Vol. 165, No. 1, 1989
November 30, 1989

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 319-326

# LONG-TERM PHORBOL ESTER TREATMENT DISSOCIATES PHOSPHOLIPASE D ACTIVATION FROM PHOSPHOINOSITIDE HYDROLYSIS AND PROSTACYCLIN SYNTHESIS IN ENDOTHELIAL CELLS STIMULATED WITH BRADYKININ

Thomas W. Martin, Dorothy R. Feldman, Keith E. Goldstein, and John R. Wagner

Department of Pathology
St. Louis University School of Medicine
St. Louis, Missouri 63104

Received August 18, 1989

SUMMARY: Bovine pulmonary artery endothelial cells (BPAEC) were prelabeled with [$^3$H]choline or [$^3$H]myristic acid to selectively label endogenous phosphatidylcholine. BPAEC were stimulated with ATP and bradykinin (BK), and phospholipase D (PLD) activation was detected as a 4-fold increase in [$^3$H]choline in cells prelabeled with [$^3$H]choline or as a 2- to 3-fold increase in [$^3$H]phosphatidylethanol in cells prelabeled with [$^3$H]myristic acid and stimulated in the presence of ethanol. Pretreatment of BPAEC with 0.1 $\mu$M phorbol 12-myristate 13-acetate (PMA) for 22 hr completely inhibited agonist-induced PLD activation, whereas prostacyclin synthesis and [$^3$H]phosphoinositide ([$^3$H]PIns) hydrolysis were enhanced in pretreated cells. Long-term PMA treatment thus dissociates agonist-induced PLD activation from [$^3$H]PIns hydrolysis, and agonist-induced prostacyclin synthesis is not dependent upon PLD activation. 

Prostacyclin synthesis in endothelial cells stimulated with $Ca^{2+}$-mobilizing agonists such as BK (1,2) and ATP (3,4) is associated with the rapid hydrolysis of PIns. BK (5) and ATP (6) also stimulate the rapid hydrolysis of PC by a mechanism that involves activation of PLD (6). PLD-catalyzed PC breakdown stimulated by agents that act independently of cell surface receptors is attenuated by PKC inhibition (7,8). We now report that receptor-dependent PLD activation by $Ca^{2+}$-mobilizing agonists is completely inhibited after prolonged exposure of BPAEC to phorbol esters under conditions known to down-regulate PKC. In contrast, agonist-induced PIns hydrolysis and prostacyclin synthesis are enhanced in pretreated cells. These findings demonstrate differential regulation of receptor-coupled activation of PLD and PIns-specific PLC and indicate that the PLD pathway of PC hydrolysis is not required for agonist-induced prostacyclin synthesis.

Abbreviations: BK, bradykinin; BPAEC, bovine pulmonary artery endothelial cells; DMSO, dimethyl sulfoxide; HBSS, Hepes-buffered salt solution, 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; $IP_1$, inositol monophosphate; $IP_2$, inositol bisphosphate; $IP_3$, inositol trisphosphate; PC, phosphatidylcholine; PDD, phorbol 12,13 didecanoate; PEt, phosphatidylethanol; PIns, phosphoinositide; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate.

0006-291X/89 $1.50

## MATERIAL AND METHODS

Material. BK, ATP, tissue culture media, fetal calf serum, 6-keto-$PGF_{1\alpha}$, PMA, and other phorbol derivatives were purchased from Sigma Chem. Co. Bio-Rex 70 and AG1-X8 ion exchange resins were obtained from Bio-Rad. Silica gel plates (LK6D) were supplied by Whatman. Antiserum to 6-keto-$PGF_{1\alpha}$ was obtained from Advanced Magnetics, Inc. [9,10-(n)-$^3$H]Myristic acid (40-60 Ci/mmole), [methyl-$^3$H]choline chloride (76 Ci/mmole), and myo-[2-$^3$H]inositol (22.8 Ci/mmole) were from Amersham. Aquasol-2 scintillation fluid and 6-[5,8,9,11,12,14,15-$^3$H(n)]-$PGF_{1\alpha}$ (157 Ci/mmole) were from DuPont-New England Nuclear.

Methods

Cell culture and phorbol ester pretreatment. BPAEC were obtained from American Type Culture Collection at passage 17. The cells were grown in T-25 flasks in Eagle's MEM with Earle's salts, nonessential amino acids, 2 mM glutamine, 50 units/ml penicillin, 50 $\mu$g/ml streptomycin, and 10% fetal calf serum (5,6). For individual experiments, the cells were split 1:8 into gelatin-coated 35 mm dishes on day 0 and grown to confluence in the presence or absence of radiolabeled precursors (see below). Phorbol derivatives were added to the cell cultures from stock solutions in DMSO on day 3. Control cultures received an equivalent volume of DMSO (0.2%, v/v final concentration). Phospholipid metabolism and prostacyclin synthesis were assayed in duplicate confluent monolayers on day 4.

Assay of PLD-catalyzed PC hydrolysis. Agonist-stimulated PLD activity was measured in cells prelabeled with [$^3$H]choline or [$^3$H]myristic acid using slight modifications of previously published methods (6). Cells were labeled with [$^3$H]choline (5 $\mu$Ci/dish) for 3 days. The medium was removed, and the cells were cultured for an additional 24 hr in medium without label. Alternatively, cells were grown for 3 days without label, and [$^3$H]myristic acid (2 $\mu$Ci/dish) was added to the culture medium for 24 hr.

Prelabeled monolayers were washed 3 times with 2 ml of HBSS (148 mM NaCl/10mM Hepes/5.6 mM KCl/2mM $CaCl_2$/1 mM $MgCl_2$/pH 7.35). Washings and incubations were performed at 37°C. Fresh HBSS (1.5 ml) was added, and the monolayers were equilibrated for 10 min. Ethanol (0.75%, v/v) was added to cell monolayers prelabeled with [$^3$H]myristic acid 5 min prior to the addition of stimuli. BK, ATP or HBSS was added in 25 $\mu$l. Incubations were terminated by aspirating the medium and adding 2.1 ml of methanol/10 mM glycine, pH 3.0 (1.6:0.5, v/v). The cells were harvested from the dishes, and lipids were extracted (6). Radioactivity in aliquots of medium, aqueous phase, and chloroform extract was determined. Intracellular [$^3$H]choline in the aqueous phase of cell extracts was measured by cation exchange chromatography (6). [$^3$H]Myristic acid incorporated into [$^3$H]PEt was determined after separation of the extracted lipids by thin-layer chromatography (6).

Assay of [$^3$H]PIns hydrolysis. BPAEC were seeded into dishes and grown in glucose-free MEM with 10% fetal calf serum containing 10 $\mu$Ci/dish myo-[$^3$H]inositol. Prelabeled monolayers were washed as above with HBSS, and the cells were preincubated with 1.5 ml of HBSS containing 15 mM LiCl for 10 min. BK or HBSS was added in 25 $\mu$l. Reactions were quenched by aspiration of the medium and addition of 2.1 ml of methanol/0.5 M HCl (1.6:0.5, v/v). [$^3$H]PIns were extracted (6), and $^3$H in the chloroform and aqueous extract was measured. The residual aqueous phase was dried by vacuum centrifugation, and the residue was made to 10 ml with water and neutralized with Trizma base. [$^3$H]Inositol phosphates were separated by anion exchange chromatography (9). In selected experiments, total [$^3$H]inositol phosphates were eluted from the AG1-X8 columns as a single fraction with 0.1 M formic acid/1M ammonium formate. Aliquots (1 ml) of the column fractions were counted in 10 ml of Aquasol-2.

Prostacyclin synthesis. BPAEC were seeded into dishes and grown for 3 days in the absence of radiolabel. The cells were refed and pretreated with DMSO or phorbol derivatives. On day 4 the monolayers were washed, and the cells were

exposed to BK or HBSS as described above. The stable metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, released into the medium was quantitated by radioimmunoassay (10).

## RESULTS

Under the labeling conditions used, [$^3$H]choline and [$^3$H]myristic acid are preferentially incorporated into [$^3$H]PC (5,6). Agonist-induced PLD activity can be assayed as an increase in [$^3$H]choline in BPAEC prelabeled with [$^3$H]choline or as an increase in [$^3$H]PEt in cells prelabeled with [$^3$H]myristic acid and stimulated in the presence of ethanol (6). [$^3$H]PEt is formed from [$^3$H]PC by a transphosphatidylation reaction unique to PLD (6), and this provides a specific assay for the PLD pathway of PC hydrolysis (11,12).

PLD-catalyzed [$^3$H]PC hydrolysis stimulated by maximally effective concentrations of BK (1 $\mu$M) or ATP (120 $\mu$M) was completely inhibited by pretreatment of BPAEC for 22 hr with 0.1 $\mu$M PMA (Table I). The extent of inhibition was the same using both assays to measure PLD. In contrast to the inhibitory effect on PLD activation, PMA pretreatment increased BK-stimulated hydrolysis of [$^3$H]PIns ($p<0.05$). Pretreatment with PMA did not change the extent of labeling of BPAEC lipids with any of the precursors, and no difference in the distribution of the labels among lipid metabolites was identified in pretreated cells.

In Table I, [$^3$H]PIns hydrolysis was measured as the accumulation of [$^3$H]inositol phosphates after an 8 min incubation with BK in the presence of 15

Table I

Effect of PMA pretreatment on agonist-induced PLD activation and [$^3$H]PIns hydrolysis

| Pretreatment | Stimulus | [$^3$H]Choline (5 min) | [$^3$H]PEt (2 min) | [$^3$H]Inositol phosphates (8 min) |
|---|---|---|---|---|
| DMSO | HBSS | 0.25 ± .01 | 0.11 ± .02 | 2.91 ± .16 |
| | BK | 1.01 ± .05 | 0.26 ± .01 | 17.8 ± 0.6 |
| | ATP | 1.11 ± .03 | 0.22 ± .01 | ND* |
| PMA | HBSS | 0.24 ± .01 | 0.17 ± .01 | 2.57 ± .14 |
| | BK | 0.23 ± .01 | 0.17 ± .02 | 20.1 ± 0.8 |
| | ATP | 0.25 ± .02 | 0.16 ± .01 | ND |

*ND, not determined.
BPAEC were prelabeled with [$^3$H]choline, [$^3$H]myristic acid, or [$^3$H]inositol, and labeled cells were pretreated with 0.2% DMSO (control) or 0.1 $\mu$M PMA for 22 hr. The cells were washed and incubated with HBSS (control), 1 $\mu$M BK, or 120 $\mu$M ATP for the time specified. Intracellular [$^3$H]choline, [$^3$H]PEt, and total [$^3$H]inositol phosphates were quantitated in cells prelabeled with [$^3$H]choline, [$^3$H]myristic acid, and [$^3$H]inositol, respectively. Data are expressed as the percent of total incorporated $^3$H. Each value shown is the mean ± S.E. of at least 5 separate experiments.

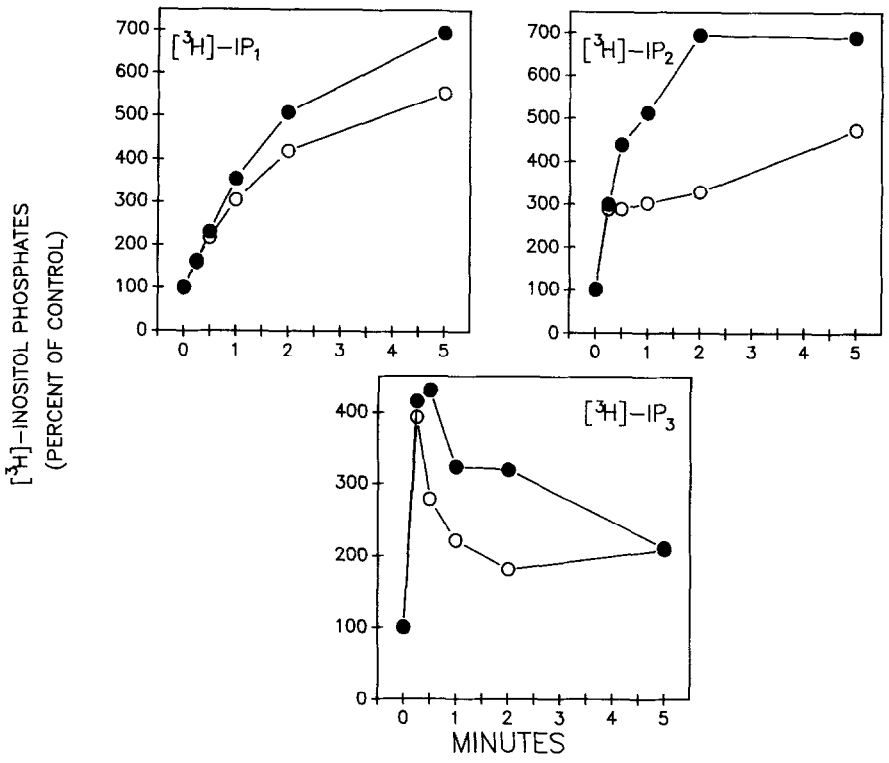


Figure 1. Effect of PMA pretreatment on BK-induced [$^3$H]inositol phosphate formation. BPAEC prelabeled with [$^3$H]inositol were pretreated with 0.2% DMSO (open circles) or 0.1 $\mu$M PMA (solid circles) for 22 hr. The cells were washed and incubated with 1 $\mu$M BK. At the times indicated the reactions were quenched, and the $^3$H content of inositol phosphates was determined. Data are expressed as a percent of control (zero time). Control values calculated as a percent of total incorporated $^3$H were as follows: $IP_1$, 2.07%; $IP_2$, 0.45%; and $IP_3$, 0.33%. Control values remained constant during the 5 min incubation. Data points are the means of duplicate determinations from one of two similar experiments.

mM LiCl. The effect of PMA pretreatment on BK-stimulated [$^3$H]PIns breakdown was examined in more detail (Fig. 1). The initial rate of BK-induced formation of [$^3$H]$IP_3$, [$^3$H]$IP_2$, and [$^3$H]$IP_1$ was similar in control and PMA-pretreated cells. A higher maximum level of accumulation was observed for each inositol phosphate metabolite in cells pretreated with PMA. The greatest relative increase was in the formation of [$^3$H]$IP_2$. These data establish that the increase in [$^3$H]inositol phosphate accumulation measured in Table I is due to an increase in PIns-specific PLC activity and is not due to a change in the time course of enzyme activation.

In view of the differential effect of PMA pretreatment on agonist-stimulated PC and PIns hydrolysis, it was of interest to determine the effect of PMA on prostacyclin synthesis (Fig. 2). The inhibitory effect of PMA on PLD activation was dose-dependent and associated with a 2- to 3-fold increase in BK-stimulated prostacyclin synthesis. The concentration dependence observed for the inhibitory

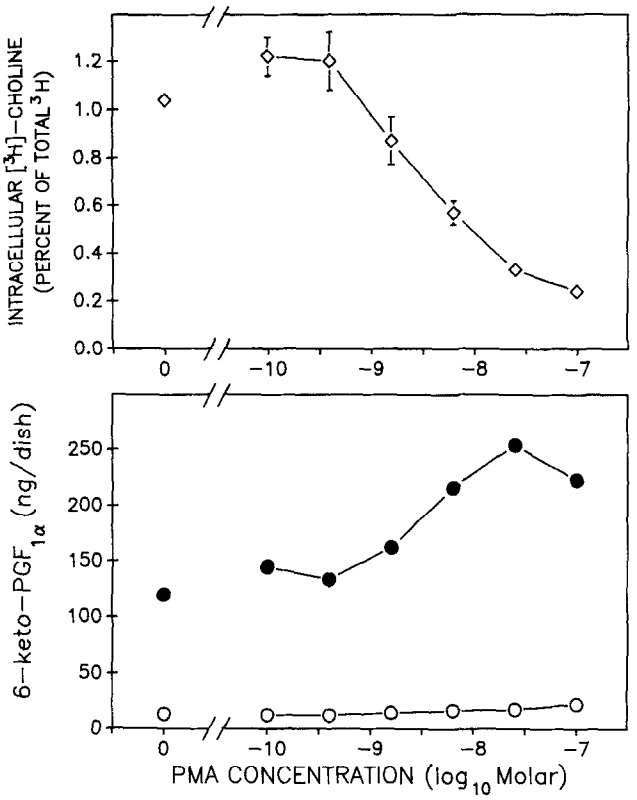


Figure 2. Effect of PMA pretreatment on BK-induced PLD activation and prostacyclin synthesis. Above: BPAEC prelabeled with [$^3$H]choline were pretreated with DMSO or the indicated concentration of PMA for 22 hr. Intracellular [$^3$H]choline was measured after a 5 min incubation of washed cells with 1 $\mu$M BK. Data are expressed as the mean ± S.E. (n=5) percent of total incorporated $^3$H. At all PMA concentrations tested, control intracellular [$^3$H]choline was 0.25 ± .03 % of the total incorporated $^3$H. Below: Parallel cultures of unlabeled BPAEC were pretreated for 22 hr with DMSO or PMA. The cells were washed, and immunoreactive 6-keto-$PGF_{1\alpha}$ released into the medium was measured after a 5 min incubation with HBSS (open circles) or 1 $\mu$M BK (closed circles). Data are the means of duplicate determinations from one of three similar experiments.

effect of PMA on PLD activation was similar to its enhancement of prostacyclin synthesis ($EC_{50}$ = 3.2 nM).

The specificity of the effects of phorbol ester pretreatment on inhibition of BK-induced PLD activation and enhancement of prostacyclin synthesis was investigated (Table II). PMA and 4-$\beta$-PDD acted similarly to inhibit PLD activation and enhance prostacyclin synthesis. These effects were not observed in cells pretreated for 22 hr with equivalent concentrations of 4-$\alpha$-phorbol or 4-$\alpha$-PDD which do not interact effectively with PKC (13).

Pretreatment of BPAEC with 0.1 $\mu$M PMA for 4 hr inhibited agonist-stimulated PLD activity by 86 ± 9% (data not shown). Experiments performed to investigate

Table II

Specificity of phorbol ester effects on BK-induced PLD activation and prostacyclin synthesis

| Pretreatment | Stimulus | [$^3$H]Choline (5 min) | [$^3$H]PEt (2 min) | 6-keto-PGF$_{1\alpha}$ (ng/dish) |
|---|---|---|---|---|
| DMSO | HBSS | 0.17 ± .01 | 0.08 ± .01 | 3.3 ± 1.3 |
| | BK | 0.64 ± .01 | 0.19 ± .02 | 62 ± 4 |
| PMA | HBSS | 0.18 ± .01 | 0.11 ± .02 | 2.9 ± 0.6 |
| | BK | 0.18 ± .01 | 0.11 ± .01 | 191 ± 13 |
| 4-$\beta$-PDD | HBSS | 0.19 ± .02 | 0.12 ± .02 | 2.3 ± .06 |
| | BK | 0.19 ± .01 | 0.12 ± .01 | 195 ± 16 |
| 4-$\alpha$-PDD | HBSS | 0.19 ± .01 | 0.10 ± .01 | 7.1 ± 2.5 |
| | BK | 0.65 ± .08 | 0.21 ± .02 | 69 ± 1 |
| 4-$\alpha$-Phorbol | HBSS | 0.17 ± .02 | 0.10 ± .01 | 3.8 ± 1.1 |
| | BK | 0.78 ± .01 | 0.23 ± .02 | 84 ± 4 |

BPAEC prelabeled with [$^3$H]choline or [$^3$H]myristic acid were pretreated for 22 hr with 0.2% DMSO or 0.1 $\mu$M of the indicated phorbol derivatives. Cells were washed and incubated for the specified times with HBSS or 1 $\mu$M BK. Intracellular [$^3$H]choline and [$^3$H]PEt were quantitated as in Table I. Values are expressed as the percent of total incoporated $^3$H (mean ± S.E., n = 4). Parallel cultures of unlabeled BPAEC were pretreated, washed, and incubated for 5 min with HBSS or 1 $\mu$M BK. Immunoreactive 6-keto-PGF$_{1\alpha}$ released into the medium was quantitated as in Fig. 2.

the effectiveness of PMA at shorter pretreatment intervals were difficult to interpret because PMA alone stimulated PLD activity. The inhibitory effect of PMA at longer intervals was not due to consumption of [$^3$H]PC substrate, however, because long-term pretreatment with PMA did not decrease the incorporation of either [$^3$H]choline or [$^3$H]myristic acid into [$^3$H]PC.

## DISCUSSION

In BPAEC, agonist-induced PC breakdown is mediated principally by a PLD (6). The time course, concentration dependence, and structural specificity of agonist-induced PLD activation and prostacyclin synthesis suggested that this pathway of PC breakdown was involved in signal transduction (5,6). The primary objective of the experiments described here was to investigate the effect of long-term phorbol ester treatment on agonist-induced PLD activation in the hope that this would provide a means to examine its involvement in signal transduction.

The data clearly demonstrate the ability of long-term phorbol ester pretreatment to completely inhibit agonist-induced PLD activation. Similar levels of inhibition were observed using two independent methods to assay PLD-catalyzed PC hydrolysis. Since phorbol ester pretreatment did not inhibit BK-

induced PIns breakdown, this provided a means to dissociate these two pathways of phospholipid metabolism stimulated by BK.

As shown in Fig. 2 and Table II, inhibition of PC breakdown via PLD was associated with an increase in BK-dependent prostacyclin synthesis. These observations provide strong evidence against the hypothesis that PC hydrolysis via PLD plays an obligatory role in the process of signal transduction leading to prostacyclin synthesis in BPAEC. The data do not exclude the possibility that some product(s) of PC hydrolysis might modulate stimulus-response coupling in BPAEC. For example, a PC degradation product might exert negative feedback on agonist-stimulated prostacyclin synthesis, perhaps at the level of PIns hydrolysis. This would explain the parallel enhancement in BK-induced PIns hydrolysis and prostacyclin synthesis after prolonged exposure of cells to phorbol esters.

Evidence has arisen to support the involvement of PKC in the activation of PLD by receptor-independent pathways (7,8). Our data suggest that activation of PLD by receptor-dependent pathways may also involve PKC. Of the phorbol derivatives tested, only those capable of interacting effectively with PKC inhibited BK-induced PLD activation. In endothelial cells (14) and other cells (15-17), PKC is down-regulated following prolonged interaction with phorbol esters. Thus, down-regulation of PKC seems a likely explanation for the inhibitory effect of long-term phorbol ester treatment on BK-induced PLD activation.

PLD may be coupled to receptors by G-proteins (6,8,18). This suggests that the mechanisms for receptor-dependent activation of PLD and PIns-specific PLC may be similar. The data presented here establish a major difference between receptor-dependent activation of PLD and PIns-specific PLC, however. In contrast to an inhibitory effect, BK-induced PIns-specific PLC activity was enhanced by long-term phorbol ester treatment. Similar findings have been reported in other cells (19-21). This effect of phorbol esters may be due to sensitization of the receptor-G-protein-PLC complex rather than to down-regulation of PKC (21). By analogy, it is conceivable that the inhibitory effect of phorbol esters on PLD activation may result from desensitization or uncoupling of a putative receptor-G-protein-PLD complex, and this may or may not involve down-regulation of PKC.

The precise mechanisms underlying the effects of phorbol ester pretreatment on agonist-induced activation of PIns-specific PLC and PLD and prostacyclin synthesis remain to be established. Nevertheless, our data demonstrate that the two major pathways of phospholipid metabolism stimulated by BK in BPAEC are differentially regulated. Furthermore, it is clear that PLD activation is not required for agonist-induced prostacyclin synthesis. Phorbol ester pretreatment may be of general usefulness in studies in intact cells to determine the roles of PIns-specific PLC and PLD in the biochemical events of cell activation triggered by $Ca^{2+}$-mobilizing agonists.

ACKNOWLEDGMENT

This work was supported by Grants HL38214 and HL30572 from the National Heart, Lung, and Blood Institute.

REFERENCES

1. Derian, C.K. and Moskowitz, M.A. (1986) J. Biol. Chem. 261, 3831-3837.
2. Lambert, T.L., Kent, R.S., and Whorton, A.R. (1986) J. Biol. Chem. 261, 15288-15293.
3. Forsberg, E.J., Feurerstein, G., Shohami, E., and Pollard, H.B. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 5630-5634.
4. Pirotton, S., Raspe, E., Demolle, D., Erneux, C., and Boeynaems, J.-M. (1987) J. Biol. Chem. 262, 17461-17466.
5. Martin, T.W. and Michaelis, K.C. (1988) Biochem. Biophys. Res. Commun. 157, 1271-1279.
6. Martin, T.W. and Michaelis, K.C. (1989) J. Biol. Chem. 264, 8847-8856.
7. Cabot, M.C., Welsh, C.J., Zhang, Z., and Cao, H. (1989) FEBS Lett. 245, 85-90.
8. Billah, M.M., Pai, J.-K., Mullmann, T.J., Egan, R.W., and Siegel, M.I. (1989) J. Biol. Chem. 264, 9069-9076.
9. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983) Biochem. J. 212, 473-482.
10. Sprague, R.S., Stephenson, A.H., and Lonigro, A.J. (1984) J. Appl. Physiol. 56, 1246-1251.
11. Pai, J.-K., Siegel, M.I., Egan, R.W., and Billah, M.M. (1988) Biochem. Biophys. Res. Commun. 150, 355-364.
12. Bocckino, S.B., Wilson, P.B., and Exton, J.H. (1987) FEBS Lett. 225, 201-204.
13. Ashendel, C.L. (1985) Biochim. Biophys. Acta 822, 219-242.
14. Uratsuji, Y. and DiCorleto, P.E. (1988) J. Cell. Physiol. 136, 431-438.
15. Solanki, V., Slaga, T.J., Callaham, M., and Huberman, E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1722-1725.
16. Rodriquez-Pena, A. and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. 120, 1053-1059.
17. Blackshear, P.J., Witters, L.A., Girard, P.R., Kuo, J.F., and Quamo, S.N. (1985) J. Biol. Chem. 260, 13304-13315.
18. Bocckino, S.B., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987) J. Biol. Chem. 262, 15309-15315.
19. Pandiella, A., Vicentini, L.M., and Meldolesi, J. (1987) Biochem. Biophys. Res. Commun. 149, 145-151.
20. Hepler, J.R., Earp, H.S., and Harden, T.K. (1988) J. Biol. Chem. 263, 7610-7619.
21. Stassen, F.L., Schmidt, D.B., Papadopoulos, M., and Sarau, H.M. (1989) J. Biol. Chem. 264, 4916-4923.