Effects of Ca, Mg, and EDTA on Creatine Kinase Activity in Cerebrospinal Fluid

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For one to obtain a precise estimate of creatine kinase (CK) activity in cerebrospinal fluid, the sample fraction is increased by about 10-fold over that used for serum. This increases the concentration of interfering substances, Ca being especially important. Therefore, the relationship between Ca, Mg, and EDTA was examined. Enzyme activity was maximal with 15 mmol of Mg per liter in the presence of 3 mmol of EDTA per liter, otherwise according to the (Scandinavian) recommended conditions for determination of CK activity in serum. These modifications increased the activity of CK by 35% for CK-MM and by 60% for CK-BB. Counteraction of Ca-induced inhibition was the main reason to this increase. We describe a practical and sensitive method for determining CK in cerebrospinal fluid.

The activity of creatine kinase (CK, EC 2.7.3.2) in human cerebrospinal fluid (CSF) is normally less than a fifth that of plasma, but it increases in a variety of neurological diseases (1–4). Usually the increase is moderate, <50 U/L, but sometimes it can be extremely high, as much as 10 000 U/L. As a diagnostic tool, CK activity in CSF has as yet gained little interest, mainly because clinical studies have shown conflicting (5) and inconsistent findings (1). There may be at least two main reasons for this: (a) The methods used have been too nonspecific and insensitive for accurate measurements in the near-normal range. (b) The clinical materials examined have been heterogeneous and the time of sampling in relation to the onset of the disease has not been standardized. Recent observations suggest that assay of CK activity in CSF, especially when combined with CK isoenzyme estimation, may be of considerable value in the detection and prognosis of cerebral damage (4, 6, 7).

The low CK activity in CSF makes it necessary to increase the sample fraction by about 10-fold as compared to that used in serum assays, to obtain measurable reaction rates for samples in the near-normal range. In so doing, considerably more potentially interfering substances are added. Such substances may include heavy metals (Fe, Cu, Zn), Mg, and Ca. Ca competes with Mg as a CK activator (8). Thus, alterations in the optimal conditions are to be expected when the volume fraction is increased to that extent.

We present here a sensitive and practical method for determining CK activity in CSF. Most of the interference is counteracted by increasing the concentration of Mg and introducing EDTA into the reaction mixture. The results also shed light on the stimulatory effect of EDTA on CK activity (9, 10).

Materials and Methods

Materials

Imidazole was obtained from Koch-Light Laboratories, England; magnesium acetate, triethanolamine, EDTA (disodium salt), and lyophilized antibody toward human CK-MM (cat. no. 11642) from Merck, F.R.G.; and 2,2-bis(hydroxymethyl)-2',2'-nitrotrithanol ("Bis/Tris"), hexokinase in sodium citrate (cat. no. H 4502), and crystallized glucose-6-phosphate dehydrogenase (cat. no. G 6378) from Sigma Chemical Co., St. Louis, MO 63178. The rest of the reagents were from Boehringer, Mannheim, F.R.G.

Most of the studies were done with partly purified CK-BB and CK-MM, diluted to an activity of 5–100 U/L immediately before use, either with CSF having low CK activity or with imidazole acetate, Bis/Tris, or triethanolamine (10 mmol/L, pH 6.7 at 25 °C). The tissue extracts were prepared from human brain or psos muscle as described by Leroux et al. (11), modified by replacing EDTA with mercaptoethanol (10 mmol/L) in the medium. The supernatant fluid of the muscle extract (CK about 400 000 U/L) and brain extract (CK about 44 000 U/L) showed, on agarose gel electrophoresis and fluorescence visualization, practically only CK-MM and CK-BB, respectively. The extracts were stored at −80 °C until used.

Cerebrospinal fluid was obtained by lumbar puncture of patients. Cisternal fluid with high CK-BB activity was collected postmortem from patients with non-cerebral diseases. The cisternal fluid was centrifuged (10 000 × g, 10 min, +4 °C), and the clear, uncolored supernatant fluid (CK activity between 2000 and 6000 U/L) was used for stability studies.

Methods

The activity of CK was measured at 37 °C according to the Scandinavian recommended method for CK in serum (12), except for the substances that were experimentally varied (see legends). The CK-B activity of CSF was measured with the suggested assay (see Results) after immunoinhibition of the CK-M monomer with a specific antibody (anti-M) as described by Gerhardt et al. (13). The activity was measured with either an LKB 8600 enzyme analyzer or a GEMSAEC centrifugal analyzer. Preincubation was for at least 10 min at 25 °C before the reaction was started by adding creatine phosphate. The steady-state reaction rate was monitored after 90 to 120 s, thus allowing for the lag phase. Reagents were either prepared in the laboratory from individual ingredients or purchased from Sigma Chemical Co., St. Louis, MO 63178.

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1 Nonstandard abbreviations used: CK, creatine kinase; CSF, cerebrospinal fluid; and EDTA, ethylenediaminetetraacetate.

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Results

Methodological Studies

Heavy metals. Iron, copper, and zinc are present in CSF in concentrations of about 7, 2, and 6 μmol/L, respectively. However, on adding up to 10 times the amounts present in 0.5 mL of CSF, we saw no inhibition of CK activity in the case of iron or copper, and only a slight inhibition in the case of zinc.

Magnesium. Increasing the concentration of Mg in the reaction mixture increases the CK activity of CSF (Figure 1). Some activity is present without extra Mg addition, obviously due to the Mg and Ca already present in the CSF. In the case of CK-MM, maximal activity is reached at about 20 mmol of Mg per liter, whereas CK-BB activity increases steadily in the range examined (up to 50 mmol/L). In the serum assay, 10 mmol of Mg per liter is optimal (12). This discrepancy is most likely due to the different amount of Ca added in the sample itself in the two assays.

EDTA. EDTA complexes Ca more strongly than it complexes Mg. Addition of EDTA stimulates CK activity of serum by about 10% at 37 °C (14). In the CSF method this stimulatory effect of EDTA is even more pronounced (see below). In the presence of 3 mmol of EDTA per liter, Mg concentration curves for CK-BB and CK-MM are essentially identical and demonstrate an optimal Mg concentration of about 15 mmol/L (Figure 1).

The maximum EDTA effect in the presence of 15 mmol of Mg per liter is reached at a concentration of about 3 mmol/L in the case of both CK-BB and CK-MM (Figure 2A). At this EDTA concentration, CK-BB is stimulated by about 35% and CK-MM by about 20% (Figure 2B and Figure 1). At 10 mmol of Mg per liter, as used in the serum assays, the percentage stimulation will be higher (Figure 1), about 60 and 35% for BB and MM, respectively. The degree of EDTA stimulation is the same whether the enzyme is dissolved in imidazole buffer containing 1.2 mmol of Ca per liter or in CSF (Figure 2B). Thus, other possible interfering substances in CSF seem to play no significant role.

Calcium. The activity of CK-BB and CK-MM dissolved in imidazole buffer decreases with increasing concentration of Ca (Figure 3, curve 3), an effect more pronounced for isoenzyme BB than for MM. The inhibition by Ca can partly be counteracted by increasing the Mg concentration from 15 to 50 mmol/L (Figure 3, curve 2). This high concentration of Mg, however, has an additional inhibitory effect, as shown when Ca is not added (Figure 3, curve 2). EDTA (3 mmol/L) increases the activity more than can be obtained by adding 50 mmol of Mg per liter (Figure 3, curve 1 and 2). This effect can only partly be explained by EDTA complexing of Ca, because stimulation by EDTA is revealed even in the absence of added Ca (Figure 3, curves 1 and 3). We do not believe that the latter effect can be attributed to the infinitesimal amounts of Ca found to be present as contaminants in the reagents. In the presence of EDTA the CK activity also decreases gradually with increasing concentration of Ca (Figure 3, curve 1).

Buffer. In accordance with the Scandinavian recommended method for CK in serum, we used imidazole acetate as buffer (12). A recent report (15) has shown that higher CK activities were obtained with Bis-Tris than with imidazole as buffer. We find that EDTA has a markedly less stimulatory effect when the former buffer is used, but in both cases the effect is more pronounced in the presence than in the absence of Ca in the reaction mixture (Table 1). In fact the CK activity was es-
The enzyme was dissolved in imidazole acetate to a final high activity (open symbols) or low activity (closed symbols). One hundred per cent activity represents 83 or 10 U/L and 92 or 22 U/L for CK-MM (O, •) and CK-BB (A, △), respectively. The activities were measured in the presence (curve 1) or absence (curve 3) of EDTA (3 mmol/L) or with increased Mg-concentrations (50 mmol/L) in the absence of EDTA (curve 2).

Fig. 3. Effect on CK-activity of increasing concentrations of Ca. The enzyme was dissolved in imidazole acetate to a final high activity (open symbols) or low activity (closed symbols). One hundred percent activity represents 83 or 10 U/L and 92 or 22 U/L for CK-MM (O, •) and CK-BB (A, △), respectively. The activities were measured in the presence (curve 1) or absence (curve 3) of EDTA (3 mmol/L) or with increased Mg-concentrations (50 mmol/L) in the absence of EDTA (curve 2).

sentially the same whether imidazole/EDTA or Bis/Tris was used (results not shown).

Suggested Assay Procedure

Based on the above results, we found that the following modifications of the German and Scandinavian recommended method for CK in blood are suitable for determination of CK in CSF:

1. Sample fraction was increased 10-fold, from 0.043 to 0.430 (0.50 mL of CSF to a final volume of 1.15 mL).
2. Magnesium acetate was increased from 10 to 15 mmol/L.
3. EDTA was added to a final concentration of 3 mmol/L.

In routine work we have found it convenient to make a suitably concentrated buffer containing the adjusted amounts of Mg and EDTA. Working reagents are prepared by dissolving kit reagents (Baker, Boehringer) for blood in an appropriate volume of this buffer. As compared to conventional final serum assay conditions (10 mmol of Mg per liter and no EDTA) the CK activity of CSF was increased by these modifications by 35 and 60% for CK-MM and CK-BB (cf. Figure 1), respectively. Sample blank reactions (i.e., omitting creatine phosphate) amounted regularly to less than 1 U/L. Higher sample blank activity than 2 U/L was observed only in the case of macroscopic contamination with blood or high activity of CK.

The assay is linear up to 100 U/L. For measuring CK activities in CSF greater than 100 U/L, we suggest use of the protocol for serum assay (12).

The within-series precisions of the method were found to be 1.7% (CV) and 2.9% and the day-to-day reproducibility 3.4 and 9.3% with test activities of 50 and 6 U/L, respectively.

Reference values were obtained by assaying CSF from 22 patients (age 11 to 70 years) subjected to myelographic examination because of low back pain, and 25 patients (50 to 83 years old) given spinal anesthesia before operation (adenoma of the prostate, fracture of the colli femoris). The distribution was skewed upwards, with a range of 0.3–6.0 U/L and an arithmetic mean of 1.5 U/L. Of the results, 97.5% were below 4 U/L and 90% below 2.0 U/L.

Stability tests show that there is a linear decline of about 1% per hour in CK activity during the first 24 h when the CSF is kept at room temperature. At 4 °C the decline is less pronounced, the activity of CK averaging 95 and 93% of the initial value after four and nine days, respectively. At −20 °C, only 85% of the initial activity was recorded after nine days, but at −80 °C essentially no alterations in activity were seen during two months.

Inhibition by Anti-M

Preincubation of CSF, to which partly purified isoenzyme CK-MM or CK-BB had been added, in the presence of anti-M gave a 98–99% inhibition of the CK-MM and 0–2% inhibition of the CK-BB. With the present method, therefore, isoenzyme estimation, even in the near-normal range, is easily accomplished.

Discussion

It is well known that Ca may act as an activator and may replace Mg in the reaction: creatine phosphate + Mg-ADP = creatine + Mg-ATP. The Ca-ADP complex binds more strongly to the enzyme, but gives only about an eighth the activity obtained by using Mg-ADP as substrate (8). In a system with optimum concentration of Mg, therefore, Ca acts as a competitive inhibitor. Of minor practical importance is a noncompetitive inhibition, which becomes apparent at high Ca concentrations (8).

The predominance of CK-BB in CSF and CK-MM in serum points to the possibility of different optimal conditions for these two assays. There are differences in substrate affinity among human CK isoenzymes (16), but the established op-
timized conditions for CK-MM in serum are essentially opti-
mal also for CK-BB (17). Therefore, a detailed examination
of all the variables in the reaction mixture was not considered
necessary.

The CK of CSF may be derived from nervous tissue (CK-
BB) or blood (CK-MM). Consequently an identification of
the isoenzyme pattern is often of clinical interest. Considering
the low CK activity in the near-normal range, an electropho-
retic technique is too insensitive and a chromatographic
technique (18) too cumbersome to be used routinely. However,
we find the immunological procedure to be convenient for the
estimation of CK isoenzymes in CSF. In fact this procedure
may be better suited for CSF than for serum, because the
B-monomer regularly constitutes a comparatively high fraction
of the total CK, and significant CK-MB activity hardly ever is present in CSF.

Studies are in progress to evaluate the clinical significance
of CK activity determination in CSF, applying the present
method.

CSF samples in which the activity of CK was determined to be used
as reference values were provided from the Neuroradiological De-
partment, Ullevål Hospital, and by Dr. P. Vaagenes. For this assis-
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