Phosphorylated and non-phosphorylated connexin-32 molecules in gap junction plaques are protected against calpain proteolysis after phosphorylation by protein kinase

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Introduction

Gap junctions are formed by plaques of functionally assembled intercellular channels which are responsible for direct communication between the cytoplasm of adjacent cells in the form of ions and second messenger molecules. These channels are formed as two hexameric hemichannels of connexin (cxn) subunits that interlock head-to-head [1–6]. Connexins form an extensive family of well-conserved proteins, which exhibit differential expression in the given tissue [7]. Thus, cxn-26 and cxn-32 are the two species expressed in adult liver.

Assembly and disassembly of gap junctions occur very rapidly, and a half-life of 1–2 h for a connexin molecule has been measured (see [8] and references therein). This would be particularly true of tissues undergoing fast growth and morphogenesis, which would exhibit dramatic changes in the number of mitotic cells [9].

Little is known about the pathways for assembly and disassembly of gap junction channels. It is evident, however, that such complex processes have to be highly regulated. We have proposed that proteolytic cleavage of connexins by calpain could be relevant for the concomitant and/or subsequent disassembly of gap junctions. Thus, phosphorylation of connexin by protein kinase C (PKC) may play a prominent regulatory role in this process, preventing unwanted connexin degradation [10]. Conversely, connexin dephosphorylation could be a signal for the initiation of gap junction disassembly [10]. We now describe that the proteolytic effect mediated by phosphorylation by PKC is efficiently spread to neighboring non-phosphorylated connexin molecules.

Experimental

Membrane fractions from male Swiss albino mice liver were prepared as previously described [10], and gap junctions were prepared from these membranes by an alkali precipitation step and sucrose gradient centrifugation [11]. Slab gel electrophoresis in a 5–20% (w/v) polyacrylamide linear gradient gel in the presence of 0.1% (w/v) SDS at pH 8.3 was performed following the method of Laemmli [12]. The method of Lowry et al. [13] was used for the determination of protein concentration in the membrane preparation, using BSA as a standard, after a precipitation step with 10% (w/v) trichloroacetic acid. In contrast, the concentration of protein in preparations of purified connexin was determined densitometrically after staining the electrophoretic gels with Coomassie Brilliant Blue R-250 and using a calibration with BSA. µ-Calpain was purified from fresh human erythrocytes as previously described [14,15]. The activated 80 kDa subunit of m-calpain from rabbit skeletal muscle, and the catalytic subunit of protein kinase A (PKA) (from bovine heart) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); PKC (from rat brain) was purchased from CalBiochem (LaJolla, Switzerland), and [γ-32P]ATP (tributylammonium salt) (3000 Ci/mmol) was obtained from Amerham (Bucks, U.K.) All other chemicals in this work were of analytical grade.

Effect of phosphorylation on proteolysis by calpain

We have characterized the phosphorylation parameters of mouse liver cxn-32 by PKC and PKA in vitro and have demonstrated that µ-calpain and m-calpain cleave cxn-32 at its C-terminal end [10]. Cxn-26, however, is not cleaved by either calpain isoform [10]. A striking differential effect was found when the phosphorylation was done using PKA or PKC. Thus, phosphorylation by PKC but not by PKA efficiently prevents proteolysis of phosphorylated cxn-32 (P-cxn-32) by either calpain isoform [10].

Figure 1 presents an autoradiograph obtained from a typical experiment, where mouse liver cxn-32 was incubated in the absence (−) or presence (+) of m-calpain after phosphorylation with PKA or PKC. The protective effect of phosphorylation by PKC is readily observable. In contrast, phosphorylation by PKA does not prevent proteolysis.
Phosphorylation of ccr-32 by PKC but not by PKA
prevents the proteolytic effect of m-calpain

Mouse liver gap junctions (0.98 μg of protein) were incubated
at 37°C for 5 min in 100 μl of a medium containing 20 mM Na-
Hepes, pH 7.4, 5 mM MgCl₂, 0.2 mM EGTA, 1.7 mM CaCl₂,
(1.5 mM free Ca²⁺), 2 mM dithiothreitol, 0.25% (w/v) Triton
X-100, 250 μg/ml phosphatidylserine, 25 μg/ml t-Boc-
Lys(CHO)₆, 10 μM (γ-³²P)ATP, and 1.5 units 0.15 μg of PKA
(1 unit = 1 pmol/min at pH 6.5, 30°C) or 0.2 units 125 ng of PKC
(1 unit = 1 pmol/min, room tempera-
ture). Theses the mixture was incubated for 1 min at 4°C
in the absence (-) and presence (+) of 0.03 units (0.8 μg)
of m-calpain (1 unit = 0.01 mg/mg/min at pH 7.5, 30°C). The reac-
tion was stopped with 10% (w/v) trichloroacetic acid (final concen-
tration) and the precipitated proteins were processed by
gel electrophoresis and autoradiography.

PKA  PKC

Ccr-32 Fragment

- 4 -

Ccr-32

We also determined the amount of ccr-32 protein in vitro
before and after phosphorylation with either PKA
or PKC (Figure 2). In all cases, phosphorylation of PKC-
phosphorylated ccr-32 was significantly lower than
proteolysis of PKA-phosphorylated or unphos-
phorylated ccr-32. No significant differences were
found in extent of proteolysis when ccr-32 was
phosphorylated by PKA.

Comparison between protein kinases

We judged that the protection of ccr-32 molecules
induced by phosphorylation by PKC amounted to
90%-80% of total ccr-32. Therefore, we decided
to evaluate the molal stoichiometry of phosphoryla-
tion in both cases, to see whether the degree of
phosphorylation differed for different protein kinases.
The stoichiometry was low in both cases,
even after 30 min of phosphorylation, and of similar
magnitude to that described by others [16]. Fur-
thermore, the phosphorylation stoichiometry with
PKC was even lower (0.005 mol P/10000 mol)
bound/mol of ccr-32) than with PKA (0.022 mol P/10000 mol
of ccr-32).

These experiments raise the possibility that
phosphorylation by PKC of only a fraction of ccr-
32 molecules protected a large number of
unphosphorylated ccr-32 molecules against proto-
ylisis. To ascertain this possibility, we performed a
series of stoichiometric phosphorylation experi-
ments where we determined the ratio of protected
non-phosphorylated ccr-32 molecules to protected
PKC-phosphorylated ccr-32 molecules after treat-
ment with μ-calpain or m-calpain. As shown in
Table 1, the total number of non-phosphorylated ccr-32 molecules protected against proteolysis by
either calpain isoform was far higher (19-133-fold)
than the number of phosphorylated ccr-32 mole-
cules present in the preparations. Additionally, we
determined that the non-phosphorylated ccr-32-PK

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Table 1

The protective effect of PKC phosphorylation of cnx-32 against proteolysis by μ- or m-calpain spread to neighboring non-phosphorylated cnx-32 molecules.

The indicated amount of mouse liver cnx-32 was phosphorylated by PKC or PMA as described in the legend to Figure 1. The phosphorylated preparations were treated with μ- or m-calpain as indicated and the protective effect produced by PKC was measured in the phosphorylated and non-phosphorylated cnx-32 molecules using as a control the experiment performed with PMA. The protection ratio is defined as amount of non-phosphorylated cnx-32 (mol)/amount of phosphorylated cnx-32 (mol).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Protease</th>
<th>Cnx-32 in the assay (nmol)</th>
<th>Phosphorylated cnx-32 (nmol)</th>
<th>Total cnx-32 protected (nmol)</th>
<th>Protection ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>μ-Calpain</td>
<td>55000</td>
<td>84</td>
<td>1180</td>
<td>133</td>
</tr>
<tr>
<td>2</td>
<td>μ-Calpain</td>
<td>24000</td>
<td>55</td>
<td>5520</td>
<td>100</td>
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<tr>
<td>3</td>
<td>m-Calpain</td>
<td>49000</td>
<td>49</td>
<td>6020</td>
<td>122</td>
</tr>
<tr>
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<td>m-Calpain</td>
<td>6700</td>
<td>78</td>
<td>1880</td>
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<tr>
<td>5</td>
<td>m-Calpain</td>
<td>4500</td>
<td>46</td>
<td>900</td>
<td>19</td>
</tr>
</tbody>
</table>

Figure 3

Effect of different parameters on the non-phosphorylated cnx-32/phospho-cnx-32 protection ratio.

(a) The non-phosphorylated cnx-32/phospho-cnx-32 protection ratio is plotted against the total amount of cnx-32 used in the assay system. The data are derived from Table 1 and the experiments were performed with μ-calpain (M) or m-calpain (A). (b) Mouse liver gap junctions (1.5 μg of protein) were incubated at 37°C for 5 min in 200 μl of a medium identical to that described in Figure 1 except that it contained 2.2 mM diethylenetriamine-N,N,N',N'-tetraacetic acid, 20 μM b-glycerophosphate, 150 μM ATP, and 10 mM of PMA or 25 ng of PKC. Thereafter, the indicated amount of m-calpain was added to the mixture and this was incubated at 4°C for 1 min. A control was performed in the absence of m-calpain. The reaction was stopped with 10% (v/v) trichloroacetic acid (final concentration) and the precipitated proteins were processed for gel electrophoresis and autoradiography. The phosphorylation stoichiometry was determined by measuring the amount of 32P bound to the cnx-32 band with a scintillation counter and using as standard [γ-32P]ATP of known concentrations. The non-phosphorylated cnx-32/phospho-cnx-32 protection ratio obtained with PKC, using as control the experiment performed with PMA, is plotted against the amount of m-calpain in the assay system. (c) Mouse liver gap junctions (16.5 μg of protein) were incubated at 37°C in 1.4 ml of a medium identical to that described in the legend to (b) except that 100 μM (15 μg) γ-32P-ATP, and 0.3 μg of PMA or 0.3 μg of PKC were used. At the indicated times, 200 μl aliquots were taken, added to 2.5 μl of m-calpain and incubated at 4°C for 1 min. The reaction was stopped with 10% (v/v) trichloroacetic acid, and the precipitated proteins were processed for gel electrophoresis and autoradiography. The non-phosphorylated cnx-32/phospho-cnx-32 protection ratio obtained in the experiment with PKC was determined using as control the experiment performed with PMA. The protection ratio is plotted against the time of phosphorylation.

![Graphs](image)

The cnx-32 protection ratio is higher when the total amount of substrates present in the assay system increases (Figure 3a), using either μ-calpain or m-calpain. In contrast, this ratio significantly drops when the amount of calpain used increases (Figure 3b) or when the time of phosphorylation increases (Figure 3c). It is important to mention that the presence in the assay systems of Triton X-100, which is required for efficient phosphorylation, does not solubilize gap junction plaques [17]. Therefore, the protection phenomenon takes place in non-disorganized gap junctions.
Promote engineering for the elucidation of the mechanism of electron transfer in redox proteins

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Introduction
Redox reactions in living organisms constitute the transfer of energy for all of their biological functions. In these processes, the electrons are taken up from reduced substrates and transferred by a mechanism that involves, in many cases, protein-protein interactions. Paradoxically, among the most important components of these electron transfer events, participating as they do in photosynthesis, respiration, microsomal electron transfer, nitrogen fixation, and other biosynthetic and degradative reactions. These proteins contain a tightly bound flavin group, which is bright yellow in colour in its oxidized state and bleaches when it becomes reduced. The reduction can take place in a single step, in which the uptake of two electrons at one time produces the hydroquinone form of the flavin, or in two sequential steps, each involving the uptake of one electron, in which case the intermediate semiquinone is formed. This characteristic change in the colour of flavoproteins, which is dependent on their redox state, makes it possible to follow their participation in enzymic reactions by simply measuring A530, an increase of which is a measure of the reduction of the oxidized form. The reaction can also be followed at 600 nm, where the semiquinone form exhibits maximal absorbance. This unique ability of flavoproteins to exchange

480 | 488
486 | 494
492 | 500
508 | 516
514 | 522
520 | 528
526 | 534