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The interference of EDTA in searching for heat-stable low-M_r inhibitors of protein kinases

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Recently Pribilla et al. [1] have reported the partial purification of a heat-resistant, low-M_r inhibitor of both protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II from bovine brain homogenates, using DEAE-cellulose chromatography with a buffer system containing EDTA.

Rabbit skeletal muscle phosphorylase kinase, another Ca^{2+}/calmodulin-dependent protein kinase [2] has been shown to be inhibited *in vitro* by a number of small molecules [3–6], but no regulatory mechanisms involving inhibitory effector molecules have been found until now. In an attempt to search for heat-stable inhibitors of phosphorylase kinase in a number of muscle tissues, we observed that EDTA present in the homogenization buffer eluted as a main inhibitory peak from the DEAE-cellulose column giving the impression of an endogenous heat-stable inhibitor in muscle. Briefly, after DEAE-cellulose chromatography of the tissue homogenate (in this case we have examined the smooth muscle cytosol; Fig. 1 A), the phosphorylase-kinase-inhibitory fractions were heated at 100 °C, the precipitate was removed by centrifugation, and the supernatant was lyophilized then chromatographed on a Sephadex G-25 column. The inhibitory activity eluted behind the exclusion volume and significantly ahead of AMP and NaCl (Fig. 1 B), while it showed the same chemical properties as those of the low-M_r inhibitor described by Pribilla et al. [1]: it exhibits increased conductivity, has no ultraviolet absorption in the range where adenine nucleotide absorbs, is not digested by proteases, is alkali-resistant and was not inactivated by incubation with 10% NH_4OH nor by 10% trifluoroacetic acid at 50 °C for 3 h. The purified inhibitor was able to reduce both Ca^{2+}-dependent and Ca^{2+}-independent activity of phosphorylase kinase. These inhibitory fractions were disappeared by high (millimolar) concentrations of Mg^{2+} or Ca^{2+} (not shown). When the DEAE-cellulose column equilibrated with 30 mM Tris/HCl, pH 7.0, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 0.1% 2-mercaptoethanol and 2% glycerol (buffer A), without loading the tissue homogenate, was eluted with the same gradient, an elution profile of inhibitory activity, similar to that obtained with muscle cytosol was observed (Fig. 1 A). The inhibitor thus obtained exhibits chemical and chromatographic properties identical to those of the inhibitor purified when the muscle extract was used. In contrast, when the DEAE-cellulose column was equilibrated with buffer A without EDTA no inhibition was observed.

In conclusion, it must be emphasized that EDTA usually contained in homogenization buffers is strongly retained by DEAE-cellulose columns, and is subsequently released highly concentrated at high ionic strength. In this case, by chelating Mg^{2+} and Ca^{2+}, EDTA could be a predominant source of inhibitory phenomena observed when searching for heat-stable low-M_r inhibitors of both Ca^{2+}-dependent and Ca^{2+}-independent protein kinases. On the other hand, it is obvious that the high concentration of EDTA in such column fractions will not only inhibit protein kinase activity but also the activity of other enzymes which are dependent on divalent cations.

Based on the above observations, it appears that the results of Pribilla et al. [1], concerning the inhibition of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II by a heat-resistant inhibitor, could possibly be explained by assuming that EDTA was present in their low-M_r inhibitory fraction. Accordingly, chelation of Mg^{2+} ions by EDTA may explain why the authors [1] observed an inhibition of protein kinase C competitive with ATP, even after removal of the regulatory domain by limited proteolysis. On the other hand the inability of their inhibitory fraction to affect the activity of cAMP-dependent protein kinase, or to completely inhibit Ca^{2+}-independent protein kinase activity from synaptosomal membranes [1], may be due to an excess of Mg^{2+} ions present in their kinase assay mixtures.

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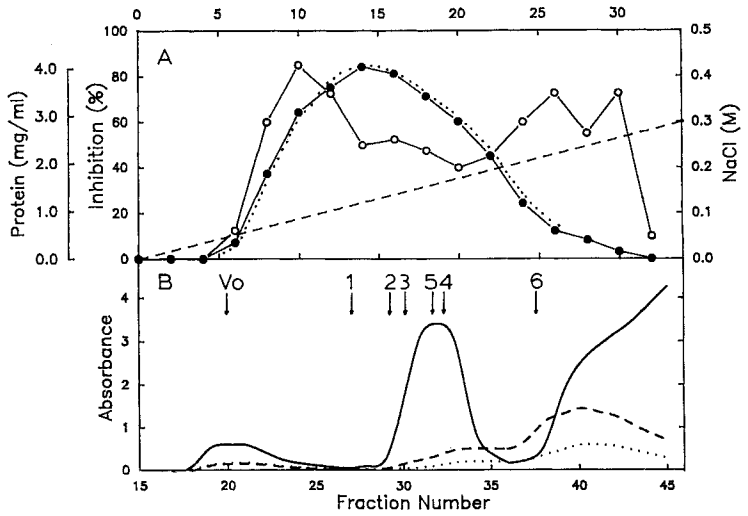

Fig. 1. *DEAE-cellulose chromatography and Sephadex G-25 gel filtration of phosphorylase kinase inhibitory activity.* (A) Bovine stomach smooth muscle (50 g) was homogenized in 2.5 vol. buffer A. The homogenate was centrifuged for 45 min at 30000 × *g* and the supernatant was loaded onto a DEAE-cellulose column (19 cm × 2 cm) equilibrated in buffer A. The column was washed with buffer A then developed with a 400-ml linear gradient of 0–0.5 M NaCl. Fractions of 7.5 ml each were collected and tested for inhibitory activity (●——●) and protein (○——○). Inhibition of rabbit skeletal muscle phosphorylase kinase at pH 8.2 was determined by measuring phosphorylase activity using 45-µl portions of fractions [5]. (·····) Inhibitory activity determined after elution of the column without loading the muscle cytosol. Protein was estimated by Bradford [7]. (B) The inhibitory fractions from DEAE-cellulose chromatography were heated at 100 °C and after centrifugation the supernatant was concentrated eightfold by lyophilization. 0.8 ml of this preparation was loaded onto a Sephadex G-25 column (1.1 cm × 53 cm) and eluted with 40 mM formic acid. Fractions of 0.7 ml each were collected then analyzed for absorbance at 230 nm (——), 260 nm (– – –) and 280 nm (·····), and for inhibitory activity. (1) Maltoheptose, (2) maltopentose, (3) bacitracin, (4) maltotriose and (6) AMP were used as molecular mass standards. Vo, void volume; (5) peak of inhibitory activity

RETRACTION

In reply to the preceeding paper by Zevgolis et al., the authors of the paper 'Heat-resistant inhibitors of protein kinase C from bovine brain' [Pribilla, I., Krüger, H., Buchner, K., Otto, H., Schiebler, W., Tripier, D. & Hucho, F. (1988) *Eur. J. Biochem. 177*, 657–664] have made the following statement:

'In some of the low-M_r fractions inhibiting the protein kinase C we identified EDTA. We observed that EDTA is a potent inhibitor of protein kinase C and of Ca^{2+}/calmodulin-dependent protein kinase, even at concentrations far below the concentration required to remove Ca^{2+} and Mg^{2+}. We do not have positive evidence for the presence of a low-M_r protein kinase C inhibitor in bovine brain. We therefore would like to retract all data published by us concerning such a low-M_r protein kinase C inhibitor. This retraction does not include the experiments describing the inhibitory action of calmodulin in the same paper.'