An Amphitropic cAMP-Binding Protein in Yeast Mitochondria. 3. Membrane Release Requires both Ca^{2+}-Dependent Phosphorylation of the cAMP-Binding Protein and a Phospholipid-Activated Mitochondrial Phospholipase†

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ABSTRACT: The amphitropic cAMP-binding protein in mitochondria of the yeast *Saccharomyces cerevisiae* is released from the inner membrane into the intermembrane space by the degradation of its lipid membrane anchor consisting of or containing phosphatidylinositol. The releasing reaction depends on the presence of an *N*-ethylmaleimide-sensitive protein (releasing factor) in the intermembrane space and is controlled by Ca^{2+} and phospholipid (or lipid derivatives). Here we demonstrate that these two effector molecules act on different activation steps within a complex releasing pathway involving both the cAMP receptor and the releasing factor: Ca^{2+}-dependent phosphorylation of the receptor protein seems to be prerequisite for its subsequent lipolytic liberation from the inner membrane. In the presence of phospholipid (or lipid derivatives) the previously soluble releasing factor, which may be identical with a soluble diacylglycerol-binding protein in the mitochondrial intermembrane space, associates with the inner membrane. This change in the intramitochondrial location of the releasing factor, which thus exhibits amphitropic behavior itself, may be required for (direct or indirect) activation of the mitochondrial phospholipase which then releases the cAMP receptor from the inner membrane in a form liable to dissociation from the C subunit by cAMP.

Proteins which occur both in a soluble cytoplasmic form and associated with cellular membranes belong to the newly recognized class of amphitropic proteins (Burn, 1988a). They exhibit properties of both cytoplasmic and peripheral or integral membrane proteins. The translocation of amphitropic proteins from a membrane to the cytoplasm of cells or vice versa is regarded as one form of information transfer from one cellular compartment to another.

The understanding of how translocation of amphitropic proteins is effected and of the enzymes involved in this process is poor. At the level of the membrane, local changes in the lipid composition may cause the direct insertion of an amphitropic protein at that site. The cytoskeletal linker protein α-actinin, for instance, has a high affinity for a certain subset of phospholipids of the plasma membrane (Burn, 1988b; Meyer et al., 1982; Rotman et al., 1982). Profilin, an actin-binding protein, interacts with phosphatidylinositol bisphosphate but has no affinity for its product of hydrolysis by phospholipase C, diacylglycerol (Lassing & Lindberg, 1985, 1988). Protein kinase C is translocated from the cytoplasm to the cytoplasmic side of the plasma membrane as soon as diacylglycerol becomes available in the lipid bilayer (Kraft et al., 1982; Cascales et al., 1984; Nishizuka, 1984). The interaction of lipids or lipid derivatives with these amphitropic proteins may induce a conformational change which results in the exposure of a hydrophobic structure on the protein's surface, originally hidden in the interior of the soluble version of the protein.

An increase in hydrophobicity could also be caused by dissociation of a cytoplasmic carrier protein which masks the membrane-insertion domain of the amphitropic protein. Such a mechanism has been demonstrated for the oncogenic tyrosine kinase $pp60^{v\text{-}src}$. In its inactive soluble state this protein is complexed by the soluble stress protein HSP90 (Courtneidge & Bishop, 1982; Brugge et al., 1983).

Alternatively, the trigger for a change in the cellular location of a protein may arise from the protein itself. Phosphorylation/dephosphorylation of a protein may cause the conformational change necessary for spontaneous membrane association without the need for prior interaction with specific lipids. The cytosolic form of the CTP:phosphocholine cytidylyltransferase of rat liver is translocated to the endoplasmic reticulum membrane upon dephosphorylation (Sleight & Kent, 1983; Cornell & MacLennan, 1985; Terce et al., 1988). Specific phosphorylation effects dissociation from the membrane (Pelech & Vance, 1982; Gilfillan et al., 1985; Radika & Possmayer, 1985). Another posttranslational modification which can provide the driving force for membrane association represents the covalent attachment of fatty acids, either directly via acylation [for review, see Schmidt (1983) and Sefton and Buss (1987)] or indirectly within a complete lipid moiety [for reviews, see Cross (1987) and Low (1987)].

In the preceding papers (Müller & Bandlow, 1989a,b) we demonstrate the amphitropic nature of a cAMP[1]-binding protein in mitochondria of the yeast *Saccharomyces cerevisiae*. This receptor is anchored in the inner membrane via a covalently attached phosphatidylinositol moiety. In the presence of Ca^{2+}, phospholipids, and an NEM-sensitive protein of the intermembrane space (releasing factor) this structure is degraded, and the receptor protein is released into the intermembrane space.

In this study we resolve the releasing process into several sequential steps and characterize the posttranslational mod-

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[1] Abbreviations: AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate; PDB, phorbol dibutyrate. For other abbreviations, see accompanying paper (Müller & Bandlow, 1989a).

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ifications involved. Ca^{2+}-dependent phosphorylation of the cAMP receptor and phospholipid-dependent binding of the amphitropic releasing factor to the inner mitochondrial membrane precede the final lipolytic cleavage reaction. Thus, both covalent modifications and noncovalent interactions play a role in a complex pathway which ultimately leads to a change in the intramitochondrial location of the cAMP-binding protein and results in the activation of a protein kinase A.

Materials and Methods

Materials. AMPPCP was purchased from Boehringer Mannheim; N^6-(2-aminoethyl)-cAMP–Sepharose was bought from Pharmacia, Freiburg; [^{3}H]PDB (2 Ci/mmol) was provided from Amersham-Buchler, Braunschweig; all other substances were obtained as reported previously (Müller & Bandlow, 1989a).

Yeast Strains, Growth Conditions, and Preparation of Mitochondria. Mitochondria were prepared from the protease-deficient yeast strain ABYS-66 (haploid), grown at 30 °C in lactate medium, as described (Rödel et al., 1985; Müller & Bandlow, 1987) and subsequently purified by equilibrium density centrifugation on 28% Percoll gradients (Müller & Bandlow, 1989a).

Subfractionation of mitochondria was carried out as described (Müller & Bandlow, 1989a). In general, incubations with Ca^{2+} (50 μM) and TPA (500 ng/mL) were performed for 10 min at 25 °C and terminated by addition of EGTA (final concentration 0.1 mM). Reisolated mitochondria were subjected to limited osmotic shock by dilution with five volumes of 25 mM MOPS/KOH–1 mM EDTA, followed by incubation on ice and mechanical shear by five strokes in a loosely fitting Dounce homogenizer. Mitochondria having an intact inner but disrupted outer membrane ("shocked mitochondria") were separated into mitoplasts and intermembrane space proteins by two-step sucrose gradient centrifugation (1.5 M/0.4 M sucrose). The interface between the two sucrose layers contained mitoplasts and the supernatant above the sucrose soluble intermembrane space proteins. The two fractions were concentrated by centrifugation or precipitation with PEG, respectively, and analyzed for cAMP-binding activity.

cAMP-Binding Assay. Specific binding of cAMP to mitochondria or mitochondrial subfractions was determined by testing the retention of protein-bound [^{3}H]cAMP on mixed-ester cellulosic filters (Müller & Bandlow, 1989a).

Assay for Membrane Association of the Releasing Factor. For preparation of mitoplasts deprived of cAMP-binding protein (MP_{-cAMP}), mitochondria were treated with Ca^{2+} plus TPA and subjected to limited osmotic shock, mechanical shear, and two successive two-step sucrose gradient centrifugations (Müller & Bandlow, 1989a). The MP_{-cAMP}, recovered from the interface of the second run, were diluted 4-fold with 20 mM MOPS/KOH (pH 7.2)–1 mM EDTA and centrifuged through a cushion of 0.1 M sucrose (1 mL/10 mg of protein). The resuspended MP_{-cAMP} (5 mg/mL in assay buffer) had retained less than 15% of the total mitochondrial cAMP-binding activity. They were incubated with intermembrane space proteins (10 μg/50 μg of mitoplasts) (10 min, 25 °C) in order to associate releasing factor with the membranes. After supplementation with EGTA (final concentration 0.1 mM), excess soluble proteins were removed by two-step sucrose gradient centrifugation, subsequent passage of the vesicles through a 1-mL column of Sephadex G-10, and centrifugation through a 1-mL cushion of 0.1 M sucrose. Hybrid vesicles were generated by ultrasonic treatment (Branson sonifier, 10 pulses of 5 s each, intensity 3, microtip, on an ice–salt mixture) of these mitoplasts, deprived of cAMP-binding protein, and enriched in membrane-associated releasing factor (400 μg in assay buffer) and an equal amount of untreated mitoplasts, having cAMP-binding protein. After incubation (10 min, 25 °C) and addition of EGTA (final concentration 0.1 mM), soluble proteins were separated from the vesicles by two-step sucrose gradient centrifugation and analyzed for cAMP-binding activity. The cAMP-binding activity of the soluble proteins (released) is given as the percentage of the sum of binding activities of the membrane vesicles and soluble proteins together.

Photoaffinity Labeling. Purified mitochondria or mitoplasts were incubated with 8-N_3-[^{32}P]cAMP in the dark and subsequently irradiated with UV_{260} on microtiter plates. After centrifugal gel filtration through Sephadex G-25 columns, the samples were precipitated with 5% TCA and analyzed by SDS–PAGE and fluorography.

Phosphorylation of the cAMP Receptor. Mitochondria (500 μg), solubilized in column buffer (25 mM MOPS/KOH, pH 7.0, 100 mM NaCl, 4 mM $MgCl_2$, 0.4 mM EGTA, 0.5 mM DTT, 0.5% MEGA 10, 5 mM NaF, 10 mM glycerol 3-phosphate, 2.5 mM sodium molybdate, 0.1 mM IBMX, 0.1 mM PMSF) (2 mg/mL), were applied to a 2-mL column of N^6-(2-aminoethyl)-cAMP–Sepharose, equilibrated with column buffer, at 4 °C. The column was washed five times with 2 mL of washing buffer (binding buffer supplemented with 125 mmM KCl and 10% glycerol; Müller & Bandlow, 1989a) and eluted with 2 mL of elution buffer (washing buffer supplemented with 100 mM cAMP) in the cold (Bandlow et al., 1985). The initial 250 μL of the eluate was centrifuged through a 1-mL column of Sephadex G-25 equilibrated with kinase buffer [25 mM MES/KOH, pH 7.0, 5 mM $MgCl_2$, 10 mm DTT, 50 μM EDTA, 50 μM PMSF, 0.4% MEGA 10, 5 mM NaF, 10 mM glycerol 3-phosphate, 5 mM $NaPP_i$ ($Na_4P_2O_7$), 2.5 mM sodium molybdate, 5% glycerol] and precipitated with an equal volume of 40% PEG 4000 in 10 mM MOPS/KOH (pH 7.2) and 1 mM EDTA. After centrifugation (15 min, 4 °C, microfuge) the pellet was dissolved in 40 μL of kinase buffer and incubated in the presence of 2 μCi of [γ-^{32}P]ATP (20 min, 30 °C). The reaction was terminated by the addition of two volumes of quench solution (10% TCA, 1% BSA, 1% SDS, 2 mM ATP, 40 mM NaH_2PO_4, 5 mM $NaH_2P_2O_7$, 100 mM NaF, 5 mM EDTA), rapidly mixed, incubated (30 min, 4 °C), and centrifuged (5 min, 4 °C, 75000 rpm, Beckman TL-100, fixed-angle rotor). The pellet was suspended in 500 μL of quench solution and centrifuged again for 1 min. The washed pellet (twice with acetone, once with ethanol) was dissolved in sample buffer and analyzed by SDS–PAGE and autoradiography.

Analytical Procedures. Polyacrylamide gel electrophoresis [5% (w/v) acrylamide stacking gels and 12% or 20% (w/v) acrylamide running gels, presence or absence of SDS and urea], Western blotting, fluorography, protein determination, and enzymic assays were carried out as reported in the accompanying papers (Müller & Bandlow, 1989a,b).

Results

Release of Mitochondrial cAMP-Binding Protein Can Be Resolved into a Ca^{2+}-Dependent and a Phospholipid/Releasing Factor Dependent Step. Efficient removal of the lipid membrane anchor from the cAMP-binding protein in yeast mitochondria in vitro requires Ca^{2+}, phospholipids, and a NEM-sensitive protein (releasing factor) of the intermembrane space. Since Ca^{2+} alone causes only very limited receptor release and phospholipids plus releasing factor in the absence of Ca^{2+} are completely ineffective, all three effector molecules seem to act in a synergistic rather than an additive fashion.

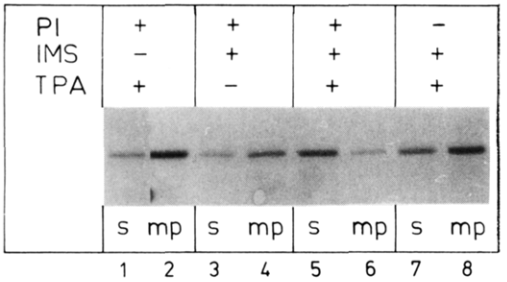

FIGURE 1: Two-step release of the cAMP-binding protein. Photoaffinity labeled mitochondria (1.6 mg) were incubated with Ca^{2+}, supplemented with phosphatase inhibitors (final concentrations: NaF, 1 mM; G-3-P, 2 mM; sodium molybdate, 5 mM; $NaPP_i$, 10 mM) and EGTA (final concentration 100 μM), and converted to mitoplasts. After centrifugation through a 2-mL cushion of 0.1 M sucrose, four aliquots of the resuspended mitoplasts (4 mg/mL in assay buffer plus 0.5 mM EGTA) were incubated in the presence or absence of 80 μg of intermembrane space proteins with or without TPA and phosphatase inhibitors (PI) (see above). The mixtures were separated into mitoplasts (mp) and soluble proteins (s) by two-step sucrose gradient centrifugation and precipitated with 66% $(NH_4)_2SO_4$. The TCA- and acetone-washed pellets were analyzed by SDS-PAGE (12%) and fluorography.

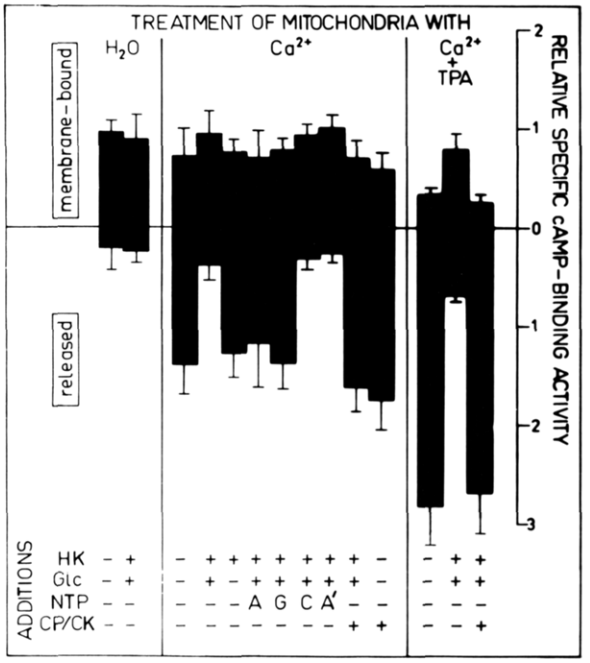

FIGURE 2: Energy requirement of the releasing process. Mitochondria (2 mg/mL in SEM buffer) were incubated in the presence or absence of 25 units/mg hexokinase (HK) with or without 10 mM glucose (Glc) (10 min, 30 °C) and subsequently supplemented with either 2 mM (final concentration) ATP (A), 2 mM GTP (G), 5 mM CTP (C), 10 mM AMPPCP (A′), or 20 mM creatine phosphate, 5 μg/mL creatine kinase, and 2 mM ADP (CP/CK). After further incubation (5 min, 30 °C) the samples were cooled on ice and aliquots treated with or without Ca^{2+} and TPA and centrifuged through a 0.5-mL cushion of 0.4 M sucrose. The resuspended mitochondria (2 mg/mL in assay buffer) were separated into mitoplasts (membrane bound) and intermembrane space proteins (released). Both fractions were assayed for cAMP-binding activity. The relative specific cAMP-binding activity of untreated solubilized mitochondria was set as 1 (0.37 pmol/mg of protein).

However, in previous work it remained unclear whether they all participate in the lipolytic cleavage reaction or whether there are several distinct steps within a complex releasing pathway which depend on one or another of these effectors. Therefore, we tried to resolve the overall releasing process into Ca^{2+}-dependent and phospholipid/releasing factor dependent steps.

To elucidate the sequence of events, the overall reaction was divided into two parts. In the first incubation whole mitochondria, photoaffinity labeled with 8-N_3-[^{32}P]cAMP, were treated with Ca^{2+} in the presence of an energy-regenerating system. After removal of free Ca^{2+}, the mitochondria were converted to mitoplasts. These were supplemented with a combination of TPA, intermembrane space proteins, and phosphatase inhibitors in the absence of Ca^{2+} in the second incubation. Subsequently, the mixtures were separated into mitoplasts and soluble proteins. Figure 1 illustrates that the releasing process can, in fact, be divided into two consecutive steps. The presence of TPA and intermembrane space proteins during the second incubation caused some solubilization of the receptor from the mitoplast membrane (lane 7) which was dramatically increased after the addition of phosphatase inhibitors (lane 5). By contrast, when the second incubation was performed with either TPA or intermembrane space fraction alone, it was significantly less effective in stimulating receptor release despite the presence of phosphatase inhibitors (lanes 1 and 3). The amount of receptor solubilized under these conditions was comparable to that released in the presence of Ca^{2+} alone. Since Ca^{2+} is indispensable for the release (Müller & Bandlow, 1989a), we conclude that the first incubation in our two-step releasing process includes a Ca^{2+}-dependent reaction which is distinct from the phospholipid/releasing factor dependent, but Ca^{2+}-independent reaction occurring during the second incubation. The dramatic increase of releasing efficiency in the presence of phosphatase inhibitors suggests that protein phosphorylation may be involved in one or another step of the releasing pathway.

We wanted to confirm this latter point by demonstrating the need for ATP hydrolysis as a prerequisite for the release of the cAMP receptor. Mitochondria were depleted of ATP by preincubation with limiting amounts of hexokinase/glucose and subsequently supplemented with an excess of nucleoside triphosphates or an energy-regenerating system, consisting of creatine kinase, creatine phosphate, and ADP, or were not supplemented. After incubation with (or without) Ca^{2+} and TPA, mitochondria were separated into mitoplasts and intermembrane space proteins. Both fractions were assayed for cAMP-binding activity (Figure 2). Absence of ATP during the incubation (preincubation with hexokinase plus glucose, but not with hexokinase alone) inhibited the (moderate) Ca^{2+}- as well as the (efficient) Ca^{2+}/TPA-dependent release of cAMP-binding protein from the inner membrane. Solubilization of the receptor was restored by the presence of an energy-regenerating system, ATP, or GTP but not of CTP. These data and the failure of the nonhydrolyzable ATP analogue AMPPCP to effect the release demonstrate the requirement for hydrolysis of ATP or GTP. Since the Ca^{2+}-induced release, which represents the Ca^{2+}-dependent step in the two-step releasing process, failed to occur after ATP depletion, a Ca^{2+}-dependent phosphorylation may be one prerequisite for the phospholipid/releasing factor dependent step. This view was confirmed by the observation that the Ca^{2+}-dependent step could be reversed by dephosphorylation (with protein phosphatase) only as long as the phospholipid/releasing factor dependent step had not yet occurred (data not shown). We propose that the Ca^{2+}-dependent reversible phosphorylation is followed by the phospholipid/releasing factor dependent reaction which renders the release highly efficient and irreversible.

Release from the Inner Membrane of the cAMP-Binding Protein Is Accompanied by Its Ca^{2+}-Dependent Phosphorylation. Next we investigated whether the cAMP receptor

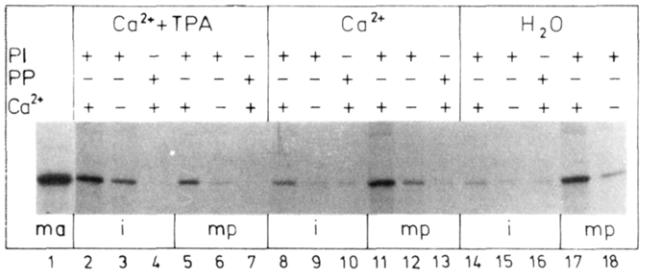

FIGURE 3: Ca^{2+}-dependent phosphorylation of the cAMP receptor. Mitochondria (1.2 mg) were incubated with H_2O, Ca^{2+}, or Ca^{2+} plus TPA and separated into mitoplast (mp) and intermembrane space fraction (i). Both fractions, recovered by centrifugation or precipitation, respectively, and solubilized in column buffer plus 0.5% MEGA 10, were subjected to cAMP–Sepharose affinity chromatography. Three aliquots of the protein of each fraction, eluted by 0.1 M cAMP, received, after dialysis, PEG precipitation, and solubilization in kinase buffer, either 100 μM Ca^{2+} plus phosphatase inhibitors (PI), 100 μM Ca^{2+} plus 2 mg/mL protein phosphatase 2A (PP), or 50 μM EGTA plus phosphatase inhibitors (PI) (see legend to Figure 1). All samples were incubated with 250 nCi of [γ-^{32}P]ATP (30 min, 30 °C). The reaction was terminated by precipitation with 5% TCA, and the pellets, after being washed, were analyzed by SDS–PAGE (12%) and autoradiography. Total protein from Ca^{2+}/TPA-treated and photoaffinity labeled mitochondria was run on the same gel as a marker (ma).

itself is a target for the putative Ca^{2+}-dependent phosphorylation. Mitochondria were incubated with or without Ca^{2+} and TPA and separated into mitoplasts and intermembrane space proteins. After solubilization both fractions were subjected to cAMP affinity chromatography. The eluates were incubated with [γ-^{32}P]ATP in the presence or absence of Ca^{2+}, protein phosphatase, and phosphatase inhibitors.

Figure 3 demonstrates that, in fact, a protein was radiolabeled, indicating that kinase and target must have been coeluted from the cAMP affinity column. The labeled protein comigrated with the authentic photoaffinity labeled cAMP receptor (ma). The intramitochondrial distribution of the phosphorylated protein after incubation of mitochondria with Ca^{2+} and TPA was similar to that previously observed for the photoaffinity labeled cAMP receptor: Treatment with Ca^{2+} plus TPA led to the appearance of the major fraction of the labeled protein in the intermembrane space (compare lanes 3 and 6). By contrast, Ca^{2+} alone caused only a moderate increase of label in this compartment (compare lanes 9 and 12) relative to the distribution in control mitochondria where virtually all of the labeled protein was recovered from the mitoplast fraction (compare lanes 15 and 18). Furthermore, the extent of phosphorylation of the receptor protein under all three incubation conditions, i.e., in both mitochondrial locations, was stimulated significantly by Ca^{2+} (lanes 2 vs 3, 5 vs 6, 8 vs 9, 11 vs 12, 14 vs 15, and 17 vs 18). The nature of the radiolabel as phosphate incorporation into the protein was confirmed by the ability of protein phosphatase 2A to remove the label from the protein, even in the presence of Ca^{2+} (lanes 4, 7, 10, and 13). Both comigration in SDS–PAGE and similar intramitochondrial distribution of the photoaffinity labeled cAMP receptor and the phosphorylated protein suggest their identity. Furthermore, the two differently labeled proteins shared many peptides, generated by limited proteolysis with endoprotease V8, which comigrated during one-dimensional SDS–PAGE (data not shown). Thus it seems very likely that the mitochondrial cAMP-binding protein can be phosphorylated in a Ca^{2+}-dependent fashion. Coelution from a cAMP affinity column of the R subunit together with the R subunit phosphorylating kinase suggests autophosphorylation at least as one mechanism to achieve this modification.

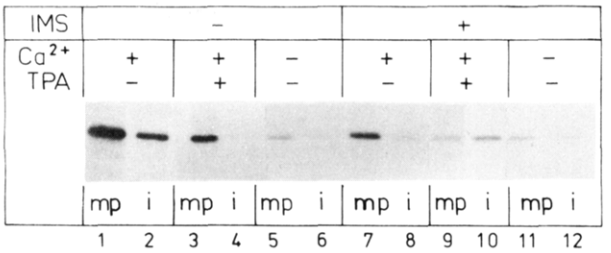

FIGURE 4: Phosphorylation of the cAMP receptor during its release. Two aliquots of mitoplasts (1.2 mg each) (2.5 mg/mL in kinase buffer plus 50 mM sucrose, 2 μCi of [γ-^{32}P]ATP, and phosphatase inhibitors, see legend to Figure 1) were supplemented with 480 μg of intermembrane space proteins (+IMS) or not supplemented (–IMS). Both samples were divided into three aliquots and incubated with either Ca^{2+} alone or Ca^{2+} plus TPA or 50 μM EGTA (30 min, 30 °C). Mitoplasts (mp) and intermembrane space proteins (i), recovered from a two-step sucrose gradient, were supplemented with column buffer plus 0.5% MEGA 10, applied to a cAMP–Sepharose column (1 mL), eluted, and analyzed by SDS–PAGE (12%) and autoradiography.

Now it was of interest whether the cAMP receptor is phosphorylated in a Ca^{2+}-dependent manner only after its partial purification by affinity chromatography or also in situ in whole mitochondria during incubation with Ca^{2+} (and TPA). In addition, we studied whether this modification is correlated with a change of the intramitochondrial location of the receptor.

For this purpose mitoplasts were treated with a combination of Ca^{2+}, TPA, and isolated intermembrane space proteins and then incubated with [γ-^{32}P]ATP. The separated mitoplast and intermembrane space fractions were applied to a cAMP affinity column and the eluates analyzed by SDS–PAGE (Figure 4). In the presence of Ca^{2+} the cAMP receptor was strongly phosphorylated whether TPA or intermembrane space proteins had been added or not (lanes 1–4 and 7–10). Without Ca^{2+} only very weak labeling of the receptor was obtained (lanes 5, 6, 11, and 12). TPA plus intermembrane space fraction (lanes 9 and 10), but none of these components alone (lanes 3, 4, 7, and 8), caused significant release of the Ca^{2+} dependently radiolabeled protein from the inner membrane into the intermembrane space. This experiment demonstrates that Ca^{2+}-dependent phosphorylation of the cAMP receptor occurs during its release and strongly suggests a functional relationship between these two events.

Releasing Factor Binds to Diacylglycerol Analogues. Since TPA stimulates receptor release only in the presence of the releasing factor (see Figure 1), we speculated that TPA and this NEM-sensitive protein together effect the Ca^{2+}-independent step of the releasing process, perhaps by interacting with each other. To test this hypothesis, we resolved the phospholipid-dependent and releasing factor dependent steps into a reaction which does not involve the cAMP receptor directly and into the final lipolytic cleavage of the receptor from the membrane. This was achieved by preincubation of native or NEM-treated intermembrane space proteins with or without TPA. Excess unbound TPA was subsequently removed by passage through a Sephadex G-10 column. Photoaffinity labeled mitoplasts were added to the eluate and incubated in the presence of various combinations of Ca^{2+}, TPA, and untreated intermembrane space proteins. Subsequently, the mixtures were separated into mitoplasts and soluble proteins and analyzed by SDS–PAGE.

Figure 5 shows that native intermembrane space proteins, incubated with TPA before addition to the mitoplasts (preincubation), caused the release of the major portion of photoaffinity labeled receptor from the mitoplasts. However, this happened only when Ca^{2+} was present during the second

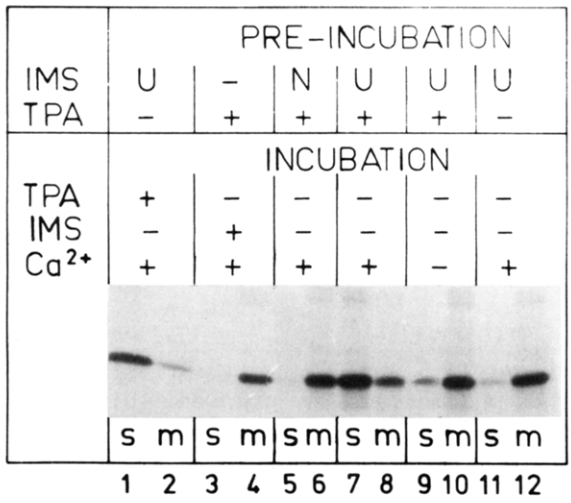

FIGURE 5: Interaction of the releasing factor with TPA. Photoaffinity labeled mitochondria were separated into mitoplast and intermembrane space fractions. Preincubation: One out of five aliquots of intermembrane space proteins (+IMS) (125 μg each) was treated with 1 mM NEM (N) (15 min, 0 °C, terminated with 2 mM DTT). The others remained untreated (U). All samples and a control, lacking intermembrane space proteins (–IMS), were incubated with TPA (5 min, 4 °C) and then passed through a 2-mL column of Sephadex G-10. The eluate (500 μL) was concentrated 5-fold by ultrafiltration through an Amicon XM-300 membrane. Incubation: Six aliquots of purified mitoplasts (400 μg each) were supplemented with 80 μg of pretreated or untreated intermembrane space proteins (IMS) or with the control sample (–IMS) and incubated with or without Ca^{2+} plus TPA. All samples were separated into mitoplasts (m) and soluble proteins (s) by two-step sucrose gradient centrifugation and analyzed by SDS–PAGE (12%) and fluorography.

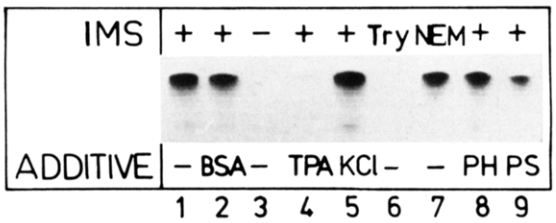

FIGURE 6: Identification of a phorbol ester binding protein in the intermembrane space. A total of 200 μg of intermembrane space proteins (2 mg/mL in assay buffer), either untreated (+IMS) or pretreated with trypsin (Try) or NEM, was supplemented with 40 nM [^{3}H]PDB (200 nCi), 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin, 10 μg/mL α_2-macroglobulin, and 100 μM EDTA, incubated in the presence of either 0.5% BSA, 3 μM TPA, 250 mM KCl, 3 μM phorbol acetate (PH), or 5 μM phosphatidylserine (PS) in a total volume of 200 μL (15 min, 4 °C), and then spun through a 1-mL column of Sephadex G-10. The eluate (250 μL) was concentrated 5-fold by centrifugation through an Amicon microconcentrator (XM-300 membrane) and electrophoresed (12% polyacrylamide gel) in the absence of SDS and mercaptoethanol and the presence of 200 μM PMSF overnight at 4 °C. Separated proteins were transferred to a nitrocellulose filter (Towbin et al., 1979) for 15 min at 4 °C (absence of SDS and urea, presence of 200 μM PMSF). The dried filter was autoradiographed.

incubation (compare lanes 7 and 9). Omission of TPA (lanes 11 and 12) or provision of NEM-treated intermembrane space proteins (lanes 5 and 6) in the preincubation left most of the receptor with the mitoplasts (lanes 6 and 12). This demonstrates that the two temporally and spatially dissected reactions of the releasing pathway together mimicked the overall reaction. TPA in the absence of intermembrane space proteins failed to exceed receptor solubilization caused by Ca^{2+} alone despite the addition of intermembrane space proteins to the subsequent incubation (lanes 3 and 4). By contrast, the presence of intermembrane space proteins alone during the preincubation and the subsequent addition of TPA released most of the labeled receptor (lane 1). This demonstrates that free TPA was efficiently removed from the intermembrane space protein fraction by the gel filtration. Thus, TPA, coeluting with intermembrane space proteins and then inducing receptor release, interacted with a NEM-sensitive component of the intermembrane space which was probably identical with the releasing factor. These data strongly suggest that the phospholipid/releasing factor dependent step involves the binding of phospholipid to the soluble releasing factor prior to the releasing factor dependent lipolytic removal of the membrane anchor from the cAMP receptor.

In an attempt to demonstrate an interaction between phospholipid (or lipid derivatives) and a protein of the intermembrane space in a more direct fashion, intermembrane space proteins which had or had not been treated with trypsin or NEM were incubated with the ^{3}H-labeled diacylglycerol analogue PDB in the presence of certain additives. Subsequently, the soluble proteins were separated from free PDB by passage through a Sephadex G-10 column and analyzed by native gel electrophoresis, transfer to nitrocellulose, and autoradiography. As can be seen from Figure 6, a unique band was radiolabeled when the native intermembrane space fraction had been incubated with labeled PDB (lane 1). No band was observed in the absence of intermembrane space proteins (lane 3) or with trypsin-treated proteins (lane 6). This demonstrated binding of PDB to a protein of the intermembrane space. The efficiency of labeling was not affected by high salt (lane 5) or the presence of BSA (lane 2) but was completely competed for by excess unlabeled TPA (lane 4), indicative of the specificity of this binding. NEM treatment of intermembrane space proteins or unlabeled TPA analogues (ineffective in membrane release) slightly diminished binding of labeled PDB. Although this experiment does not disclose the nature of the PDB-labeled protein, the specificity of the binding, the observed inactivation of both the soluble releasing factor and the PDB-binding protein by identical concentrations of trypsin, and their common intramitochondrial location suggest the identity of both proteins.

TPA Induces Association with Mitochondrial Membranes of the Releasing Factor. Since the soluble releasing factor of the intermembrane space seems to bind diacylglycerol analogues prior to releasing factor dependent cleavage of the membrane anchor of the membrane-bound cAMP receptor, we suspected that this interaction ultimately leads to an association of the releasing factor with the inner mitochondrial membrane. Such behavior has been observed for some amphitropic proteins interacting with the plasma membrane (see introduction).

To demonstrate the amphitropic nature of the releasing factor, we used mitoplasts deprived of the cAMP-binding protein by the previous combined action of Ca^{2+} and TPA. If our hypothesis is correct, these membranes should have incorporated releasing factor from the intermembrane space upon treatment with TPA and should consequently be able to serve as a source of releasing factor for liberation of the cAMP receptor from subsequently added untreated membranes. Thus, after reisolation of deprived mitoplasts from the interface of a two-step sucrose gradient, the association of the releasing factor with these vesicles was examined by assaying their ability to release the receptor from untreated mitoplasts after membrane fusion during a second incubation in the absence of intermembrane space proteins.

Figure 7 shows that mitoplasts lacking the receptor caused significant receptor release from untreated mitoplasts after fusion when Ca^{2+} plus TPA was present during both incuba-

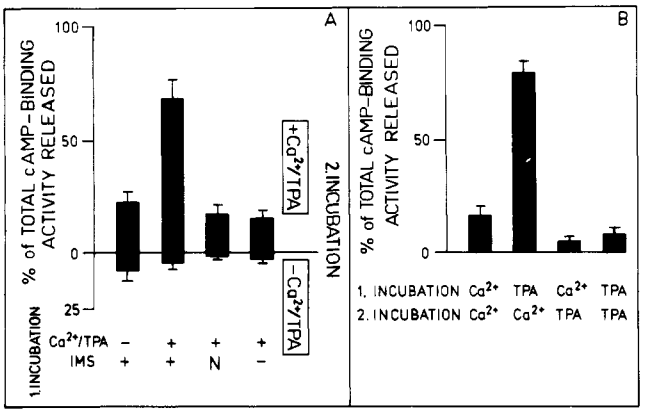

FIGURE 7: Association of the releasing factor with mitochondrial membranes. Panel A: Mitoplasts, deprived of cAMP-binding activity (400 μg each), were supplemented with untreated (+IMS) or NEM-treated (N) (1 mM, 15 min, 0 °C, stopped with 2 mM DTT) intermembrane space proteins or were not supplemented (−IMS) and were incubated with or without Ca^{2+} plus TPA (1. INCUBATION). The mitoplasts, recovered from two-step sucrose gradients, were assayed for association of the releasing factor in the presence or absence of Ca^{2+} plus TPA (2. INCUBATION). Panel B: The experiment followed that in panel A except that both incubations were carried out in the presence of untreated intermembrane space proteins and either Ca^{2+} or TPA.

tions (panel A). Omission of intermembrane space proteins in the first incubation or addition of NEM-treated intermembrane space proteins led to limited release which was caused by Ca^{2+} alone. Deprived mitoplasts which had been incubated first in the presence of intermembrane space protein but in the absence of Ca^{2+}/TPA failed to stimulate the release of receptor from the hybrid vesicles during the second incubation above the level observed in the presence of Ca^{2+} alone. Ca^{2+}/TPA present during the second incubation should guarantee efficient release if releasing factor were present, copurified with the deprived mitoplasts. Thus the releasing factor dependent liberation of the cAMP receptor occurred in the second incubation only if the factor had become previously bound to the mitoplasts in a Ca^{2+}/TPA-dependent fashion.

This experiment suggests that the releasing factor binds to mitochondrial membranes after interaction with phospholipid (or lipid derivatives) and, in a membrane-associated form, causes the release of the cAMP receptor. The observations that diacylglycerol analogues bind to a protein of the intermembrane space, most likely the releasing factor (Figures 5 and 6), and that Ca^{2+} stimulates phosphorylation of the cAMP receptor (Figures 3 and 4) tempted us to speculate that phospholipids mediate membrane binding of the releasing factor whereas Ca^{2+}, through phosphorylation, renders the membrane-bound cAMP receptor protein susceptible to lipolytic cleavage. We were able to verify this hypothesis, since the first incubation represents the interaction between releasing factor, phospholipids, and mitochondrial membranes and the second incubation the phosphorylation of the receptor and the cleavage of its membrane anchor.

Figure 7 (panel B) demonstrates that efficient release of the receptor occurred only when TPA was present during the first and Ca^{2+} during the second incubation, thus confirming our view. The reverse combination or only one effector present during both incubations resulted in an extent of receptor release which did not exceed the Ca^{2+}-dependent/releasing factor independent liberation.

Discussion

Four distinct reactions, characterized in this paper, are involved in the Ca^{2+}- and phospholipid-controlled release of the amphitropic cAMP-binding protein from the inner membrane into the intermembrane space of yeast mitochondria.

(*i*) *Binding of Phospholipids to a Protein of the Intermembrane Space.* The unique PDB-binding protein of the intermembrane space may be identical with the NEM-sensitive releasing factor in this compartment, which has been shown to be required for the lipolytic cleavage of the receptor's membrane anchor (Müller & Bandlow, 1989b). Binding of PDB to NEM-treated intermembrane space protein is not necessarily in conflict with this assumption, since phospholipid binding and catalytic activity may concern different domains of the releasing factor. Surprisingly, the binding of this diacylglycerol analogue is, to a certain degree, competed for by TPA analogues which are completely ineffective in releasing the receptor. One explanation could be that activation of the releasing factor not only involves the occupation of the lipid-binding site but also requires the interaction of the ligand with some other functional region, possibly the catalytic or activation domain of the phospholipase. Since Ca^{2+} is not required for phospholipid binding, it presumably acts at a different level within the releasing pathway.

(*ii*) *Binding of Releasing Factor to the Inner Mitochondrial Membrane.* Reconstitution experiments with mitoplasts from which the cAMP receptor had been removed demonstrated that the releasing factor of the intermembrane space becomes associated with mitochondrial membranes in a phospholipid-dependent fashion. Again, this event does not depend on Ca^{2+}. Since mitoplasts consist of both inner and outer membranes, the intramitochondrial site of the association remains unclear. However, the topology of the cAMP receptor strongly suggests the inner membrane for this event. The sequential order of the interactions between the releasing factor, phospholipids, and mitochondrial membranes has not yet been determined. Lipid vesicles with membrane-embedded releasing factor may fuse with the inner membrane. Alternatively, lipid molecules bound to releasing factor may mediate its spontaneous membrane association by causing local disturbance of the lipid bilayer. In any case, phospholipids or derivatives thereof seem to participate exclusively at the level of membrane binding of the releasing factor. In the case of identity of the putative mitochondrial phospholipase with the releasing factor (Müller & Bandlow, 1989b), phospholipid-dependent insertion into the inner membrane may either cause its activation or, if active in the soluble as well as in the membrane-associated state, allow its access to the membrane anchor of the cAMP receptor. Alternatively, the releasing factor activates, through membrane insertion, a phospholipase which is bound to the inner mitochondrial membrane.

(*iii*) *Ca^{2+}-Dependent Phosphorylation of the Receptor.* Besides the reconstitution experiment (Figure 7), several other approaches succeeded in dissecting the phospholipid-dependent and releasing factor dependent steps from the Ca^{2+}-dependent step: Mitochondria which had previously been incubated with Ca^{2+} are predisposed to release of the receptor in the presence of TPA even after removal of Ca^{2+} (Figure 1). Conversely, intermembrane space proteins, preincubated with TPA, stimulate receptor release from mitoplasts in a Ca^{2+}-dependent fashion even after the lipid analogue had been removed by column chromatography (Figure 5). The Ca^{2+}-dependent step is reversed by removal of Ca^{2+}, provided TPA is absent (data not shown).

Several lines of evidence indicate that the Ca^{2+}-dependent reaction is a Ca^{2+}-dependent phosphorylation of the cAMP receptor. First, affinity-purified cAMP receptor is phospho-

rylated in a Ca^{2+}-dependent manner (Figure 3). Second, the Ca^{2+}-dependent step depends on the hydrolysis of ATP or GTP (Figure 2) and is reversed by protein phosphatase (data not shown). Third and most convincing, the mitochondrial cAMP receptor is heavily phosphorylated in the presence of Ca^{2+} in isolated mitoplasts. TPA and intermembrane space proteins cause the efficient release of the phosphorylated receptor from the mitoplast membrane (Figure 4). However, the functional relationship between the Ca^{2+}/phospholipid-controlled amphitropism and the Ca^{2+}-dependent phosphorylation of the receptor remains to be established, and the protein kinase involved has to be identified. It seems clear that the protein kinase involved resides in the inner mitochondrial compartment since in the absence of intermembrane space proteins receptor phosphorylation also takes place and is even more pronounced, indicative of a protein phosphatase within the intermembrane space compartment. It is likely that the kinase is identical or forms a complex with the cAMP-dependent protein kinase, since it is coeluted with its target, the cAMP-binding protein, in cAMP affinity chromatography, suggesting that it acts as a Ca^{2+}-dependent kinase of the R subunit in the membrane-associated and a cAMP-dependent protein kinase in the solubilized state.

In addition to the R subunit and two inner membrane proteins of 40 and 59 kDa (Müller & Bandlow, 1989a), the kinase accepts acidic heterologous proteins for substrates like phosvitin and casein (Müller & Bandlow, 1987a,b) and thereby widely contrasts to the cytoplasmic cAMP-dependent protein kinase in yeast which preferentially phosphorylates histone fractions (Hixson & Krebs, 1980). The amino acid(s) serving as the actual target site(s) of this kinase is (are), however, not yet known. Although there is evidence for the presence of three protein kinase C like kinases in yeast and although the release of the cAMP receptor is Ca^{2+} and phospholipid dependent, such an activity is very unlikely to be involved in the phosphorylation step. The phosphorylation of the cAMP receptor was shown to require only the presence of Ca^{2+} ions and not to be influenced by phospholipids, which—as shown by the dissection of the releasing reaction—are necessary solely for the activation by membrane association of the releasing factor. The releasing factor appears to be no target for a protein kinase.

Ca^{2+} alone induces only limited receptor release in the absence of phospholipids and releasing factor. This can be explained by either of two ways: (i) The Ca^{2+}-dependent step of the releasing process may weaken the association between receptor and membrane without cleavage of the membrane anchor. (ii) Mitochondria and even mitoplasts may contain some active releasing factor which, in the absence of exogenously added phospholipids, would suffice for limited release through degradation of the membrane anchor. The reversibility of the Ca^{2+}-induced release and the presence of phosphatidylinositol in the Ca^{2+}-released receptor (Müller & Bandlow, 1989b) favor the first explanation.

(*iv*) *Lipolytic Removal of the Membrane Anchor from the Receptor.* Only phosphorylated cAMP receptor molecules seem to be substrates for the final cleavage reaction since the ratios of released to nonreleased phosphorylated and photoaffinity labeled receptor are comparable. Thus, a phosphorylation-induced conformational change of the receptor may be a prerequisite for the subsequent lipolytic degradation of the phosphatidylinositol membrane anchor of the cAMP receptor. Ca^{2+} is not required for the enzymic activity of the putative phospholipase.

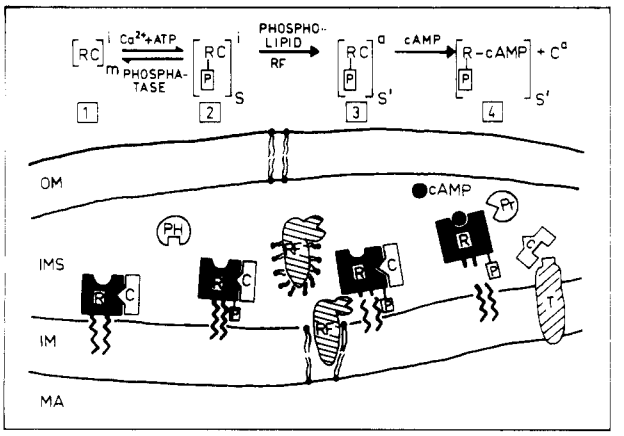

FIGURE 8: Working model for release of the mitochondrial cAMP receptor. (1) The cAMP-dependent protein kinase, consisting of a cAMP-binding regulatory subunit (R) and a catalytic subunit (C), is associated with the inner membrane (IM) of yeast mitochondria via the phosphatidylinositol membrane anchor of the R subunit. This membrane-bound (m) complex, [RC], is inactive (i) because it fails to dissociate into the subunits despite its ability to bind cAMP. (2) The interaction of the R subunit with the inner membrane is weakened by its Ca^{2+}-dependent phosphorylation, causing partial release (S) of the still inactive complex [RC] and an increased accessibility of the lipid anchor for lipolytic attack. This process is reversed by a soluble phosphatase (PH). (3) The soluble releasing factor (RF) of the intermembrane space binds to phospholipids, presumably at the inner face of the outer membrane (OM). The lipids are then transferred to the outer face of the inner membrane, whereby the releasing factor itself associates with the inner membrane. The intrinsic phospholipase activity of this NEM-sensitive protein then cleaves the lipid anchor of the R subunit, causing irreversible release (S′) of the complex [RC] into the intermembrane space. (4) The soluble complex [RC] can be activated (a) since binding of cAMP now causes its dissociation. The R subunit is then readily digested by proteases (Pr), and the active C subunit phosphorylates a 40-kDa target protein (T) of the inner membrane, thereby causing signal transmission into the matrix (MA).

Thus the Ca^{2+}- and phospholipid-controlled amphitropism of the mitochondrial cAMP receptor protein is based on two different principles: Ca^{2+}-mediated susceptibility of the receptor for cleavage by a mitochondrial phospholipase (presumably caused by Ca^{2+}-dependent phosphorylation) and phospholipid-mediated activation of this phospholipase. A hypothetical model for the complex releasing pathway which compiles our findings including those from the preceding papers is presented in Figure 8.

A central point, emerging from the activation of the mitochondrial phospholipase by phospholipids, concerns their physiological origin. Lipid biosynthesis is associated with specific membranes; e.g., in yeast phosphatidylinositol (Cobon et al., 1976), phosphatidylcholine (Dils & Hübscher, 1961; Guarnieri et al., 1971), and phosphatidylserine (Cobon et al., 1974) are made in the endoplasmic reticulum and outer mitochondrial membranes, whereas syntheses of phosphatidylethanolamine via decarboxylation of phosphatidylserine and of mitochondrial cardiolipin occur exclusively in the inner mitochondrial membrane (Hostetler & Van den Bosch, 1972; Jelsema & Morre, 1978; Dygas et al., 1980; Zborowski et al., 1983). Therefore, translocation of phospholipids from their site of synthesis to other membranes is necessary. Little is known about intraorganellar transport of lipids (Wirtz & Zilversmit, 1968; Wojtczak et al., 1971; Wirtz, 1974; Yaffe & Kennedy, 1983; Megli et al., 1986) and nothing about their transfer between the outer and inner mitochondrial membrane. It is tempting to speculate that a (additional) role of the described phospholipid-binding releasing factor consists of the shuttling of phospholipids across the soluble mitochondrial

intermembrane space compartment (Blok et al., 1971; Baranska & Wojtczak, 1984).

Registry No. Ca^{2+}, 7440-70-2; cAMP, 60-92-4; phospholipase, 9013-93-8.

References

Bandlow, W., Schwarz, U., Rödel, G., Strobel, G., & Wachter, C. (1985) *Biol. Chem. Hoppe-Seyler 366*, 545.

Baranska, J., & Wojtczak, L. (1984) *Biochim. Biophys. Acta 773*, 23.

Blok, M. C., Wirtz, K. W. A., & Scherphof, G. L. (1971) *Biochim. Biophys. Acta 233*, 61.

Brugge, J., Yonemoto, W., & Darrow, D. (1983) *Mol. Cell Biol. 3*, 9.

Burn, P. (1988a) *Trends Biochem. Sci. 13*, 79.

Burn, P. (1988b) *J. Cell. Biochem. 36*, 15.

Cascales, S. C., Martin, A., & Brindley, D. N. (1984) *Biochem. J. 219*, 911.

Cobon, G. S., Crowfoot, P. D., & Linnane, A. W. (1974) *Biochem. J. 144*, 265.

Cobon, G. S., Crowfoot, P. D., Murphy, M., & Linnane, A. W. (1976) *Biochim. Biophys. Acta 441*, 255.

Cornell, R., & MacLennan, D. M. (1985) *Biochim. Biophys. Acta 835*, 567.

Courtneidge, S. A., & Bishop, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7117.

Cross, G. A. M. (1987) *Cell 48*, 179.

Dils, R. R., & Hübscher, G. (1961) *Biochim. Biophys. Acta 46*, 505.

Dygas, A., Zborowski, J., & Wojtczak, L. (1980) *Acta Biol. Pol. 27*, 153.

Gillfillan, A. M., Smart, D. A., & Rooney, S. A. (1985) *Biochim. Biophys. Acta 835*, 141.

Guarnieri, M., Stechmiller, B., & Lehninger, A. L. (1971) *J. Biol. Chem. 246*, 7526.

Hixson, C. S., & Krebs, E. G. (1980) *J. Biol. Chem. 255*, 2137.

Hostetler, K. Y., & Van den Bosch, H. (1972) *Biochim. Biophys. Acta 260*, 380.

Jelsema, C. L., & Morre, D. J. (1978) *J. Biol. Chem. 253*, 7960.

Kraft, A. S., Anderson, W. B., Cooper, H. L., & Sando, J. J. (1982) *J. Biol. Chem. 257*, 13193.

Lassing, I., & Lindberg, U. (1985) *Nature 314*, 472.

Lassing, I., & Lindberg, U. (1988) *J. Cell. Biochem. 37*, 255.

Low, M. G. (1987) *Biochem. J. 244*, 1.

Megli, F. M., De Lisi, A., van Amerongen, A., Wirtz, K. W. A., & Quagliariello, E. (1986) *Biochim. Biophys. Acta 861*, 463.

Meyer, R. K., Schindler, H., & Burger, M. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4280.

Müller, G., & Bandlow, W. (1987a) *Yeast 3*, 161.

Müller, G., & Bandlow, W. (1987b) *Z. Naturforsch. 42C*, 1291.

Müller, G., & Bandlow, W. (1989a) *Biochemistry* (first paper of three in this issue).

Müller, G., & Bandlow, W. (1989b) *Biochemistry* (second paper of three in this issue).

Nishizuka, Y. (1984) *Nature 308*, 693.

Pelech, S. L., & Vance, D. E. (1982) *J. Biol. Chem. 257*, 14198.

Radika, R., & Possmayer, F. (1985) *Biochem. J. 232*, 833.

Rödel, G., Müller, G., & Bandlow, W. (1985) *J. Bacteriol. 161*, 7.

Rotman, A., Schindler, H., & Linder, S. (1982) *Biochemistry 21*, 1713.

Schmidt, M. F. G. (1983) *Curr. Top. Microbiol. Immunol. 102*, 101.

Sefton, B. M., & Buss, J. E. (1987) *J. Cell Biol. 104*, 1449.

Sleight, R., & Kent, C. (1983) *J. Biol. Chem. 258*, 824.

Terce, F., Record, M., Ribbes, G., Chaps, H., & Douste-Blazy, L. (1988) *J. Biol. Chem. 263*, 3142.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350.

Wirtz, K. W. A. (1974) *Biochim. Biophys. Acta 344*, 95.

Wirtz, K. W. A., & Zilversmit, D. B. (1968) *J. Biol. Chem. 243*, 3596.

Wojtczak, L., Baranska, J., Zborowski, J., & Drahota, Z. (1971) *Biochim. Biophys. Acta 249*, 41.

Yaffe, M. P., & Kennedy, E. P. (1983) *Biochemistry 22*, 1497.

Zborowski, J., Dygas, A., & Wojtczak, L. (1983) *FEBS Lett. 157*, 179.