Activation of the heme-stabilized translational inhibitor of reticulocyte lysates by calcium ions and phospholipid

(polypeptide chain initiation/inhibition of translation/protein phosphorylation/Ca\textsuperscript{2+} and phospholipid in translational control)

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ABSTRACT Hemin-supplemented reticulocyte lysates can be activated for translational inhibition by addition of Ca\textsuperscript{2+} or phospholipid. The fact that this inhibition is prevented or decreased in both cases either by the Ca\textsuperscript{2+} chelator EGTA or by polymyxin B, an inhibitor of the recently described Ca\textsuperscript{2+}- and phospholipid-dependent protein kinases, suggests the involvement of both Ca\textsuperscript{2+} and phospholipid in this activation. The inhibition by Ca\textsuperscript{2+} or phospholipid is accompanied by phosphorylation of the 38-kilodalton subunit of the eukaryotic initiation factor 2 (eIF-2) and the 90-kilodalton band of the heme-controlled translational subunit of the eukaryotic initiation factor 2 (HCl) and can be reversed by high concentrations of Ca\textsuperscript{2+} or GTP. When incubation is conducted at 30°C, the inhibition produced by Ca\textsuperscript{2+} is not reversed by EGTA after 15 min. However, at 20°C, Ca\textsuperscript{2+} inhibition can be fully reversed as late as 90 min from the start of incubation and phosphorylation of the eIF-2 \(\alpha\)-subunit is correspondingly decreased. These results are consistent with the idea that, like heme deprivation, the activation by Ca\textsuperscript{2+} and phospholipid promotes the first step of the reaction proinhibitor \(\Leftrightarrow\) reversible inhibitor \(\Leftrightarrow\) irreversible inhibitor and suggest that, in the presence of heme albeit by a different mechanism, this activation affects the same inhibitor that is activated in the absence of heme—namely, HCl. Whether this activation is direct or indirect—e.g., via a separate Ca\textsuperscript{2+}- and phospholipid-dependent protein kinase—remains to be determined.

While isolating a heat-stable factor (HS) from reticulocyte lysates that, like a factor described from bovine heart (1), inhibits translation in heme-supplemented lysates and promotes phosphorylation of the eukaryotic initiation factor 2 (eIF-2) \(\alpha\)-subunit, we used Ca\textsuperscript{2+} for a negative control. Ca\textsuperscript{2+} is known to inhibit protein synthesis, but we did not expect this metal ion to promote eIF-2 \(\alpha\)-subunit phosphorylation. However, much to our surprise, it did so. It also clearly promoted the phosphorylation of a polypeptide that comigrates with the 90-kilodalton (kDa) band of the heme-controlled translational inhibitor (HCl) on electrophoresis in dissociating polyacrylamide gels (2). At 30°C, translational inhibition by Ca\textsuperscript{2+} was not reversed by the Ca\textsuperscript{2+} chelator EGTA after 15 min of incubation, but at 20°C, the inhibition was fully reversed by EGTA, even after incubation for 90 min, and phosphorylation of the eIF-2 \(\alpha\)-subunit was correspondingly decreased (2). Other metal ions, including Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, Cd\textsuperscript{2+}, Co\textsuperscript{2+}, and Cu\textsuperscript{2+}, inhibited the heme-dependent translation but of these only Co\textsuperscript{2+} promoted significant phosphorylation of the eIF-2 \(\alpha\)-subunit. The effect of Co\textsuperscript{2+} has not been further investigated.

We first thought that the Ca\textsuperscript{2+}-promoted activation of HCl might be mediated by calmodulin. This hypothesis had some support in the fact that reticulocyte HS, unlike the one from bovine heart (1), resembles calmodulin in molecular weight and other properties. However, calmodulin antibody had no effect on the inhibition produced by Ca\textsuperscript{2+}, and authentic calmodulin did not affect translation even in the presence of limiting amounts of Ca\textsuperscript{2+} (2). The involvement of a different system was indicated by the finding that the translational inhibition and the eIF-2 \(\alpha\)-subunit phosphorylation induced by Ca\textsuperscript{2+} were both prevented or decreased not only by EGTA but also by polymyxin B, a bacterial antibiotic that inhibits the activity of Ca\textsuperscript{2+}- and phospholipid-dependent protein kinases (3). Moreover, a 10:1 mixture of phospholipid (phosphatidylserine) and diacylglycerol (1,3-diolein) inhibited translation and promoted phosphorylation of the eIF-2 \(\alpha\)-subunit in heme-supplemented reticulocyte lysates at concentrations similar to those required to activate Ca\textsuperscript{2+}- and phospholipid-dependent protein kinase (4, 5). The effect of both Ca\textsuperscript{2+} and phospholipid was prevented or decreased by either EGTA or polymyxin B. The results are consistent with the idea that Ca\textsuperscript{2+} and phospholipid activate, directly or indirectly, the proinhibitor form of HCl.

MATERIALS AND METHODS

Assays. Translation was assayed in 30-\(\mu\)l samples essentially as described by Hunt et al. (6) with 18 \(\mu\)l of 1:1.5 lysate and [\(^{14}\)C]leucine as the labeled amino acid, with or without 25 \(\mu\)M hemin and other additions as indicated in the table and figure legends. Incubation was for 60 min at 30°C unless otherwise specified. In the assays for phosphorylation of the 90- and 38-kDa polypeptides, aliquots were withdrawn at the end of incubation for assay of (i) [\(^{14}\)C]leucine incorporation (6 \(\mu\)l) and (ii) phosphorylation. For the latter purpose, the aliquots were supplemented with various components to give the following final concentrations or amounts: 20 mM Hepes buffer, pH 7.8/1 mM Mg\textsubscript{2+}/0.5 mM dithiothreitol/0.3 mM [\(^{32}\)P]ATP (80 \(\times\) 10\(^{6}\) cpm), and an ATP-generating system according to Jagus and Safer (7) without or with 20 pmol of \(\approx\)50% pure eIF-2 unless otherwise stated. After incubation for the times and at the temperatures specified, the samples were subjected to Na\textsubscript{D}O\textsubscript{S}O\textsubscript{4}/polyacrylamide gel electrophoresis and analyzed by autoradiography (8). All translation values are given for a 6-\(\mu\)l aliquot of the 30-\(\mu\)l translation sample. Protein was determined by the Lowry method (9) or the Bradford (10) procedure with bovine serum albumin as the standard.

Preparations. Lysates were prepared essentially as described by Pelham and Jackson (11). EGTA, L-\(\alpha\)-phosphatidyl-\(\alpha\)-serine from bovine brain, and diolene were from Sigma. The diolene (from olive oil) contained \(\approx\)85% of the 1,3 isomer and 15% of the 1,2 isomer. Polymyxin B sulfate, from Gayoso-Well.
come Laboratories (Madrid, Spain), was the gift of A. Jimenez (Centro de Biología Molecular). The phosphatidylerine/diolen mixture was prepared according to Kikkawa et al. (12). Briefly, 2 mg of phosphatidylerine and 0.2 mg of diolen were dissolved in a minimum volume of chloroform, and the solution was dried in a stream of nitrogen. The residue was taken up in 300 μl of 20 mM Tris-HCl buffer (pH 7.6), sonicated for 5 min in a Bransonic 12 sonicator, centrifuged for 10 min in a clinical centrifuge at 3,000 rpm, and the supematant was used for the experiments. The concentration of phosphatidylerine was determined as described by Bartlett (13). Calmodulin (phosphodiesterase 3' : 5' -cyclic nucleotide activator) was from Sigma (bovine brain) or Boehringer (hag brain). Calmodulin antibody, purified by affinity chromatography, was from New England Nuclear. The antibody was dissolved in 75 mM NaCl/125 mM borate, pH 8.4/1 mM EGTA/0.2% bovine serum albumin.

RESULTS

Translational Inhibition by Ca²⁺. As shown in Table 1, Ca²⁺ is a potent inhibitor of translation in reticulocyte lysates. The extent of inhibition varies widely with different lysates. This may be due to variability of endogenous Ca²⁺ content. This idea is consistent with frequent observations that lysates that, in the absence of added EGTA, are poorly stimulated by heme are stimulated to a much greater degree when EGTA is present as will be shown later. In line with these observations, lysates that respond poorly to the addition of heme are in general inhibited by concentrations of Ca²⁺ lower than those required to inhibit lysates that are highly responsive to heme. Polymyxin B, an inhibitor of Ca²⁺- and phospholipid-dependent protein kinases (3), has an effect similar to that of EGTA (see next section). The effect of Ca²⁺ described here is not mediated by calmodulin, because calmodulin antibody is without effect on the Ca²⁺ inhibition (data not shown).

Like the translational inhibition due to heme deficiency (see refs. 14–16 for review), Ca²⁺ inhibition is largely prevented by high levels of added eIF-2 or GTP. Thus, in one experiment, 0.1, 0.2, and 0.4 mM Ca²⁺ inhibited heme-dependent translation by 9, 52, and 88%, respectively. About 90 pmol of eIF-2 (which increased translation in the absence of heme...
Translational Inhibition by Phospholipid. We thought at first that the unsuspected activation of HCl by Ca²⁺ might be due to one or both of the following causes: (i) limited proteolysis by a Ca²⁺-activated protease (17) and (ii) Ca²⁺ inhibition of the protein phosphatase(s) that dephosphorylates eIF-2 phosphorylated in its α (38-kDa)-subunit [eIF-2 (αP)] (cf. also ref. 1). However, both of these possibilities appeared to be ruled out (data not shown). First, protease action is excluded by the reversibility of the Ca²⁺ inhibition observed at 20°C. Moreover, protease inhibitors (phenylmethylsulfonyl fluoride, soybean trypsin inhibitor) had no effect on the Ca²⁺ inhibition, and sensitive tests for proteolysis in lysates revealed no effect of Ca²⁺. Second, Ca²⁺ was also without effect on the rapid dephosphorylation of eIF-2 (α²P) that occurs in lysates. The possibility that Ca²⁺ might in some way activate the double-stranded RNA-activated translational inhibitor also seemed to be ruled out because high levels of double-stranded RNA (which block activation) did not affect the translational inhibition by Ca²⁺.

The report by Kuo and co-workers (3) that polymyxin B is a potent inhibitor of Ca²⁺, and phospholipid-dependent protein kinases prompted us to try this compound. As shown in Table 2, the strong translational inhibition promoted by Ca²⁺ was largely prevented by polymyxin B. Table 2 also documents the statement made in the preceding section that lysates that respond poorly to the addition of hemin are stimulated by the porphyrin to a much greater degree when HCl or, to a lesser extent, polymyxin B is present. Consistent with this observation, we found phospholipid to be a strong inhibitor of hemin-depen-
translational inhibition. Preproteins were synthesized in reticulocyte lysates (Fig. 4 A and B), and this inhibition was prevented by polymyxin B (Fig. 4 C). Furthermore, as shown in Fig. 5, phospholipid promoted phosphorylation of the 35-kDa subunit of eIF-2 in the presence of hemin (lane 3), to an extent similar to that found without phospholipid in the absence of hemin (lane 1), and this phosphorylation was prevented by either EGTA (lane 4) or polymyxin B (lane 5). Phospholipid also promoted significant phosphorylation of the 90-kDa band of HCl (data not shown).

**DISCUSSION**

In this paper, we present evidence that suggests that the heme-controlled translational inhibitor in reticulocyte lysates can be activated in the presence of hemin by a mechanism that depends on the availability of Ca\(^{2+}\) and phospholipid. Although activation occurs when either Ca\(^{2+}\) or phospholipid is added alone, such activation is probably possible because of the presence of small amounts of endogenous Ca\(^{2+}\) and phospholipid in lysates. There is in fact some proof for this in the observed stimulation of hemin-dependent translation by the addition of either the Ca\(^{2+}\) chelator EGTA or the phospholipid antagonist polymyxin B to lysates that are poorly stimulated by hemin (Table 2). The most convincing proof for the involvement of both Ca\(^{2+}\) and phospholipid is that activation by either one can be largely prevented by either EGTA or polymyxin B (Table 2 and Fig. 5). We have also occasionally observed synergistic effects of added Ca\(^{2+}\) and phospholipid on translation in lysates. Moreover, with crude pro-HCl preparations (18) we have seen that Ca\(^{2+}\) alone is insufficient; both Ca\(^{2+}\) and phospholipid must be added to activate HCl.

The existence of Ca\(^{2+}\)- and phospholipid-dependent protein kinases (Nishizuka's protein kinase C) has only recently been described (4, 5, 19, 20). Release of diacylglycerol from phosphatidylinositol by a specific phospholipase (21, 22) may be involved in the activation of these enzymes at physiological concentrations of Ca\(^{2+}\) and phospholipid. Diacylglycerol has been reported to lower the \(K_m\) of both Ca\(^{2+}\) and phospholipid for kinase C activation (5), and we have observed that omission of diolein from our phospholipid mixture decreases the phospholipid effect by 50%. Our observations are consistent with the view that HCl can be activated by Ca\(^{2+}\) and phospholipid in the presence of hemin, but further studies with purified pro-HCl are needed to ascertain whether this activation is direct or indirect.

There are a number of ways in which heme-stabilized HCl can be activated (16), including high hydrostatic pressure, increased temperatures, sulfhydryl reagents (e.g., N-ethylmaleimide), or low levels of oxidized glutathione, although the mechanism of activation is unknown in all cases. The activation by oxidized glutathione is of interest because of its possible physiological significance.

The Ca\(^{2+}\)-phospholipid mechanism cannot be involved in the activation of HCl due to heme lack, because neither EGTA nor

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**Table 2. Effect of EGTA and polymyxin B on translation**

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<th>Exp. no.</th>
<th>Addition(s)</th>
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<td>183</td>
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</tbody>
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**Fig. 4. Translational inhibition by phospholipid in reticulocyte lysates and reversal by polymyxin B.** (A) Inhibition as a function of the concentration of phospholipid. (B) Kinetics. With hemin; without hemin; with hemin and phospholipid (4 \(\mu\)M). (C) Polymyxin B reversal of translational inhibition by phospholipid. Without polymyxin B; with polymyxin B (50 \(\mu\)M). Unless otherwise indicated incubations were for 60 min at 30°C. Curves in A and C show the hemin-dependent translation. Incorporations with and without hemin, respectively, were 39.1 and 5.4 cpm \(\times 10^3\) in A and 49.3 and 7.3 cpm \(\times 10^3\) in C. The phospholipid solution contained 1.5 \(\mu\)g of phosphatidylserine and 0.15 \(\mu\)g of diacylglycerol (1,3-diolein) per \(\mu\)l.
polymyxin B can relieve the effect of heme deficiency. The biological significance, if any, of this mechanism remains to be determined. C kinases are largely membrane associated (22, 23) and a Ca\(^{2+}\)-phospholipid mechanism might be involved in control of protein synthesis in the cell membrane.

Little is known about eIF-2 \(\alpha\)-subunit kinases in cells other than reticulocytes, but the presence of eIF-2 \(\alpha\)-subunit kinases or related enzymes has been reported in Ehrlich ascites tumor cells (24), Friend leukemia cells (25), rat liver (26), and bovine adrenal cortex (27). Further, there are indications that eIF-2 \(\alpha\)-subunit kinases may be present in Artemia salina embryos (28), wheat germ (28), Drosophila larvae (M. G. Mateu and J. M. Sierra, personal communication), and Xenopus laevis oocytes (unpublished observations). It will be of interest to see whether a Ca\(^{2+}\)-phospholipid mechanism of translational control exists in nucleated cells.

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