Stimulation of Calcium Uptake in *Saccharomyces cerevisiae* by Bovine Protein Kinase C α*

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Ca**+** plays essential roles as a second messenger often in synergism with the calcium- and phospholipid-dependent phorbol ester receptor, protein kinase C (PKC), which stimulates Ca**+** influx in various cell types in a potential positive feedback mechanism. To address the compatibility of these mechanisms between lower eukaryotes and mammals, we have stably expressed bovine PKCα in the yeast *Saccharomyces cerevisiae*. We find that phorbol ester binding sites are created which stimulate a specific calcium- and phospholipid-dependent catalytic activity in vitro. Phorbol ester activation *in vivo* stimulates PKC down-regulation, uptake of extracellular Ca**+**, Ca**+** dependence of cell viability, and changes in cell morphology. This may represent activation of a putative PKC-mediated signaling pathway utilized by functional yeast homologs of mammalian PKC isoforms. These are suggested by some protein data; however, their genes have not yet been characterized (Simon, A. J., Milner, Y., Saville, S. P., Dvir, A., Mochly-Rosen, D., and Orr, E. (1991) *Proc. R. Soc. Lond. B* 243, 165–171). Our findings indicate that bovine PKCα is functional in yeast and stimulates calcium uptake in a manner similar to some of its responses in mammalian cells, which suggests compatible aspects of higher and lower eukaryotic signaling pathways and the feasibility of dissecting parts of the action of common signaling mediators in a simple genetic model.

Ca**+** is an important second messenger in eukaryotic cell cycle regulation, hormone action, neurotransmitter release, muscle contraction, fertilization, and lymphocyte activation (reviewed by Alkon and Rasmussen (1988) and Campbell (1983)). Various stimuli induce a transient rise in cytoplasmic [Ca**+**] by opening Ca**+** channels in the plasma membrane for influx of extracellular Ca**+** or by membrane phosphoinositide lipid hydrolysis to diacylglycerol and inositol 1,4,5-trisphosphate (reviewed by Berridge (1987)). Inositol 1,4,5-trisphosphate stimulates release of free Ca**+** from internal stores, whereas, in a synergistic pathway, diacylglycerol activates the calcium- and phospholipid-dependent phorbol ester receptor protein kinase C (PKC), which plays a role in extracellular Ca**+** influx in various cell types (reviewed by Berridge (1987), Alkon and Rasmussen (1988), and Campbell (1983)).

In the lower eukaryote *Saccharomyces cerevisiae* intracellular Ca**+** is essential for cell cycle progression (Iida et al., 1990a), which is comparable to its obligatory role in mediating G1 events in mammalian cells (reviewed by Alkon and Rasmussen (1988) and Campbell (1983)). During the late stage of the mating pheromone response Ca**+** influx is stimulated (Ohsumi and Anraku, 1985) and cell viability becomes dependent on extracellular Ca**+** (Iida et al., 1990b). In *S. cerevisiae* a PKC-related, diacylglycerol-stimulated but phorbol ester-unresponsive protein activity with distinct substrate specificity has been described (Ogita et al., 1990; Iwai et al., 1992), as well as putative mammalian-like PKC isoforms in a separate study (Simon et al., 1991, 1992). Independently a related, essential gene PKC1 has been identified with a cell cycle-specific role in osmotic stability and perhaps in bud morphogenesis, which is, however, not complemented by mammalian PKC isoforms when disrupted (Levin et al., 1990; Levin and Bartlett-Heubusch, 1992).

To begin to address the compatibility of these signaling mechanisms between higher and lower eukaryotes we have stably expressed bovine PKCα in *S. cerevisiae*. We find that phorbol ester binding sites are created which stimulate a specific calcium- and phospholipid-dependent catalytic activity *in vitro*. Phorbol ester activation *in vivo* stimulates PKC down-regulation, uptake of extracellular Ca**+**, Ca**+** dependence of cell viability, and changes in cell morphology, suggesting that bovine PKCα is fully functional in yeast and stimulates calcium uptake in a manner similar to some of its responses in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**cDNA Construction**—The complete protein-coding region of bovine protein kinase C α (Parker et al., 1986) was isolated and joined at the NcoI site at the translation initiation codon with a synthetic HindIII-NcoI adapter 5'-AGCTTAAAAA-3' and 3'-ATTTTTTGATAC-5' to optimize the sequence upstream of the ATG codon for maximum efficiency (Cigan and Donahue, 1987). The cDNA was truncated at the 3'-end by exonuclease Bal31 digestion and joined with the synthetic blunt end-XbaI adaptor 5'-TAACTAACTAAT-3' and 3'-ATTTTTTGATAC-5', which provides translation stop base pairs of the 3'-untranslated sequence (PKCα) and includes 10 base pairs of the 3'-untranslated sequence (PKCα) and a truncated cDNA lacking coding sequences for 149 (an 824-672 carboxy-terminal aa (CD149) were inserted into the HindIII and XbaI sites under control of galactose-inducible transcriptional elements in the high copy number yeast epimonal expression plasmid YEp62 containing the LEU2 gene for selection (Brosch et al., 1983).

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1 The abbreviations used are: PKCs, bovine protein kinase C α; aa, amino acid(s); ODiso, optical density at 600 nm; PBS, phosphate-buffered saline; PDBu, phorbol-12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; CD149, bovine PKCα deletion of an 824-672 (149 carboxy-terminal aa); MEF, 4-morpholinoethanesulfonic acid.
After ligation plasmids were amplified in Escherichia coli DH5α and were identified and confirmed by restriction analysis and DNA sequencing.

 Yeast Transformation and Culture Conditions—PKC expression plasmids and YEp52 (Broach et al., 1983) as a control were introduced into S. cerevisiae strain 354 (MAT a; pep4–5; prb1–1122; ura5–52, leu3–1121, rbi1–1200, lys2–801; [Lys2]m, [Lys2]m) by YEp52 transformation (Ito et al., 1985). Cells were routinely grown in leucine-free synthetic medium containing 2% glucose in liquid culture or on 1.5% agar plates to select for stable propagation of the expression plasmids. Transformation of mammalian PKC cDNAs was induced for 20 h with 2% galactose, which results in up to 1000-fold induction of transcription within 6 h in strain 354 (Hovland et al., 1989). PKC activity was routinely measured in response to a single dose of 1 μM PMA or in controls (−PMA) in response to 1 μM inactive isomer 4α-PMA (both LC Services, Woburn, MA) which was added at the start of each experiment. Cells were harvested from 100-ml cultures at a density of 0.5 OD600. All experiments were performed several times with comparable results, and representative data are shown.

Phorbol Ester Binding—Expressing cells were washed twice in 10 mM potassium phosphate buffer, pH 7.5, 20 mM EDTA, 10 mM dithiothreitol, washed once in 1 M sorbitol, 10 mM potassium phosphate buffer, pH 7.0, 1.1 M sorbitol, 0.5 mM CaCl2. Cells were incubated with 200 μl (1 unit) of glucosidase (β-glucuronidase/arylsulfatase from Helix pomatia, Boehringer Mannheim) for about 1 h under shaking at 30 °C to remove the cell wall and were resuspended in 5 ml PBS, pH 7.4 and cell lysis was performed at 4 °C (Beggia, 1978).

Phosphorylation of 3 pg of the specific pseudosubstrate derivative was determined by liquid scintillation spectroscopy.

RESULTS

To create yeast expression constructs, we inserted the complete protein coding cDNA of bovine PKCa (Parker et al., 1986) or a truncated cDNA lacking coding sequences for 149 (CD149) carboxy-terminal amino acids (aa) under the control of a galactose-inducible transcriptional promoter element into the high copy number episomal plasmid YEp52 (Broach et al., 1983). Yeast cells were transformed and plasmids were stably propagated by selection in leucine-free 2% glucose medium. Transcription was routinely induced with 2% galactose for 20 h in most experiments, and control-transformed cells were used to determine the experimental background.

To test whether normal and truncated PKC protein products were properly expressed, proteins from detergent cell extracts were separated on SDS-polyacrylamide gels and analyzed with PKC-specific antibodies in immunoblots as shown in Fig. 1A. Bovine PKCa, co-migrated with PKC isolated from rat brain (not shown) at M, 80,000 and the mutant CD149 lacking the carboxy-terminal 149 aa migrated at Mr, 60,000 as a consequence of the truncation. Expression levels of normal and truncated PKC forms appeared to be overall comparable based on band intensities observed on the original immunoblot in Fig. 1A and based on comparable phorbol ester binding levels to expressing yeast cells (Fig. 1B).

To directly test the function of the regulatory PKC domain, phorbol ester binding sites were determined after enzymatic removal of the yeast cell wall by exposure of yeast spheroplasts to [3H]phorbol-12,13-dibutyrate ([3H]PDBu). As shown in Fig. 1B, expression of normal PKCa as well as the truncation mutant CD149 resulted in [3H]PDBu binding levels that were at least 13-fold elevated over background levels of control-transformed yeast cells. A similar increase in PDBu binding has been observed upon PKC expression in transfected mammalian fibroblasts (Knopf et al., 1986). These data suggest that the truncation not unexpectedly does not interfere with the function of the PKC regulatory domain and that expression levels are comparable between both PKC forms. Scatchard analysis shown in Fig. 1C suggests 10-fold higher binding sites/yeast cell (10-μm diameter) with a low affinity of Kd, 100 nM for normal PKCa. Affinity constants have been reported in mammalian cell types depending on the experimental conditions from subnanomolar Kd values to low affinities of Kd, 60 nM in PB-3c murine mast cells (Mazurek et al., 1987), suggesting that the yeast plasma membrane may not provide an optimal environment to detect high affinity PDBu binding.

To characterize the enzymatic activity of bovine PKCa expression products in yeast, phosphorylation of the PKC-specific [Ser-25]PKCa(19–31) substrate peptide, a derivative of the pseudosubstrate sequence (House and Kemp, 1987) was measured in detergent cell extracts. Compared to control cell extracts, PKCa led to a 50-fold increase in peptide phosphorylation, which was clearly calcium- (Fig. 2A) and phospholipid-dependent with a Kd of 50 μg/ml for phosphatidylserine (Fig. 2B) comparable to the characteristics reported in
Calcium Uptake by Bovine Protein Kinase Cα in Yeast

**FIG. 1.** Functional expression of bovine PKCα in yeast. A, immunoblot of normal and truncated bovine PKCα. Detergent extracts of yeast cells expressing control plasmids (Cont.), normal PKCα (PKCa), or a truncation mutant lacking 149 aa of the carboxyl terminus (CD149) were separated on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and analyzed by immunoblotting with a specific antibody to mammalian PKCα provided by Oliver et al. (1991). Size markers are indicated in kDa. B, [3H]PDBu binding to yeast spheroplasts expressing normal and truncated PKCα. [3H]PDBu binding to yeast spheroplasts transformed with control plasmids (Cont.) or expressing normal PKCα (PKCa) or truncation mutant CD149 was compared. Background binding determined in the presence of a 2000-fold excess of unlabeled PDBu has been subtracted. C, Scatchard analysis of [3H]PDBu binding to yeast spheroplasts expressing PKCα.

mammalian cells (Hannun et al., 1985). As observed for PKCfrom mammalian cells (Hannun and Bell, 1990), at low calcium concentrations catalytic activity was stimulated by the activators diacylglycerol (not shown) and the phorbol ester phorbol 12-myristate 13-acetate (PMA) 20-fold (Fig. 2A). Deletion of the carboxyl-terminal 149 aa in CD149, which likely leads to inactivation of the kinase, resulted in the complete loss of catalytic activity (Fig. 2A). PMA insignificantly affected the $K_m$ (from 34 to 64 μM) and caused a 30-fold increase in the $V_{max}$ (from 1,800 to 57,000 cpm/min) for peptide substrate phosphorylation (Fig. 2C). Catalytic activity was also stimulated by other PKC activators including the indol alkaloid indolactam V (Fujiki et al., 1985) comparable to PMA, while the PMA response was abolished by PKC inhibitors such as the antibiotic polymyxin B (Zhereleva, 1989) (Fig. 2D). These results are consistent with proper expression and calcium- and phospholipid-dependent and

**FIG. 2.** Catalytic activity of normal (PKCa) and truncated (CD149) bovine PKCα in yeast. A, phorbol ester (PMA) and calcium (Ca++) dependence (PS, phosphatidylserine; Cont., cells transformed with control plasmids). B, phosphatidylserine dependence of PKCα. C, kinetics of PKCα substrate phosphorylation. D, stimulation of PKCα by indolactam V (Indo.) or by PMA and inhibition by polymyxin B (Plmx.). The phosphatidylserine concentration varied from 25 ng/ml to 2.5 μg/ml in B and was 160 μg/ml in all other experiments. The concentration of substrate peptide RFARKGSLRQKNV varied from 50 nM to 250 μM in the presence of 1 μM PMA (+PMA) or the inactive 4α-PMA (−PMA) in C.
phorbol ester-stimulated function of bovine PKCα in yeast cells.

Upon exposure to phorbol esters, mammalian cells respond by down-regulation of PKC which may play a role in PKC action (Rodriguez-Pena and Rozengurt, 1984; Young et al., 1988; Droms and Malkinson, 1991). To test whether a compatible cellular mechanism exists in yeast, expressing cells were exposed to PMA, cytosolic and membrane cell fractions were enriched, and equal amounts of total protein of each fraction were separated on SDS-polyacrylamide gels and analyzed in immunoblots. As shown in Fig. 3A, upon PMA treatment disappearance of PKC was observed from both cell fractions. In the membrane fraction after 5 h of PMA treatment, significant loss of PKCα was observed to less than half of the original levels, and after 18 h of PMA treatment, PKCα was hardly detectable. In the cytosolic fraction much weaker PKC protein levels were detected, proportional to the lower ratio of PKC to total protein in this fraction, whereas the same amount of total protein had been loaded on the gel for each fraction. After PMA treatment for 5 or 18 h, PKC was no longer detectable. In the membrane fraction two major PKC bands of M, 80,000 and 77,000 were observed, which similarly disappeared upon phorbol ester treatment. In the cytosolic fraction a major protein band was detected by the polyclonal antibody at M, 81,000 with constant intensity independent of PMA treatment. This band probably represents a different cytosolic protein that obscured much of the M, 80,000 PKC form, which was only marginally detectable below the background band. The traces of this M, 80,000 form disappeared in response to PMA treatment similar to the more prominent M, 77,000 PKC form in this fraction. Similar size heterogeneity has been described for PKC isolated from mammalian cells related to different levels of PKC phosphorylation (Borner et al., 1989); however, we have not addressed this question. Our findings suggest that bovine PKCα interacts with a cellular mechanism in yeast, which results in PKC down-regulation in response to phorbol esters compatible to responses observed in mammalian cells.

To test whether PKCα activation might result in other biological responses in yeast which affect cell morphology, cell proliferation was followed under the phase contrast microscope. In the absence of PMA cell morphology was always normal (not shown); however, PMA stimulation caused morphological changes similar to the mating factor-induced "shmoo" phenotype in up to 10% of PKCα-expressing cells, which were never observed in control cells (Fig. 3B).

Ca2+ influx has been reported in response to PKC activation in a variety of eukaryotic cells such as N. crassa plasmodial (Zherlova, 1989), Aplysia bag cell neurons (Strong et al., 1987), rat ventricular myocytes (Lacerda et al., 1988), and rat pituitary cells (Stojilkovic et al., 1988). To investigate whether mammalian PKCα expression leads to a similar response in yeast, 45Ca2+ uptake was measured after PMA stimulation (Fig. 4). While control cells did not respond, PMA-activation of PKCα led to an up to 4-fold increase, which was abolished by a defective catalytic domain (CD149) lacking 149 carboxyl-terminal aa as expected, suggesting that this response depends on activation of the kinase (Fig. 4A). PMA did not affect uptake of tracer 86Rb+ mixed with K+, indicating that the ion uptake response is specific for divalent Ca2+ ions as opposed to monovalent ions (Fig. 4B). At the same time glucose stimulates uptake of divalent as well as monovalent ions (Fig. 4B) by a mechanism including hyperpolarization of the cell membrane (Borst-Pauwels, 1981; Eilam and Othman, 1990).

The PMA stimulation of PKCα-mediated Ca2+ uptake was abolished by La3+ (Fig. 4C), an inhibitor of carrier mediated Ca2+ influx (Eilam and GROSSOWICZ, 1982), but it was largely unaffected by other divalent ions such as Mn2+ (not shown). Increasing Mg2+ concentrations resulted in decreased basal Ca2+ uptake (not shown), consistent with the idea that both ions may compete with a shared transport system in yeast (Borbolla and Pena, 1980). Our findings indicate that we are observing specific ion uptake rather than uptake due to permeabilization or other damage of cell membranes (Borbolla and Pena, 1980; Eilam and Othman, 1990). As shown in Fig. 4D, PMA stimulation of PKCα-mediated Ca2+ uptake was little affected by the monovalent ions Na+ and K+ or by amiloride which blocks Na+/H+ and Na+/Ca2+ exchange mechanisms, but the response was significantly increased even in control cells by Cd2+, which raises the cation permeability of the yeast cell membrane and results in intracellular acidification (Borbolla and Pena, 1980; Kessels et al., 1987). Only PMA-stimulated responses have been shown in Fig. 4D for control and PKCα-expressing cells. Responses of control or PKCα-expressing cells in the absence of PMA have been omitted for clarity, since they were indistinguishable from PMA-stimulated control cells as indicated in Fig. 4A. Part of the Ca2+ uptake response was measured in DEAE-dextran-permeabilized cells (not shown) suggesting that it involves Ca2+ uptake into vacuoles, the major cellular stores for Ca2+.

**Fig. 3. Biological responses to PKCα expression in yeast.** A, down-regulation of PKCα. PKCα-expressing cells were exposed to PMA (+) or the inactive 4α-PMA (−) for the times indicated, and membrane or cytosolic cell fractions were separated on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and analyzed by immunoblotting with a specific antibody to mammalian PKCα (Oliver et al., 1991). Size markers are indicated in kDa. B, yeast morphology changes by PMA activation of PKCα. Cells transformed with control plasmid (Cont.) or PKCα-expressing cells (PKCα) were cultured in normal 2% galactose medium for 7.5 h in the presence of 1 μM PMA, fixed in 3.7% formaldehyde, and photographed in phase contrast. The length of the panels corresponds to 0.5 mm.
Ca2+ dependence of yeast cell growth and viability after PMA activation of PKCa

Control or PKCa expressing cells were cultured with 2% galactose in the absence (−PMA) or presence (+PMA) of 1 μM PMA in Ca2+-supplemented (1 mM) (+Ca2+) or Ca2+-free (−Ca2+) synthetic minimal medium (Iida et al., 1990b). Loss of cell viability (% of stained cells) was evaluated after 10 h by vital staining in 0.01% methylene blue, 2% sodium citrate (Rose, 1975). Cell growth was measured by following the cell density photometrically for 2 weeks and a ODmax reading of 10-fold diluted cultures is shown.

| Nonviable cells (% of total) | Cell density (ODmax) |
|-----------------------------|----------------------|
| −Ca2+ +PMA                  | 4  2  0.52  0.53   |
| −Ca2+ −PMA                 | 3  5  0.55  0.49   |
| +Ca2+ −PMA                 | 4  13 0.53  0.52   |
| +Ca2+ +PMA                 | 4  35 0.54  0.00   |

Our findings indicate that bovine PKCα is fully functionally expressed in yeast and creates cellular phorbol ester binding sites. Expression products display substrate-specific calcium-, phospholipid-, and phorbol ester-responsive enzymatic activity in cell extracts in vitro and result in proportional biological consequences. Four responses which were stimulated by phorbol esters in vivo (PKC down-regulation, 45Ca2+ net uptake, Ca2+ dependence of cell viability, and altered cell morphology) all correlate well with the enzymatic activities measured in vitro.

We have detected PKCα in yeast whole cell detergent extracts (Fig. 1A) as well as in cytosolic and membrane fractions (Fig. 3A) consistent with its localization in mammalian cells (Kraft and Anderson, 1983). We have demonstrated disappearance of the protein from yeast membrane and cytosolic fractions upon PMA stimulation, which is consistent with PKC down-regulation. This has been described in mammalian cells and involves PKC proteolysis (Rodriguez-Pena and Rosengurt, 1984; Young et al., 1988). These findings indicate a compatible mechanism in yeast, which interacts with PKCα to cause its down-regulation. This may play a role in PKC action in mammalian cells, although this has not been demonstrated (Droms and Malkinson, 1991; Young et al., 1988).

Ca2+ influx has been described in response to PKC stimulation in various eukaryotic experimental systems (Strong et al., 1987; Lacerda et al., 1988; Stojilkovic et al., 1988; Zheleva, 1989). In higher eukaryotic cells, including mammalian cells, the activation of specific ion channels has been implicated in this response, with durations ranging from readily reversible (<5 min) to persistent (>30 min) PKC effects.

**DISCUSSION**

Ca2+ influx has been described in response to PKC stimulation in various eukaryotic experimental systems (Strong et al., 1987; Lacerda et al., 1988; Stojilkovic et al., 1988; Zheleva, 1989). In higher eukaryotic cells, including mammalian cells, the activation of specific ion channels has been implicated in this response, with durations ranging from readily reversible (<5 min) to persistent (>30 min) PKC effects.
Calcium Uptake by Bovine Protein Kinase Cα in Yeast

(Reisman et al., 1988). In yeast an energy-independent cell surface Ca²⁺ uptake mechanism has been reported that exploits an electrochemical gradient (Eilam et al., 1990). Ca²⁺ is subsequently transported into the yeast vacuole in an energy-dependent process via the Ca²⁺/H⁺ antiporter (Ohsumi and Anraku, 1983), which is fueled by a proton gradient generated by the vacuolar H⁺-ATPase (Eilam et al., 1990).

The reported yeast Ca²⁺ uptake mechanism is not inconsistent with our own measurements in yeast cells. In our assay we measure the accumulation of extracellularly added ⁴⁵Ca²⁺ in the yeast cell from the technical short time limit of our experiments (10 min, not shown) up to 2 h. This accumulation is proportional to the activity of expressed PKCa. It is increased by phorbol ester stimulation and decreased to background levels if the kinase is inactivated as shown in Fig. 4A. Part of the Ca²⁺ uptake response was measured in DEAE-dextran-permeabilized cells (not shown), suggesting that it involves Ca²⁺ uptake into vacuoles, the major cellular stores for Ca²⁺ in yeast (Theuvenet et al., 1986). Our standard assay does not allow us to discriminate between mechanisms that may control ion fluxes across the plasma membrane or between the cytosol and yeast vacuole. It also does not normally address the direction of the ion fluxes regulated by PKC. However, the net accumulation of extracellularly added Ca⁴⁺ we are observing in the cell suggests an underlying Ca²⁺ flux across the cell membrane into the cytosol and subsequently into the vacuole. PKCa may directly modulate Ca⁴⁺ influx into the cell and/or vacuole. Alternatively, our data are consistent with a constitutive influx mechanism, where Ca⁴⁺ influx from the vacuole or across the membrane would be modulated by PKCa. Future unidirectional studies will be required to dissect the modulation of these ion fluxes in detail. As shown in Fig. 4B, the PMA stimulation of PKC-mediated Ca²⁺ net uptake is specific for divalent Ca²⁺ ions as opposed to monovalent Rb⁺/K⁺ ions. It remains largely unaffected by competition with monovalent ions Na⁺ and K⁺ and is not abolished by competition with divalent ions Mn²⁺ and Mg²⁺ (not shown). Increasing Mg²⁺ concentrations lead to reduced basal Ca²⁺ uptake, which results in a maximum PMA stimulation of up to 4-fold at 5 mM Mg²⁺. This may be due to a partially shared transport system for the divalent ions Mg²⁺ and Ca²⁺ in yeast (Borbolla and Pena, 1980). The lack of Mg²⁺ suppression of Ca²⁺ uptake by PMA-stimulated PKC may be due to modulation of a different mechanism, which remains to be identified and may involve vacuolar Ca²⁺ transport. Amiloride, a blocker of Na⁺/H⁺ and Na⁺/Ca²⁺ exchange mechanisms, does not affect PKC-mediated Ca²⁺ uptake, whereas La³⁺, an inhibitor of carrier-mediated Ca²⁺ influx, abolishes the response and Ca⁴⁺ significantly increases the uptake even in control cells by raising the cation permeability of the yeast cell membrane (Borbolla and Pena, 1980; Eilam and Grossowicz, 1982; Kessels et al., 1987) as shown in Fig. 4. Our findings suggest that activation of PKCa catalytic activity by phorbol esters stimulates net Ca²⁺ uptake into the yeast cell. The mechanisms involved may share some characteristics of a proposed cell surface channel and may include Ca²⁺ fluxes between the cytosol and the yeast vacuole, the major cellular stores for Ca²⁺ in yeast (Eilam et al., 1990; Theuvenet et al., 1988).

Several of the biological responses we observed after phorbol ester stimulation of PKCa, stimulation of net Ca²⁺ uptake, dependence on extracellular Ca²⁺ for cell viability, and changes in cell morphology have been reported upon activation of the mating pheromone response pathway (Marsh and Herskowitz, 1988; Iida et al., 1990b). Partial activation of this pathway at the initial receptor/G protein level has been shown after co-expression of mammalian β₂-adrenergic receptors with mammalian G, α protein subunits and removal of interfering yeast G α subunits (King et al., 1990). This resulted in the "shmoo" phenotype and in induction of the FUS1 gene promoter; however, actual cell mating was not stimulated, a result consistent with multiple roles of yeast pheromone receptors (King et al., 1990).

Since this is the only major signal transduction pathway characterized in yeast to date, we explored whether mammalian PKC might specifically interact with this pathway. We were unable to detect other established responses to this pathway upon PKCa stimulation, in particular, cell agglutination and induction of pathway-specific genes such as FUS1. Weak PKCa responses were occasionally observed in PKC-transformed, diploid yeast cells (not shown), whereas the mating factor response is specific to haploid cells. These findings demonstrate that bovine PKCa stimulation does not activate the mating pheromone response pathway. Cellular responses to PKCa activation may be stimulated by other mechanisms, and signaling pathways may exist in yeast which have not yet been established. PKCa activation did not affect yeast cell viability in normal culture medium; however, it rendered cells calcium-dependent, since no cell growth was observed in calcium-free medium after PKC activation (Table I). The lack of growth correlated with decreased cell viability as determined by vital staining: PMA treatment lowered cell viability 3-fold in PKC-expressing cells. The calcium dependence of cell viability and Ca²⁺ net uptake, which are both induced by PKCa activation, may be functionally related and involve common regulatory mechanisms that remain to be identified. Stimulation of net Ca²⁺ uptake appears physiologically meaningful in a state of Ca²⁺ dependence.

The endogenous PKC background in yeast is still controversial. We have not observed any evidence for phorbol ester-responsive yeast PKC activity in any of our assays. This is consistent with a described PKC-related diacylglycerol-stimulated yeast protein activity, which does not significantly respond to phorbol esters and displays distinct substrate specificity (Ogita et al., 1990; Iwai et al., 1992). In combination with the specific responses observed in our assays, these findings suggest that mammalian PKC isoforms can be selectively activated in yeast by phorbol esters and studied in the presently undefined yeast cellular background. The related, essential yeast gene PKC1 plays a role in cell cycle-specific osmotic stability; however, it is not complemented by mammalian PKC isoforms, including α, when disrupted (Levin et al., 1990; Levin and Bartlett-Heubusch, 1992) and consequently does not represent a functional yeast homolog of mammalian PKCα. The function of PKC1 characterized in yeast suggests a role in a common pathway with genes of the SKCα and BCKα locus, possibly in bud morphogenesis (Fields and Thomer, 1991; Levin and Bartlett-Heubusch, 1992; Lee and Levin, 1992).

Since the functional counterparts of mammalian PKC genes have not been identified in yeast we have expressed an available mammalian PKC isoform to exploit this advantageous, rapid, lower eukaryotic experimental model to study mammalian PKC signaling. The normal catalytic function of bovine PKCa and its biological responses in yeast suggest that it may, in response to phorbol esters, participate in a pathway shared with putative functional yeast homologs to mammalian PKC isoforms that have not yet been characterized at the gene level. The report of mammalian-like PKC isoforms in yeast suggests that they may exist (Sinon et al., 1991; 1992), and bovine PKCa may activate the putative signaling pathway they participate in. The future dissection of these interactions should be facilitated by the experimental advantages of the yeast model. The knowledge gained should help to elucidate the yeast signaling pathway involved as well as the molecular mechanisms of mammalian PKC action.
results suggest compatible aspects of higher and lower eukaryotic signaling pathways and support the perspective of dissecting parts of the action of common signaling mediators in a simple genetic model.

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Calcium Uptake by Bovine Protein Kinase C in Yeast