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SPERMINE EFFECT ON THE BINDING OF CASEIN KINASE I TO THE RAT LIVER MITOCHONDRIAL STRUCTURES

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The results indicated here, together with those previously reported, show that spermine, ubiquitous polyamine, while promoting the transmembrane translocation of casein kinase II (CKII) across the outer membrane to more internal compartments of rat liver mitochondria, promotes the binding of casein kinase I (CKI) to the external surface of outer mitochondrial membrane but inhibits its spontaneously occurring binding to more internal structures.

Casein kinase I(Ca^{2+}- and cAMP-indipendent protein kinase) an ubiquitous cell enzyme of about 40 KDa, is able to phosphorylate several substrates on Ser/Thr-residues in an acidic environment by utilizing only ATP as a phosphate donor (1).

As previously reported (2), spermine, ubiquitous polyamine, promotes the transmembrane translocation of casein kinase II (CKII) (about 130 KDa Mr) through the outer membrane of rat liver mitochondria and its binding to more internal mitochondrial structures. These results prompted us to investigate whether this polycation behaves in the same manner also with casein kinase I (CKI).

The results reported in this paper show that this is not the case, i.e. spermine promotes (at concentrations up to about 0.5 mM) the binding of CKI to the external surface of outer mitochondrial membrane, whereas, entering the mitochondrion (3), hinders the spontaneously occurring binding of CKI to more internal structures.

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Abbreviations: RLM, rat liver mitochondria; CKI, casein kinase I; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

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Methods and Materials.

Rat liver mitochondria were isolated in buffer A (5 mM Hepes buffer, pH 7.4, containing 0.25 mM sucrose) by conventional differential centrifugation as described in (4).

Mitoplasts.
Mitoplast preparation and their purity assays were performed as described in a previous paper (2).

CKI-binding to intact mitochondria and mitoplasts.
Intact mitochondria or mitoplasts (2 mg protein) were preincubated at 20°C for 15 minutes in 2 ml of the following isotonic buffer B (10 mM Hepes-HCl buffer pH 7.4, containing 200 mM sucrose, 5 mM succinate, 1.25 μM rotenone, 1 mM phosphate and, when present, variable concentrations of spermine).
After 15 min, 100 μl of casein kinase CKI (150 units) dissolved in buffer C (25 mM Hepes-HCl buffer pH 7.0, containing 10% glicerol, 25 mM NaCl, 10 mM mercaptoethanol, 1mM EDTA, 0.1% TRITON X-100, 0.05 mM PMSF and 0.02% NaN_3), or the same volume of buffer C (control), were added to the suspensions (mitochondria or mitoplasts) in buffer B, and the incubation was continued for other 30 min at 20°C.
At the end of the incubation, the samples were centrifuged for 1 min, at 15800 rpm in an Eppendorf Centrifuge 5415 C to separate the pellet from the supernatant medium.
The pellets were resuspended in 500 μl of the above isotonic buffer B and assayed for the casein kinase activity.
2 μl oligomycin (1μg/ml) were added to each sample, to block F_1F_0 ATPase activity.

CKI-binding to mitochondrial membranes.
Mitochondria (2 mg protein), prepared as described in (4), were suspended in 2 ml of hypotonic medium (25 mM Tris-HCl buffer pH 8, containing 0.03 mM PMSF and 0.02% NaN_3), disrupted by freezing-thawing and then centrifuged at 144000 x g for 50 min.
The pellet (membranes) was resuspended in 2 ml of buffer A and preincubated for 15 min at 30°C in the absence or presence of spermine. Thereafter, 100 μl of CKI (150 units) dissolved in buffer C or the same volume of buffer C (control) were added and incubation continued for other 30 min.
At the end, membranes were separated by centrifugation (144000xg for 50 min), and the pellet was resuspended in 500 μl of the 25 mM Tris-HCl buffer (pH 8).
2 μl oligomycin (1 μg/ml) were added to each sample.

Casein kinase activity assay.
Membranes (25 μl) or the corresponding supernatants (50 μl) or intact mitochondria (100 μl) or mitoplasts (100 μl), or the same volume (100 μl) of the corresponding supernatants were separately incubated for 7 min at 30°C in 125 μl reaction mixture containing 15 μM [γ-^{32}P]ATP ($6x10^6$ cpm/nmol) 100 mM Hepes-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 10 μM Vanadate and 0.65 mg/ml of casein as exogenous substrate.
The reaction was stopped by addition of 2% SDS and 1% β-mercaptoethanol (final concentration) followed by a 5-min treatment at 100°C as previously described (5)
Half of the mixture was submitted to electrophoresis in 0.1% SDS/10% Polyacrylamide slab gels, essentially according to Laemmli(6) as previously described in (5).
The slab gels were stained with Coomassie Blue according to Laemmli (6). Dried gels were autoradiographed at -80°C with intensifying screens.
1 Unit of casein kinase was defined as the amount of enzyme catalyzing the incorporation of 1 pmol ^{32}P into whole casein in 1 min, under the above standard conditions.

Purification of cytosolic casein kinase CKI.
Cytosolic casein kinase CKI (S) was prepared from human erythrocyte cytosol essentially according to (7)
After purification, the enzyme was dialyzed against buffer C for 4 hours.

Other methods and miscellaneous.
Protein concentration of mitochondria and mitoplasts was estimated according to (8), by the biuret method with bovine serum albumine as standard.

Results.

Experiments with intact mitochondria.

When rat liver intact mitochondria, pretreated with casein kinase I (CKI), were separated by centrifugation from the supernatant and then incubated with casein in the presence of [γ-^{32}P]ATP, they display negligible, if any, ^{32}P-labeling of casein (Fig. 1A, lane a), the added CKI being almost completely recovered into the corresponding supernatant (Fig. 1B, lane a).

This indicates that the added CKI, like CKII (2), does not bind spontaneously to the external membrane of rat liver mitochondria.

By contrast, when mitochondria were treated with CKI after a pretreatment with 0.1-0.5 mM spermine, and then separated by centrifugation, the ^{32}P-labeling of casein displayed by the pellet (Fig. 1A, lane b-d) was higher than in the control (i.e. mitochondria treated with CKI without previous treatment with spermine (Fig. 1A, lane a), thus indicating that the spermine has promoted a binding of CKI to the external membrane in such a way that the enzyme still remains accessible to casein substrate added for the assay.

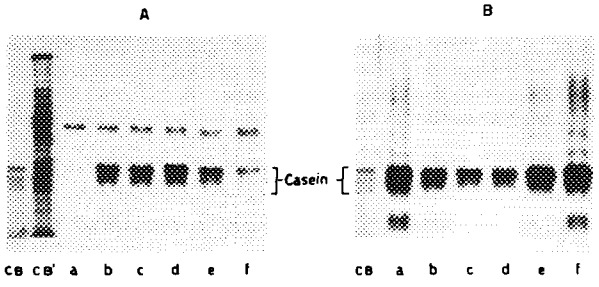

Fig. 1.
EFFECT OF INCREASING SPERMINE CONCENTRATION ON THE BINDING OF CASEIN KINASE CKI TO INTACT MITOCHONDRIA.

A. Autoradiograms showing SDS-PAGE patterns of ^{32}P-labeling of casein (casein kinase activity) displayed by intact mitochondria (200 µg proteins).
Mitochondria were treated with CKI after preincubation in the absence (lane a) or in the presence of 0.1 mM (lane b), 0.25 mM (lane c), 0.5 mM (lane d), 1 mM (lane e), 2 mM (lane f) spermine, separated by centrifugation from the corresponding supernatants and resuspended in isotonic medium for the assay of kinase activity (as described in the Methods).
100 µl of mitochondrial suspension (400 µg proteins) was used for the casein kinase assay.
Lanes CB and CB' show the Coomassie-Blue-stained gels of casein alone and of mitochondrial assay mixture, respectively.
The autoradiograms were exposed for 24 h.

B. ^{32}P-labeling of casein (casein kinase activity) displayed by corresponding supernatants (50 µl) obtained by centrifuging the above described intact mitochondria treated with CKI after preincubation in the absence (lane a) or in the presence of 0.1 mM (lane b), 0.25 mM (lane c), 0.5 mM (lane d), 1 mM (lane e), 2 mM (lane f) spermine.
100 µl of supernatants were used for the casein kinase assay.
Lane CB shows the Cooassie-Blue-stained gel of casein.
The autoradiograms were exposed for 24 h.

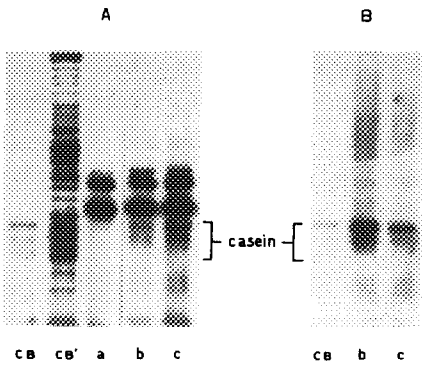

Fig. 2.
EFFECT OF SPERMINE ON CKI BINDING TO MITOPLASTS.

A. Autoradiograms showing the phosphorylation patterns on SDS-PAGE by mitoplasts (200 μg proteins).
Mitoplasts were treated with CKI after preincubation in the absence (lane b) or in the presence of 0.5 mM spermine (lane c) separated from the corresponding supernatants by centrifugation and resuspended in isotonic medium.
Lane a shows the SDS-PAGE pattern of endogenous protein phosphorylation by spermine-untreated mitoplasts incubated with casein in the absence of added casein kinase CKI.
100 μl mitoplasts (400 μg proteins) was used for the casein kinase assay (see Methods).
Lanes CB and CB' show the Coomassie-Blue-stained gel of casein alone and of mitoplast assay mixture, respectively.
The autoradiograms were exposed for 48 h.

B. ^{32}P-labeling of casein (casein kinase activity) by supernatants (50 μl) obtained by centrifuging the above described intact mitoplasts treated with CKI after preincubation in the absence (lane b) or in the presence of 0.5 mM spermine (lane c).
100 μl supernatants were used for the casein kinase assay (see Methods).
Lane CB shows the Coomassie-Blue-stained gel of casein.
The autoradiograms were exposed for 48 h.

This is confirmed also by the finding that CKI activity, recovered in the corresponding supernatant (fig. 1B, lane b-d) was much lower than that in the control (fig. 1B, lane a).

However, such a spermine-promoted binding rises to a maximum level at lower spermine concentrations (up to 0.5 mM) (fig. 1A, lanes b-d) and thereafter declines (fig. 1A, lanes e, f), as also confirmed by the increased casein kinase activity recovered in the supernatants (Fig. 1B, lanes e, f).

This suggests that spermine, as reported in the Discussion, displays two opposite effects on the binding of CKI to intact mitochondria, depending on the polycation concentration.

Experiments with mitoplasts.

When the mitoplasts, instead of their parent mitochondria, were incubated with CKI after a pretreatment with spermine, and then separated by centrifugation, the casein kinase activity (fig. 2A, lane c) displayed by the pellet was higher than that by the control (fig. 2A, lane b), i.e. spermine-untreated mitoplasts incubated with CKI.

Accordingly, the casein kinase activity displayed by the corresponding supernatant (fig. 2B, lane c) was lower than that by the control (fig. 2B, lane b).

This indicates that the mitoplasts, like their parent mitochondria, display a spermine-promoted bindig of CKI.

However, unlike their parent mitochondria (fig. 1A, lane a), the mitoplasts appear to display also a spontaneous binding of CKI in the absence of spermine (fig. 2A, lane b).

This is indicated by the finding that the pellet separated by centrifugation from spermine-untreated mitoplasts incubated with CKI (fig. 2A, lane b) displays a detectable casein kinase activity as compared with negligible activity by the control, i.e. pellet from spermine-untreated mitoplasts incubated with casein in the absence of added CKI (fig. 2A, lane a).

Such a spontaneous binding appears to be by far predominant, if not exclusive, when assayed in "membrane" preparations from disrupted mitochondria, thus suggesting that, unlike outer surface of external membrane, more internal structures does not require the spermine for the binding of CKI.

This idea is supported by the following experiments with membranes, where more internal binding sites, likely located in the inner surface of membranes, become accessible to added CKI.

Experiments with membranes.

When the membranes were treated with CKI after a pretreatment with increasing concentrations of spermine, and then separated by centrifugation, the casein kinase activity of the pellet was lower (fig. 3A, lanes b-e) than that in the control, (fig. 3A, lane a), and decreases in a dose-dependent manner in the presence of this polyamine.

Accordingly, the casein kinase activity displayed by the corresponding supernatants (fig. 3B, lanes b-e) was higher than that by control (fig. 3 B, lane a): i.e. it was negligible in the absence of spermine (fig. 3B, lane a) and increases in a dose-dependent manner in the presence of this polyamine.

This indicates that CKI can bind spontaneously to membrane preparation and such a binding is decreased by spermine in a dose-dependent fashion.

Discussion.

The above results indicate that spermine at 0.1-0.5 mM concentration promotes the binding of casein kinase CKI to the outer membrane of rat liver mitochondria.

A reasonable explanation is that spermine, because of its polycationic structure, may act as bifunctional ligand, i.e. it interacts (head-to-head) both with CKI (negatively charged, being adsorbed on DEAE-cellulose) and with negatively charged binding sites of external surface of outer membrane. Such a reversible binding of CKI to the membrane-bound spermine does not follow normal saturation kinetics being counteracted and reversed by a competing process, i.e. by a reversible binding of added CKI to the aliquot of spermine ("free" spermine) which is not bound to membranes, thus giving rise to a soluble "spermine-CKI" complex, recovered in the supernatant. This latter process becomes by far predominant when, at higher spermine concentrations, no more binding

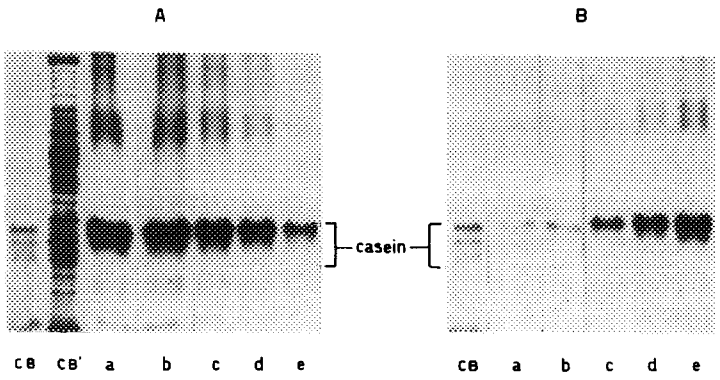

Fig. 3.
INHIBITORY DOSE-DEPENDENT EFFECT OF SPERMINE ON CKI BINDING TO MITOCHONDRIAL MEMBRANES.

A. Autoradiograms showing SDS-PAGE patterns of ^{32}P-labeled casein (casein kinase activity) by mitochondrial membranes (50 µg of proteins).
Membranes, obtained as described in the Methods, were treated with casein kinase CKI after preincubation in the absence (lane a) or in the presence of 0.5 mM (lane b), 1 mM (lane c), 2 mM (lane d), 5 mM (lane e) spermine, recovered by centrifugation and assayed (100 µg) for casein kinase activity as described in the Methods.
Lanes CB and CB' show the Coomassie-Blue-stained gels of casein and of membrane assay mixture, respectively.
The autoradiograms were exposed for 7 h.

B. Autoradiograms showing SDS-PAGE patterns of ^{32}P-labeled casein by the corresponding supernatants (25 µl).
Supernatants were obtained by centrifuging the above described membranes treated with CKI after preincubation in the absence (lane a) or in the presence of 0.5 mM (lane b), 1 mM (lane c), 2 mM (lane d), 5 mM (lane e) spermine.
50 µl supernatants were used for the casein kinase assay.
Lane CB shows the Coomassie-Blue-stained gel of casein.
The autoradiograms were exposed for 7 h.

sites on the membrane are available for the binding of spermine, so that the increasing concentrations of exceeding "free" spermine can compete with the membrane-bound spermine for the binding of added CKI, thus ultimately leading to a decrease of membrane-bound CKI (recovered in the supernatant).

In conclusion, the above results, together with those reported in a previous paper (2), show that spermine-promoted association of casein kinase CKI and CKII to the mitochondrial structures occurs through two different mechanisms.

Precisely, this polyamine, while promoting the transmembrane translocation of CKII across the outer membrane and its binding to more internal structures (2), promotes the binding of CKI to the outer membrane but inhibits its spontaneous binding to positively charged binding sites of more internal structures (perhaps positively charged phospholipids (9)).

These results suggest that this ubiquitous polycation may play an important critical role in regulating the intracellular distribution of casein kinases CKI and CKII in order to locate the enzyme close to their physiological target substrate.

By the way, it is noteworthy that (as shown by previous papers (10-12) concerning the different intramitochondrial localization of the two endogenous casein kinases), the

CKII (referred to as casein kinase TS) has been found to be localized predominantly in the soluble intermembrane compartment, whereas CKI (referred to as casein kinase S) has been found to be tightly bound to membrane structures mainly to outer membrane through electrostatic interactions, being extractable by high ionic strength solutions (12).

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