Transduction of the bradykinin response in human fibroblasts: prolonged elevation of diacylglycerol level and its correlation with protein kinase C activation

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Stimulation of quiescent human fibroblasts with the peptide mitogen bradykinin (BK) led to a biphasic elevation in cellular 1,2-diacylglycerol (DAG), as estimated by either measurement of total DAG mass or [³H]arachidonate incorporation. A rapid initial transient that peaked 15 s after BK addition was followed by a decline to near basal levels then a second rise to a plateau phase during which DAG levels remained elevated for ≤45 min. The source of the initial DAG transient appeared to be primarily polyphosphoinositides as these phospholipids were rapidly hydrolyzed after BK addition. This transient correlates well temporally with previous observations of the kinetics of inositol trisphosphate accumulation and intracellular free [Ca²⁺] observed in the same cells. Cultures preincubated with [³H]myristic acid incorporated label predominantly into the phosphatidylcholine (PC) pool. Subsequent addition of BK under these conditions caused only a relatively slow accumulation of [³H]DAG to a plateau level, without an initial transient. Together with the observation that PC was found to decrease upon BK stimulation, these observations suggest that the late phase of DAG accumulation may involve breakdown of other phospholipids including PC. To investigate the consequences of DAG elevation we examined the phosphorylation of an acidic 80 kDa protein, whose phosphorylation is solely

dependent on the activation of protein kinase C (PK-C). The 80 kDa fibroblast protein could be immunoprecipitated by an antibody to bovine brain "myristoylated and alanine-rich C-kinase substrate" (MARCKS) and phosphopeptide maps of brain and fibroblast MARCKS were similar. Stimulation of [³²P]-prelabeled fibroblasts with serum, BK, vasopressin, or 12-O-tetradecanoyl phorbol acetate, but not epidermal growth factor or calcium ionophores, resulted in the rapid phosphorylation of MARCKS. With BK or serum this phosphorylation showed an initial transient peak at <1 min then rose again to a plateau level that was sustained for ≤45 min. Removal of BK resulted in a rapid decline in MARCKS phosphorylation. These studies show that the biphasic DAG signal in BK-stimulated human fibroblasts correlates well with the state of activation of PK-C. However, the persistent activation of PK-C does not appear to require continued high levels of Ca²⁺.

Introduction

Multiple second messenger pathways become activated when quiescent fibroblasts are stimulated with a complex mitogen such as serum (for review see Rozengurt, 1986). Dissection of these pathways is facilitated by analyzing the signal transduction mechanisms utilized by individual growth factors that may be only partial mitogens. In the case of specific types of human fibroblasts we have shown that the mitogenic response to serum can be mimicked by a mixture of purified factors consisting of bradykinin (BK), vasopressin, epidermal growth factor (EGF), and insulin (Owen and Villereal, 1983). This combination of factors stimulates both Na/H exchange and DNA synthesis to approximately the same extent as 10% fetal bovine serum. Considerable progress has been made in elucidating the rapid intracellular events that transpire in response to these individual factors in various types of fibroblasts (Rozengurt, 1986). For example, BK leads to a stimulation of arachidonic acid release from human fibroblasts,

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which appears to be mediated by a phospholipase A₂-linked mechanism (Vicentini *et al.*, 1984). In addition, this growth factor rapidly activates phospholipase C leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and generation of inositol 1,4,5-trisphosphate ([ins-P₃] Jamieson and Villereal, 1987; for review see Berridge and Irvine, 1989). This intracellular messenger in turn causes release of Ca²⁺ from intracellular stores (Muldoon *et al.*, 1987b; Byron and Villereal, 1989). An important consequence of Ca²⁺ accumulation in the cytoplasm is the activation of calmodulin (CaM) and CaM-regulated processes. This has been demonstrated in human fibroblasts by showing that Ca/CaM-dependent protein kinase III (CaM-PK III) activity, as reflected in the phosphorylation of its major substrate elongation factor-2 (EF-2), is transiently elevated shortly after BK addition to quiescent cells (Palfrey *et al.*, 1987). Phosphorylation of EF-2 is maximal at 1 min and returns to baseline within 5 min of BK addition. This rapid deactivation of CaM-PK III parallels the decline of ins-P₃ and cytoplasmic [Ca²⁺] that occurs within 1 min of stimulation. Thus there is a good correlation between the generation of the second messengers (ins-P₃ and Ca²⁺) and the final biochemical response (phosphorylation of EF-2) in this case.

Breakdown of PIP₂ (and other phospholipids) by phospholipase C also results in the generation of 1,2-diacylglycerol (DAG), and it is to this second messenger that we have turned our attention in the present work. DAG, along with Ca, is the probable physiological activator of protein kinase C ([PK-C] Nishizuka, 1986, 1988) and we wished to compare the kinetics of activation and inactivation of this enzyme with the levels of these second messengers. To this end we have measured cellular DAG content in parallel with the state of phosphorylation of an *M*_r 80 000 protein (80 kDa), that is homologous with a brain protein ("87 kDa" or "MARCKS") previously shown to be exclusively phosphorylated by PK-C (Albert *et al.*, 1986; Stumpo *et al.*, 1989). Our results suggest that, in contrast to CaM-PK III, PK-C remains active for an extended period following a BK stimulus. A preliminary account of this work has been presented (Etscheid *et al.*, 1988).

Results

Measurement of DAG levels and phospholipid breakdown in BK-stimulated quiescent cells

[³H]arachidonic acid labeling. Serum-deprived cultures were labeled for 18 h with [³H]-

arachidonate, then stimulated with BK and the appearance of label in 1,2-DAG estimated by solvent extraction followed by thin-layer chromatography (TLC) and scintillation counting of DAG. As shown in Figure 1A appearance of labeled DAG after BK stimulation was distinctly biphasic. There was a rapid initial transient peak at 15 s followed by a decline to near basal levels; a more extended elevation in DAG from 5 to 20 min ensued. DAG accumulation was not markedly affected by treatment of cells with A23187, showing that the BK effect was not a secondary consequence of Ca²⁺ accumulation in the cytosol.

Mass measurement of DAG. An alternative and more quantitative method of measuring total DAG levels was used to confirm the results obtained above. In this procedure DAG is converted to [³²P]phosphatidic acid (PA) in the presence of a bacterial enzyme that is specific for 1,2-DAG; counts in PA are reflective of the DAG in the initial extract and can be converted to absolute DAG concentrations by reference to a standard curve. Results similar to those obtained with [³H]arachidonic acid labeling were obtained. DAG levels rose to an early transient peak, then declined and rose again more gradually to a second peak at 30 min (Figure 1B). Even 45 min after BK stimulation DAG was above control levels. DAG levels were determined to be 1.5 nmol/mg protein under basal conditions and 4 nmol/mg protein at peak.

Confirmation of phosphoinositide breakdown was obtained by TLC analysis of [³²P]-labeled phosphoinositides. A rapid ~50% decline in both PIP and PIP₂ label as well as a smaller decrease in PI labeling was seen immediately after BK stimulation. This was followed by a slower reincorporation of label suggesting resynthesis of these molecules (Figure 2).

[³H]Myristic acid labeling. The question arose as to whether DAG release could be attributed entirely to breakdown of PIP/PIP₂ or whether other phospholipids might be involved. As arachidonic acid becomes incorporated homogeneously among the phospholipids (Hong and Deykin, 1979), it is difficult to determine the source of released [³H]DAG. Myristic acid has been reported to preferentially label phosphatidylcholine (PC) in certain cells (see Billah and Anthes, 1990, for refs.). Thus examination of [³H]myristate labeled DAG production after BK stimulation might implicate the hydrolysis of PC in the prolonged DAG peak. Preliminary experiments demonstrated that ~85% of the

Figure 1. DAG production in fibroblasts. (A) Time course of BK stimulation of [^3H]arachidonate labeled DAG production. HSWP cells were prelabeled with [^3H]arachidonate and then stimulated with BK ($0.1\ \mu\text{M}$) or A23187 ($2.5\ \mu\text{M}$) for various times. A representative single experiment is shown; this experiment was repeated several times with similar results. (B) Time course of BK stimulated DAG production. The experiment was similar to that in (A) except that total DAG levels were measured by conversion to [^{32}P]phosphatidic acid (PA) in the presence of bacterial DAG kinase.

[^3H]myristate incorporated into phospholipid appeared in the PC pool (data not shown). Subsequent studies demonstrated that BK did stimulate the release of [^3H]myristate-labeled DAG but with a distinctly different time course to that of [^3H]arachidonate-labeled DAG (Figure 3). No rapid peak was seen; instead, a slow rise reaching a plateau after 10 min was observed. This suggests that breakdown of PC may contribute to the persistent DAG elevation seen at longer time points. Consonant with this hypoth-

esis a 30% reduction in [^{32}P]PC levels occurred 5 min after BK addition (data not shown).

Identification of the 80-kDa acidic phosphoprotein as MARCKS and stimulation of phosphorylation by mitogens

In previous experiments we showed by 2-dimensional electrophoresis that the phosphorylation of a ~ 80 kDa acidic protein was enhanced on mitogen or phorbol ester treatment

Figure 2. Breakdown of phosphoinositides after BK stimulation. HSWP cells were prelabeled with [32 P]orthophosphate, treated with BK (0.1 μ M) for various times and counts in PI, PIP, and PIP₂ quantitated after TLC separation. This experiment was repeated three times with similar results.

of human fibroblasts (Muldoon *et al.*, 1987a). As this protein seems to be an exclusive substrate for PK-C, its state of phosphorylation is potentially an accurate reflection of the state of activation of PK-C in intact cells. We used an antibody generated against bovine brain "87 kDa" (now termed myristoylated and alanine-rich C-kinase substrate [MARCKS]) to immunoprecipitate the protein from fibroblasts. Preliminary experiments showed that the anti-87kDa antibody could immunoprecipitate a protein of ~80 kDa from fibroblast extracts incubated under conditions expected to activate PK-C (i.e., in the presence of Ca + phosphatidylserine [Figure 4A]). The phosphorylated protein was more abundant in a crude membrane preparation than in the cytosol and had an acidic pI. In addition, phosphopeptide maps of immunoprecipitated [32 P]-labeled fibroblast 80-kDa protein were quite similar to their rat brain counterpart (Figure 4B). We conclude therefore that these antibodies are reacting with "fibroblast MARCKS." Immunocytochemical localization of MARCKS in human fibroblasts revealed a diffuse primarily cytoplasmic distribution, but with patches of immunoreactivity that appeared to be associated with the ruffling edge of the fibroblast surface (data not shown).

Using this antibody we immunoprecipitated MARCKS from [32 P]-prelabeled HSWP and WI38 cells before and after stimulation with different

mitogens, including BK. As shown in Figure 5, basal incorporation of 32 P into MARCKS was low in quiescent cells and was rapidly increased up to 10-fold by serum or BK stimulation. This compares to a maximal stimulation of 20- to 30-fold that could be attained when cells were incubated in TPA (0.1 μ M; 2–10 min). Vasopressin (0.1 μ M) could also increase MARCKS phosphorylation, but EGF or Ca ionophores (A23187, 2.5 μ M or ionomycin, 0.5 μ M) elicited only weak or no effect (Figure 5A and data not shown). Both BK- and serum-stimulated MARCKS phosphorylation was attenuated when cells were depleted of PK-C by long-term TPA pre-treatment (Figure 5B).

Examination of the time course of MARCKS phosphorylation in response to serum (Figure 5C) or BK (Figure 6) revealed a peak at <1 min then a decline that was followed by a sustained elevation for ≤ 45 min after mitogen addition. The decline after the initial peak was less marked in the presence of serum than with BK. No difference in these stimulation parameters between HSWP and WI38 cells was found. These results are consistent with a persistent activation of PK-C after mitogenic stimulation; however, a possible alternative explanation for the long-term phosphorylation of MARCKS

Figure 3. Time course of BK-stimulated [3 H]myristate labeling of DAG. HSWP cells were prelabeled with [3 H]myristate before stimulation by BK (0.1 μ M) for different times, then processed for measurement of labeled DAG as described in Materials and Methods. This experiment was reported three times with similar results.

Figure 4. Identification of the 80-kDa fibroblast PK-C substrate as MARCKS. (A) Extracts of HSWP cells (Palfrey *et al.*, 1987) were separated into cytosol and crude particulate (100 000 $\times g$) fractions which were incubated either in the absence or presence of Ca (0.5 mM) + phosphatidylserine (PtdSer; 50 $\mu g/ml$) with γ -[^{32}P]ATP for 2 min at 30°C. One-half of each sample was directly dissolved in SDS sample buffer and the other one-half subjected to immunoprecipitation with anti-MARCKS antibodies. Samples were separated by SDS-7.5% PAGE and the gels autoradiographed. (B) ^{32}P -labeled fibroblast 80 kDa from gels of immunoprecipitates such as those shown in (A) and ^{32}P -labeled rat synaptosomal 87 kDa from samples prepared as in Wu *et al.* (1982) were excised from dried gels and proteolyzed with thermolysin. The resultant peptides were separated in two dimensions by electrophoresis (horizontal) and chromatography (vertical). The positions of the ^{32}P -peptides were determined by autoradiography.

could be that it is only slowly dephosphorylated. That this is unlikely is suggested by experiments in which the external solution was replaced 20 min after BK stimulation to remove the hormone. This maneuver resulted in an immediate decrease in [^{32}P]MARCKS, indicating that an active phosphatase was present in the cells under these conditions (Figure 6). These results agree with those of Rodriguez-Pena *et al.* (1986)

showing that an 80-kDa acidic phosphoprotein from 3T3 cells is also rapidly dephosphorylated on removal of mitogenic stimuli.

Discussion

Previous work from this laboratory showed that BK interacts with a single receptor type on the surface of human fibroblasts (Etscheid and Vil-

Figure 5. Immunoprecipitation of fibroblast [32 P]MARCKS from intact cells. (A) HSWP cells were stimulated for 2 min with TPA, BK, vasopressin, EGF (all at 0.1 μ M), or ionomycin (0.5 μ M) and then processed for immunoprecipitation as described in Palfrey *et al.* (1987). In this experiment the fold increases in MARCKS phosphorylation over control were TPA (17), BK (6), vasopressin (6), EGF (1.5), and ionomycin (2). **(B)** HSWP cells were incubated for 24 h in the absence (-) or presence (+) of TPA (1 μ M) to deplete PK-C. They were then challenged with either BK (0.1 μ M) or serum (10%) for 2 min and MARCKS immunoprecipitated as described. TPA (0.1 μ M) was included as a control. In this experiment the fold increases in MARCKS phosphorylation were TPA (15), BK (-TPA: 8; +TPA: 1.2), serum (-TPA: 10; +TPA: 3.5). Down-regulation had no effect on the basal state of MARCKS phosphorylation (not shown). **(C)** Autoradiogram showing time course of serum-stimulated incorporation of 32 P into MARCKS. Elevation of MARCKS phosphorylation is evident throughout the 45-min incubation; however, quantitation of gel slices reveals a distinct early peak at 30 s followed by a decline then a rise to a sustained plateau at 45 min.

lereal, 1989). This receptor was characterized as a B_2 type on the basis of BK analogue studies and this single receptor appears to be responsible for both phosphatidylinositol turnover via G-protein-coupled phospholipase C activation and arachidonic acid release via phospholipase A_2 stimulation. Evidence that these processes are independently regulated comes from experiments with phorbol ester-pretreated cells; short-term incubation with TPA interfered with BK-induced ins-P_3 generation while having no effect on arachidonic acid release (Etscheid *et al.*, 1990; see also Burch and Axelrod, 1986, for 3T3 cells and Kaya *et al.*, 1989, for aortic endothelial cells).

The purpose of the present work was to analyze the consequences of phospholipase C activation by BK in more detail. We observed a biphasic increase in DAG after BK stimulation of quiescent fibroblasts using two different methods: mass measurement of total 1,2-DAG or by prelabeling phospholipids with [3 H]-arachidonate. The biphasic nature of the response suggested that DAG accumulation resulted from the breakdown of different phospholipids. In accordance with this hypothesis, analysis of phosphoinositide hydrolysis showed that the initial DAG peak correlated well with a rapid decline in PIP and PIP $_2$ content as well as with previous determinations of ins-P_3 levels in

Figure 6. Time course of MARCKS phosphorylation following BK and its rapid reversal by removal of BK. Cells were prelabeled as in Figure 5, stimulated with BK ($0.1 \mu\text{M}$) for the indicated times and the amount of ^{32}P in immunoprecipitates of MARCKS was quantitated by scintillation counting of gel pieces. In some samples BK-containing medium was removed (arrow) and replaced with fresh DMEM (dotted line).

these cells (Jamieson and Villereal, 1987). However, BK stimulation of [^3H]myristate prelabeled cells resulted in only a slow rise to a maintained plateau. Because myristate is primarily incorporated into PC, and a concomitant loss of PC was found in the present experiments, it is reasonable to suggest that the plateau phase of DAG production emanates from PC breakdown. Similar prolonged elevations in DAG have been obtained, e.g., in angiotensin II-stimulated vascular smooth muscle cells (Griendling *et al.*, 1986), bombesin-stimulated 3T3 cells (Muir and Murray, 1987), serum-stimulated chinese hamster ovary fibroblasts (Wright *et al.*, 1988), and BK-stimulated neuronal tumor cells (Francel and Dawson, 1988). Several investigators (see Billah and Anthes, 1990, for full references) have concluded that DAG release after cell stimulation cannot be entirely due to phosphoinositide hydrolysis and PC hydrolysis has been specifically implicated in several cases (e.g. Besterman *et al.*, 1986; Bocckino *et al.*, 1987; Muir and Murray, 1987; Pessin and Raben, 1989). It has also been proposed that rapid PK-C activation after

initial phosphoinositide hydrolysis may contribute a positive feedback loop to PC breakdown, as the latter is frequently stimulated by phorbol esters (Besterman *et al.*, 1986; Muir and Murray, 1987). At present, the precise mechanism of DAG generation from PC remains unknown and contributions from both phospholipase C and phospholipase D may be differentially involved in the process (Billah and Anthes, 1990).

Because hydrolysis of phospholipids generates both intracellular Ca and DAG, activation of PK-C as well as other Ca^{2+} -dependent protein kinases is expected. We have already demonstrated that the activation of CaM-PK III after BK addition to quiescent fibroblasts is a transient phenomenon, with kinetics similar to that of the measured Ca^{2+} spike itself (Palfrey *et al.*, 1987). Thus by 5 min phosphorylation of the principal CaM-PK III substrate EF-2 increases (5- to 10-fold) and returns to baseline. The lack of persistent activation of this CaM-dependent enzyme suggests that CaM-regulated protein phosphorylation and, by extrapolation, other CaM-stimulated events, are relatively short-lived. In the present study, we have used an analogous approach to probe the state of activation of PK-C after BK addition, i.e., measurement of the phosphorylation state of a well-defined substrate of PK-C. The alternative approach of measuring PK-C activity directly in cell extracts has substantial drawbacks in that second messenger concentrations inevitably change on cell homogenization.

Several groups, including our own, have noted the existence of a prominent acidic protein of apparent $M_r \sim 80\,000$ whose phosphorylation state is markedly increased following stimulation of fibroblasts with mitogens or phorbol esters (e.g., Rozengurt *et al.*, 1983; Rodriguez-Pena and Rozengurt, 1985; Blackshear *et al.*, 1986; Isacke *et al.*, 1986; Muldoon *et al.*, 1987a; Issandou and Rozengurt, 1990). This protein turns out to be similar if not identical to a protein first identified in rat brain synaptosomes, initially called 87 kDa and now MARCKS (Wu *et al.*, 1982; Albert *et al.*, 1986; Stumpo *et al.*, 1989). The similarities between the human fibroblast 80-kDa protein studied here and MARCKS include acidic pI, phosphorylation by TPA addition to intact cells, or Ca^{2+} and phosphatidylserine addition to cell fractions, indicating that it is a PK-C substrate (Figure 4A), similar phosphopeptide maps (Figure 4B) and, most importantly, recognition by antibodies against MARCKS (Figures 4A and 5). The actual M_r of MARCKS, calculated from the deduced amino acid se-

quence after cloning of the cDNA, is ~32 000 rather than the 68–87 000 found in various preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Stumpo *et al.*, 1989). The anomaly appears to be due to the elongated shape of the molecule. The phosphorylation sites in MARCKS have been identified and are confined to a 25-residue basic domain that contains four potential phosphorylatable serines (Graff *et al.*, 1989a; Stumpo *et al.*, 1989). In synaptosomes, phosphorylation *in situ* results in a shift in the location of the protein from membrane to cytosol (Wang *et al.*, 1989). Phosphorylation of the purified protein by PK-C also leads to a reduction in its ability to bind CaM (Graff *et al.*, 1989b). In addition, MARCKS is a substrate for N-terminal myristoylation (Aderem *et al.*, 1988). We are currently examining the fatty acylation of fibroblast MARCKS and assessing the possible redistribution of the protein after mitogen addition using immunological methods.

While the function of MARCKS is still unknown, the fact that this protein is exclusively phosphorylated by PK-C makes it an ideal candidate for monitoring the activity of this enzyme in intact cells. The results of the present study indicate that PK-C is rapidly and persistently activated after serum or BK stimulation of human fibroblasts, as reflected in the state of MARCKS phosphorylation. Vasopressin had essentially the same effect but EGF was inactive, in agreement with the results of Blackshear *et al.* (1986) in 3T3 cells. Because PK-C is activated *in vitro* by a combination of Ca^{2+} and DAG, it might be predicted that both second messengers would need to remain elevated for continued enzyme activity. The current experiments suggest that this is not the case in human fibroblasts. Though DAG production seems to be maintained over an extended period, measurements of either population averages (Palfrey *et al.*, 1987) or individual cells (Byron and Villereal, 1989) indicate that the intracellular free Ca^{2+} response to BK is transient in nature. The peak levels obtained soon after hormone addition to cells decline quickly, although not all the way to basal levels. In fact, our recent imaging studies on individual cells indicate that many cells maintain a plateau phase at which Ca^{2+} levels are ~20% of peak values (Byron and Villereal, 1989). We conclude, however, that continued activation of PK-C does not necessitate continued *high* levels of cytoplasmic free $[\text{Ca}^{2+}]$. Several explanations for this phenomenon can be entertained. For example, the Ca^{2+} require-

ment for PK-C activity may have decreased due to the presence of DAG (cf. Kishimoto *et al.*, 1980). Alternatively, PK-C may now be in a form that does not need a continuous supply of Ca^{2+} (e.g., bound Ca may be sequestered by the enzyme) or a completely Ca^{2+} -insensitive isozyme of PK-C (see Nishizuka, 1988 and Huang, 1989, for reviews) may be involved. At present it is not known whether the basal or slightly elevated free Ca^{2+} levels seen after BK stimulation are sufficient to maintain PK-C activity or whether Ca^{2+} -insensitive forms of PK-C are present in human fibroblasts. However, if the initial Ca^{2+} spike is sufficient to induce PK-C translocation to the membrane the enzyme may remain there in an active form if an adequate supply of DAG is maintained. Translocation of PK-C to membranes after cell stimulation has been observed in a number of cases (see Nishizuka, 1986 and TerBush *et al.*, 1988 for references), but has been difficult to observe in mitogen-stimulated fibroblasts (e.g., 3T3 cells: Halsey *et al.*, 1987). It is widely assumed that the membrane-associated form of PK-C is the active species of the enzyme as both phosphatidylserine and DAG are confined to the membrane. Most importantly, it appears that the membrane bound form of PK-C is active in the absence of Ca^{2+} and can only be rendered Ca^{2+} -sensitive by removal from the membrane with detergent (e.g., TerBush *et al.*, 1988; Bazzi and Nelsestuen, 1988).

The persistent phosphorylation of MARCKS is not due to some anomaly in its dephosphorylation. Removal of BK resulted in an immediate reduction in MARCKS phosphorylation. A similar phenomenon has been noted in 3T3 cells after mitogen removal (Rodriguez-Pena *et al.*, 1986). In addition, we have recently shown that increased phosphorylation of another PK-C substrate, α -adducin, is maintained for extended periods after BK addition to human fibroblasts (Waseem and Palfrey, 1990). The persistent phosphorylation of MARCKS observed here contrasts with data recently published by Issandou and Rozengurt (1990) while the present work was being completed. Those authors found that BK addition to quiescent Swiss 3T3 cells only transiently stimulated "80K" phosphorylation, with kinetics resembling the initial peak response seen in the present study. They did find, however, that the effects of vasopressin and bombesin on 80K phosphorylation were longer-lived. It is possible, as suggested by Issandou and Rozengurt, that the B_2 receptor in 3T3 cells exhibits homologous desensitization

by some unknown mechanism. Additionally, Price *et al.* (1989) have found that BK addition to Swiss 3T3 cells has no effect on PC hydrolysis; thus it seems that the B₂ receptor in these cells is not coupled to a phospholipase C or D that attacks PC.

Protein phosphorylation is crucial to the final physiological effects of mitogens and their second messengers. PK-C has been implicated as an essential component in the mitogenic response of some types of fibroblast in that phorbol esters can duplicate the effects of a physiological mitogen (see Rozengurt, 1986 for review). Other types of fibroblast appear to use different pathways that may depend on alternate second messengers and protein kinases (e.g., Muldoon *et al.*, 1987a). The length of time a kinase remains active is important, in that alterations in substrate function can be terminated or perpetuated as needed by the cell. It is likely that the long-term rise in PK-C activity described here plays an important role in regulating cell behavior before DNA synthesis.

Materials and methods

The sources of materials were as follows: *Staphylococcus aureus* cells (Pansorbin, Calbiochem, San Diego, CA); BK (Peninsula, Belmont, CA); bacterial DAG kinase (Lipidex, Westfield, NJ); cardiolipin (Avanti, Birmingham, AL); A23187, vasopressin and lipid standards (Sigma, St. Louis, MO); TPA (LC Services, Woburn, MA); EGF (Collaborative Research, Bedford, MA); [³²P]orthophosphate (ICN); [³²P]ATP (Amersham, Arlington Heights, IL); [³H]arachidonic and -myristic acids (Amersham).

Cells and cell culture

HSWP (skin) and WI38 (lung) fibroblasts were cultured as previously described (Muldoon *et al.*, 1987a). Serum deprivation involved lowering the fetal bovine serum concentration to 0.1% for at least 24 h before experiments. In experiments where PK-C was down-regulated, cultures were exposed to 1 μ M TPA throughout the period of serum deprivation.

[³H]fatty acid incorporation into DAG

Cultures were incubated overnight in low serum medium containing 0.1 μ Ci/ml [³H]arachidonic acid. Before mitogen treatment the monolayer was washed three times to remove unincorporated label and then incubated 1 h in 2.7 ml amino acid-free N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-Eagle's minimum essential medium (EMEM) containing 1 mg/ml fatty acid-free bovine serum albumin. Assays were started by the addition of 0.3 ml BK to the dish (100 nM). The stimulation was terminated by aspirating the medium and adding 2 ml ice-cold methanol to the dish, which was then set on ice. To each dish was added 100 μ l of methanol containing 2 nCi [¹⁴C]DAG (to permit normalization of yield) and 15 μ g unlabeled DAG (as a carrier). The cells were scraped from the dishes using a rubber policeman and were transferred to 15 ml Pyrex tubes. The dishes were rinsed once with 3.6 ml 1 M NaCl, which was added to the methanol extract. Chloroform (4 ml) was added to each tube then the

extract was vortexed and centrifuged at low speed to separate the phases. The upper phase was aspirated, 4 ml of 1 M NaCl were added, and the tubes were vortexed and centrifuged. After a second NaCl wash, the extract was dried down under N₂. The dried DAG extracts were redissolved in 100 μ l chloroform, and 40 μ l was spotted on Silica G TLC plates (Whatman) along with arachidonic acid and DAG standards. The plates were developed with ether:hexanes:glacial acetic acid (70:35:2). After drying, plates were placed in I₂ vapor to stain the lipids brown. Using the standard as a guide, the DAG spots were scraped off the plate, transferred to a scintillation vial with 2 ml scintillation fluid, and counted using a [³H]/[¹⁴C] program. [³H]DAG recovery was normalized using the [¹⁴C]DAG yield for each dish as follows (with backgrounds subtracted): [³H]DAG $\times$ ([¹⁴C]DAG added/[¹⁴C]DAG recovered) = "true" [³H]DAG.

DAG kinase assay

The protocol followed was that of Wright *et al.* (1988), which is a modification of the original procedure of Preiss *et al.* (1986). Extracts were prepared as detailed above for [³H]DAG through the step of drying the lipids under N₂ gas. The lipids were then resuspended into micelles of cardiolipin and *n*-octylglucoside, and the endogenous DAG reacted with bacterial DAG kinase in the presence of [³²P]ATP to yield [³²P]phosphatidic acid. A standard curve using known concentrations of synthetic DAG reacted with the DAG kinase permitted quantitation of the total DAG.

³²P-Labeling of phospholipids

Serum-deprived cells on 60 mm dishes were incubated with 2.7 ml of 25 μ Ci/ml [³²P]orthophosphate in phosphate-free HEPES-Hanks' balanced salt solution for 2 h. The experiments were initiated by addition of BK (0.1 μ M). At various times the reaction was quenched by adding 2 ml ice-cold methanol, and the dishes were set on ice. The cells were scraped from the culture dish with a rubber policeman and transferred to a 15-ml pyrex tube containing 4 ml chloroform and 40 μ l concentrated HCl. Dishes were rinsed once with 2 ml 0.2 M KCl/5 mM K₂EGTA, which was combined with the cell extract. Following vortexing and centrifugation the upper layer was aspirated. The organic layer was washed once with 4 ml 0.1 M KCl/50% methanol. After vortexing and centrifugation, the upper layer was discarded and the lower layer was dried under N₂. The phospholipids were separated by thin-layer chromatography on silica G plates that had been prerun in methanol:water:1% potassium oxalate:100 mM EDTA (40:34:24:2), air dried, and activated in a warm oven for 30 min immediately before loading the sample. Three solvent systems were used to separate and identify the phospholipids: (1) chloroform:methanol:acetic acid:water (25:15:4:2) for separating PC/PI; (2) chloroform:methanol:3.3 N NH₄OH (45:35:10) for separating PI, PIP, and PIP₂; and (3) chloroform:methanol:acetone:acetic acid:water (40:13:15:12:8) for resolving PA from PI. Standards were used to positively identify each of the phospholipids.

Phosphorylation and immunoprecipitation

The techniques used were essentially those described in Palfrey *et al.* (1987) with minor modifications. In the immunoprecipitation 10 μ l of polyclonal rabbit anti-bovine brain "87K" antiserum (Albert *et al.*, 1986) was used per assay tube. Phosphorylation of 87kDa in synaptosomes was performed by the method of Wu *et al.* (1982). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE),

autoradiography, and quantitation of ^{32}P incorporation into protein was done as previously described (Palfrey *et al.*, 1987). Two-dimensional phosphopeptide maps of fibroblast 80 kDa and synaptosomal 87kDa were generated using thermolysin as detailed in Albert *et al.* (1986) and Palfrey *et al.* (1987).

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