm5F cells, although to a smaller extent compared to PMA (not shown).

Taken together, the results in PC12 and RINm5F cells that we have reported strongly suggest that the effect of PMA on Ca^{2+} homeotasis is due to inhibition of the voltage-gated Ca^{2+} channel. The effective concentrations of the phorbol diester were very low, in the range that is considered specific for the activation of protein kinase C (7). Moreover the inhibition developed rapidly, but was not immediate, as it is to be expected for a process mediated by a metabolic event, such as phosphorylation by protein kinase C. Recently, De Riemer *et al.* (21) have reported electrophysiological evidence suggesting that protein kinase C phosphorylation(s) increase voltage-dependent Ca^{2+} currents in *Aplysia* bag cells. Species and cell differences might account for the discrepancy between our data and those of DeRiemer *et al.* (21). Moreover the effects observed by De Riemer *et al.* (21) took several minutes (over 15) to appear and were not dependent on the concentration of PMA in the range tested, suggesting the possibility that the effects on Ca^{2+} currents were not directly triggered by protein kinase C activation.

Previous studies carried out primarily on heart cells showed that the activity of the voltage-gated Ca^{2+} channel can be modulated by cAMP-dependent phosphorylation(s), causing an increased probability of channel opening during depolarization (1–4). In neurons, on the other hand, inhibitory modulation of the channel has been reported (22, 23). The intracellular mechanism(s) responsible for these latter effects has not been identified yet. The present results suggest that an inhibitory regulation of the Ca^{2+} channel might be triggered by the activation of those receptors which are linked to the hydrolysis of polyphosphoinositides. Diacylglycerol, one of the metabolites generated by this reaction, is in fact believed to be the physiological activator of protein kinase C (6, 7). Indeed results have been recently reported in some cell system showing decreased Ca^{2+} conductance upon activation of this class of receptors (24, 25).

In the last few months it has become increasingly clear that protein kinase C plays a primary role not only in the activation of cell functions (6, 7) but also in the feedback inhibitory mechanisms (10–15). So far stimulation by phorbol esters of protein kinase C has been shown to inhibit responses due to receptors linked to polyphosphoinositide hydrolysis, *i.e.* those which lead to protein kinase C activation. Our data suggest that protein kinase C has an even more complex modulatory effect in Ca^{2+}-activated processes. For example our results might offer a simple explanation to some contradictory observations obtained in bovine adrenal medulla cells. These cells possess both muscarinic and nicotinic acetylcholine receptors, which cause polyphosphoinositide breakdown and membrane potential depolarization, respectively. In permeabilized bovine adrenal medulla cells, PMA is synergistic with Ca^{2+} in stimulating secretion (26), while in intact cells, muscarinic stimulation (which generates diacylglycerol and thus should activate protein kinase C) inhibits neurotransmitter release (27, 28). Since the $[Ca^{2+}]_i$ rises triggered by acetylcholine in these cells are almost exclusively due to depolarization-induced voltage-gated Ca^{2+} channel opening (29), on the basis of the present findings we would suggest that the concomitant activation of muscarinic receptors should result in a net inhibition of the amplitude and/or duration of $[Ca^{2+}]_i$ transients. A similar interpretation can be offered also to a recent observation by Drummond (30) (published while this manuscript was in preparation). In GH3 cells, thyrotropin-releasing hormone, an agonist acting via polyphosphoinositide breakdown, accelerates the decay of $[Ca^{2+}]_i$ rise induced by K^+ depolarization. Thus, it can be speculated that, depending on the cell type and the intensity of protein kinase C activation, polyphosphoinositide breakdown products will cause either stimulation or inhibition of cell functions.

Acknowledgment—We are indebted to Dr. F. Wanke for stimulating discussions and suggestions

REFERENCES

1. Reuter, H. (1983) *Nature* **301,** 569–576
2. Siegelbaum, S. A., and Tsien, R. W. (1983) *Trends Neurosci.* **6,** 307–312
3. Kostyuk, P. G. (1984) *Neurosci. Res.* **13,** 983–989
4. Osterrieder, J. W., Brum, G., Heschler, J., Trautwein, W., Flokenzi, V., and Hofmann, F. (1982) *Nature* **308,** 693–698
5. Berridge, M. J., and Irvine, R. (1984) *Nature* **312,** 315–321
6. Williamson, J. R., Cooper, R. H., Joseph, S. K., and Thomas, A. P. (1985) *Am. J. Physiol.* **17,** C203–C216
7. Nishizuka, Y. (1984) *Nature* **308,** 693–698
8. Rink, T. J., Sanchez, A., and Hallam, T. J. (1983) *Nature* **805,** 317–319
9. Di Virgilio, F., Lew, D. P., and Pozzan, T. (1984) *Nature* **310,** 691–693

10. Drummond, A. H., and Mac Intire D. E. (1985) *Trends Pharmacol. Sci.* 233–234
11. Vicentini, L. M., Di Virgilio, F., Ambrosini, A., Pozzan, T., and Meldolesi, J. (1985) *Biochem. Biophys. Res. Commun.* **127,** 310–317
12. Sagi-Eisenberg, R., Lieman, H., and Pecht, I. (1985) *Nature* **8,** 59–60
13. Watson, S. P., and Lapetina, E. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 2623–2626
14. Orellana, S. A., Salski, P. A., and Brown, J. H. (1985) *J. Biol. Chem.* **260,** 5326–5329
15. Cooper, R. H., Coll, K. E., and Williamson, J. R. (1985) *J. Biol. Chem.* **260,** 3281–3288
16. Meldolesi, J., Huttner, W. B., Tsien, R. Y., and Pozzan, I. (1984) *Proc. Natl. Sci. U. S. A.* **81,** 620–624
17. Wollheim, C. B., and Pozzan, T. (1984) *J. Biol. Chem.* **259,** 2262–2267
18. Nicholls, D. G., Rugolo, M., Scott, I. G., and Meldolesi, J. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7924–7928
19. Toll, L. J. (1982) *J. Biol. Chem.* **257,** 13189–13192
20. Pozzan, T., Gatti, G., Dozio, N., Vicentini, L. M., and Meldolesi J. (1984) *J. Cell Biol.* **99,** 628–638
21. De Riemer, S. A., Strong, J. A., Albert, K. A., Greengard, P., and Katzmark, L. K. (1985) *Nature* **313,** 313–316
22. Dunlap, K., and Fishbach, G. D. (1981) *J. Physiol. (Lond.)* **317,** 519–535
23. Galvan, M., and Adams, P. (1982) *Brain Res.* **244,** 135–141
24. Belluzzi, O., Sacchi, O., and Wanke, E. (1985) *J. Physiol. (Lond.)* **358,** 109–129
25. Barker, J. L. (1985) *J. Neurochem.* **44,** 55c
26. Knight, D. E., and Baker, P. F. (1983) *FEBS Lett.* **160,** 98–100
27. Derome, G., Tzeng, R., Mercier, P., Lemaire, I., and Lemaire, S. (1981) *Biochem. Pharmacol.* **30,** 855–860
28. Swilem, A. F., Hawthorne, J. N., and Azila, N. (1983) *Biochem. Pharmacol.* **32,** 3873–3874
29. Kao, L., and Schneider, A. S. (1985) *J. Biol. Chem.* **260,** 2019–2022
30. Drummond, A. H. (1985) *Nature* **315,** 752–755