A rapid assay for the localization of protein kinase modulator (inhibitor) in complete polyacrylamide gels[1]

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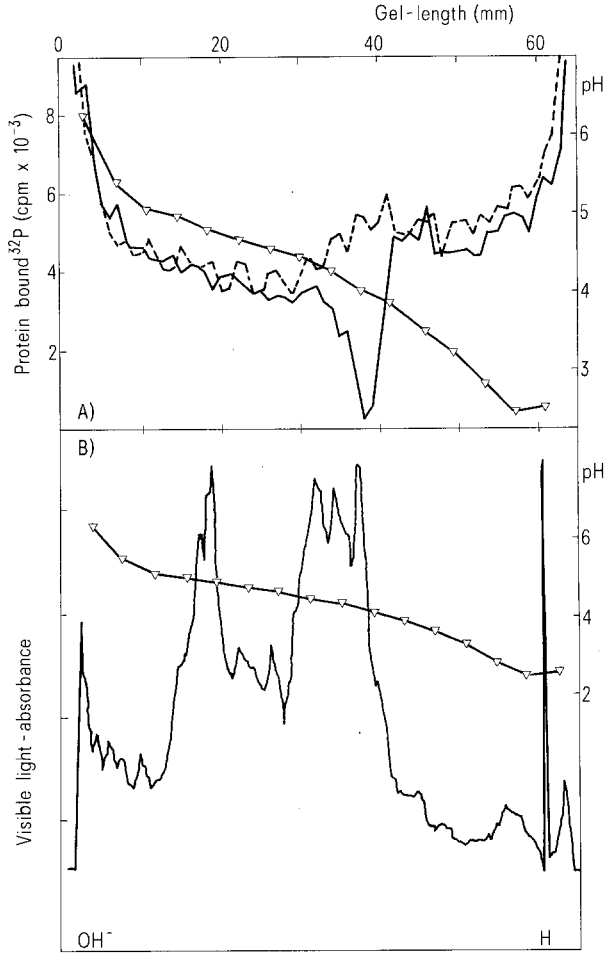
Summary. A gel assay system for determination of inhibitory proteins for protein kinase(s) on isoelectric focusing gels has been developed. Preparations of heat-stable inhibitors were applied, focused, and the complete gels incubated with protein kinase in the presence of substrate protein. At the position where inhibitory protein had focused, the phosphorylation reaction was blocked selectively.

In view of the wide variety of different regulatory functions of cAMP the question must be raised as to how the second messages might be translated specifically by the catalytic subunit of cAMP-dependent protein kinases (PK) (EC 2.7.1.37)[3]. Small thermo-stable proteins found in many tissues may help to confer this specificity[4] since such preparations contain PK-modifying, especially inhibitory activity[5]. In order to attribute inhibitory activity to particular proteins without lengthy and elaborate purification procedures, we have developed a rapid assay system for the localization of PK-inhibitory activities in complete polyacrylamide gels. It should be of particular assistance in studying the possible significance of PK-modifiers for mechanisms involved in the regulation of the specificity of protein phosphorylation.

The gel assay system for PK-inhibitory protein is based on the observation that PK-activity can be quantitatively assayed in complete polyacrylamide gels[6]. It consists, in principle, of a protein phosphorylation carried out over the entire gel which is supposedly inhibited at the sites where inhibitory proteins are located after being separated either by electrophoresis or electrofocusing. For this purpose protein kinase and substrate protein, as well as cosubstrate, have to be introduced into the gel. After the enzyme reaction the gels are fixed, extracted and sliced for determination of radioactivity. The procedure in detail is as follows:

1. For equilibration the gel was removed from the gel tube and immediately incubated in 10 ml 0.2 M MOPS (morpholinopropane sulfonic acid) buffer, pH 6.8 (in Duran glass tubes 200 × 13.5 mm) for 3 × 10 min in an ice bath with 2 changes of buffer.
2. The gel was further soaked with 10 ml 0.05 M MOPS buffer, pH 6.8, containing $5\text{–}10 \times 10^3$ units of catalytic subunit of cAMP-dependent protein kinase from rat muscle[7] and 0.05% BSA (bovine serum albumin added in order to stabilize the enzyme[8]) for 30 min at 5 °C.
3. For the introduction of the substrate protein the gel was transferred to 10 ml 0.05 M MOPS buffer, pH 6.8, containing the same amount of fresh enzyme as before, BSA, and in addition, calf thymus histone (5 mg/ml; from Sigma, No. H 9250), Mg-acetate (10 mM) and dithioerythritol (1 mM) for 15 min at 5 °C.
4. In order to remove supernatant enzyme, the gel was soaked in the cocktail as in 3., but prewarmed and without enzyme for 15 min at 30 °C. After the 4th step the pH profile of the gel varied between 6.8 and 6.4, which is in the 95–100% range of the optimum pH for the phosphorylation of histone by the particular protein kinase[6].
5. For the enzyme reaction the gel was transferred to 10 ml cocktail as in 4. but with the addition of 1 nmole [γ-^{32}P]ATP (sp. act. > 25 Ci/mmole, from New England Nuclear). Incubation was in a roller apparatus (2 rpm) at 30 °C for 30 min.
6. Fixation, extraction, slicing and measurement of the radioactivity were carried out as described earlier[6].

The result of a typical experiment is given in the figure, A. About 20 µg of rat muscle PK-inhibitor preparation, (of which 0.3 µg caused 50% inhibition of 100 units of enzyme), were focused and assayed as above (solid line). Control gels without PK-inhibitory protein were processed in an identical manner (dashed line). Despite several contaminating proteins, as detected in parallel gels by staining (figure, B), the inhibitory activity could be attributed with high accuracy to particular bands. The high radioactivity at both ends of the gels originates from the geometry of the enzyme plus



PK-inhibitor assay in complete focusing gels. *A* PK-inhibitor gel (solid line), control gel (dashed line), pH gradient (–▽–▽–); *B* protein distribution (Coomassie blue). About 20 µg PK-inhibitor (prepared according to Walsh et al.[5]) was focused in polyacrylamide gels as described recently[6] and assayed as outlined in the text. The inhibitor preparation contained neither ATPase nor phosphoprotein phosphatase activity. The final ampholine concentration was 0.98%; it was composed of the ampholines pH 5–8 (40%); pH 4–6 (40%) and pH 2.5–4 (20%) in the ratio 1:1.6:2. Gel size was 80 × 4.5 mm. Fixation and staining with Coomassie blue R-250 as well as scanning of transmission was as described earlier[6,7].

histone distribution throughout the gel since both proteins enter only the outer parts during the penetration. Here, a compromise between protein elution from the gel (i.e. of inhibitor) and degree of protein penetration (i.e. of substrate and enzyme) had to be approached. Protamine can be used as well as histone as the substrate. For this purpose, however, the gel should be adjusted to pH 7.8, which is the optimum for the protamine phosphorylation with rat muscle enzyme[6]. Taking into account the contamination with other small proteins it was estimated that about 5 µg of pure PK-inhibitory protein should be easily detectable by this procedure. Preliminary experiments have shown that some inhibitor preparations from rat muscle contain 2 closely located activities inhibitory for the catalytic subunit with regard to histone phosphorylation. The use of other PK-substrates as well as inhibitor preparations from different tissues should help to elucidate the physiological significance of these small proteins.

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Evidence against the involvement of cyclic GMP in the insulin-stimulation of lipoprotein lipase activity in fat cells[1]

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Summary. Under in vitro experimental conditions in which insulin increases adipose tissue lipoprotein lipase, cyclic GMP or dibutyryl cyclic GMP has no effect on this enzyme in rat adipose tissue fragments, or on either the intra- or extracellular forms of this enzyme in isolated fat cells. These results do not support the involvement of cyclic GMP in the insulin-stimulation of lipoprotein lipase in adipose tissue.

In a recent report, Vydelingum et al.[2] confirmed previous findings[3,4] by showing the ability of insulin to increase the 3′-5′ cyclic guanosine monophosphate (cyclic GMP) content and to enhance the lipoprotein lipase (LPL) activity of adipose tissue in vitro. Although the kinetics of these effects were markedly different[2], these authors suggested, that cyclic GMP might mediate the insulin stimulation of LPL activity in adipose tissue.

As the direct effects of cyclic GMP on adipose tissue LPL were not investigated in these studies[2], we have tested this hypothesis by studying, under the experimental conditions used by Vydelingum et al.[2], the influence of different concentrations of cyclic GMP or its stable analog, dibutyryl cyclic GMP (dcGMP), on the LPL activity of adipose tissue. Moreover, the same investigations were carried out on isolated fat cells to determine whether cyclic GMP might affect selectively the intracellular or the extracellular form[5,6] of LPL. The present data clearly indicate that, with experimental conditions under which insulin increases adipose tissue LPL, cyclic GMP and dcGMP have no effect on this enzyme, a result which therefore does not support the involvement of cyclic GMP in the mechanism of the insulin-mediated stimulation of LPL activity in adipose tissue.

Materials and methods. Before sacrifice male Wistar rats (120–140 g) were fasted overnight but given 5% (w/v) glucose in the drinking water. After decapitation, epididymal fat pads were excised and isolated fat cells prepared as previously described[7]. In studies using adipose tissue, fragments from one pad from each animal served as control, while fragments from the other pad were used as the test material. Adipose tissue (200 mg/ml) or isolated fat cells (500 µl packed cells/ml) were incubated under O_2/CO_2 (95/5, v:v) in the absence or presence of the compounds to be tested in Krebs-Ringer[8] -bicarbonate buffer (pH 7.4) containing 1.25 mM calcium, 5 mM glucose, 20 mg/ml dialysed bovine albumin (fraction V) and an amino-acid mixture (final concentration 390 nM)[9], the composition of which corresponded to the amino-acid concentration of rat plasma[9]. In experiments using fat cells, the incubation buffer was added with fresh rat serum (3.8%, v/v).

Adipose tissue fragments were incubated for 2 h at 37 °C in the absence or presence of the compounds to be tested. Fat cells were first preincubated fro 30 min at 26 °C after which the compounds to be tested were added and the incubation further extended for an additional 90-min period. Fat cells and adipose tissue fragments were then separated from the

Influence of various concentrations of dibutyryl cyclic GMP on the intra- and extracellular forms of lipoprotein lipase in isolated rat adipocytes in vitro

Concentration of dibutyryl cyclic GMP added to the incubation medium (M)	Lipoprotein lipase activity (% of basal activity)	
	Remaining in the cells	Released from the cells into the incubation medium
0	100 ± 17	100 ± 3
10^{-11}	101 ± 22	87 ± 15
10^{-9}	126 ± 11	90 ± 9
10^{-7}	97 ± 12	102 ± 4
5×10^{-4}	114 ± 13	92 ± 6

Isolated fat cells were incubated at 26 °C as described under material and methods section. After a preincubation period of 30 min, different concentrations of dibutyryl cyclic GMP were added; 90 min later, fat cells were separated from the medium and lipoprotein lipase activities determined in both the fat cells and the incubation medium as described. Lipoprotein lipase activity is expressed as percentage of the basal values obtained in cells incubated without dibutyryl cyclic GMP. Each value is the mean ± SEM of 2 experiments performed in triplicate.