Specificity of Chicken (IgY) versus Rabbit (IgG) Antibodies Raised against Cholecystokinin Octapeptide (CCK-8)

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Summary
Antibodies produced in chickens (egg yolk antibody-IgY) and rabbits against CCK-8 TyrSE (a C-terminal extended CCK-version) were compared with respect to their specificity against several modified CCK-sequences by means of radioimmunoassay and spot blot assay. The content of neuronal CCK was determined by using both an „avian“ and a „mammalian“ RIA. The IC₅₀ values obtained indicate differences between the binding capacity of rabbit and chicken antibody, respectively. Supported by the data from spot blot assay, it appears, that the avian antibody binding activity was directed primarily towards short CCK-sequences whereas the longer sequences are less well recognised in contrast to the mammalian antibody. Probably, these differences may be due to characteristics regarding the shape of the molecules (caused also by fixation processes necessary for blotting procedures) as well as to structural differences between avian and mammalian antibodies itself (both antibodies originate from quite different immune systems). By comparing the quantitative CCK data (avian versus mammalian RIA) a significant correlation could be observed. Immunohistochemical studies using avian antibodies revealed a neuronal CCK pattern different from those using rabbit antibodies. These results are discussed on the basis of the specificity studies.

Keywords: CCK, IgY, egg yolk antibody, RIA, alternative

1 Introduction
CCK-8 belongs to a so-called brain-gut peptide family (Tatemoto 1983) which includes several molecular species (e.g. gastrin, CCK-58, CCK-33, CCK-4) having peripheral (nutrition) and central (neuronal transmission) biological functions. Antibodies raised against CCK usually cross-react with other CCK/gastrin members since these peptides share the last five amino acids and the antibodies are mostly directed against the C-terminal part of the molecule. Sequence-specific antibodies are rather scarcely available. Neuronal CCK was firstly described by Vanderhaeghen et al. (1975) and subsequently, largely identical distribution patterns of CCK in mammalian brain were described by several authors by using different antibodies (Loren et al., 1979; Innis et al., 1979; Larsson and Rehfeld 1979; Hökfelt et al., 1988; Seroogy et al., 1989 a,b; Pfister et al., 1989). Perikarya and fibres with CCK-like immunoreactivity (CCK-IR) were observed in several brain regions as e.g. substantia nigra (SN), ventral tegmental area (VTA), hippocampus, amygdala, cortex, whereas only fibres were found in the nucleus accumbens, striatum and substantia olfactoria. However, occasionally single somata with CCK-IR were observed in these brain regions (rats) (Takagi et al.,...
1984; Hökfelt et al., 1988; Seroogy et al., 1989) but so far it is not defined under which experimental conditions these neurons have been visualised (perhaps it might be due to a pretreatment with colchicine). Recently, we have produced an antibody against CCK-8 in chickens which reproducibly stains perikarya in telencephalic rat brain regions (Schade et al., 1988; Pfister et al., 1989; Schade et al., 1991). Since these findings are at least partly in contrast to corresponding data from literature this study was performed to characterise the specificity of the avian antibody (IgY-antibody) in comparison to a rabbit antibody raised against the same conjugate.

2 Laboratory animals, material and methods

2.1 Animals
Chickens were housed in groups of five animals under SPF conditions in cages 2 × 2 m. The keeping parameters were in accordance with EG requirements. The experiment was performed in the „Agricultural Training- and Experimental-Station (Landwirtschaftliche Lehr- und Versuchsstation) of the Humboldt-University“. From the point of animal welfare these housing conditions are not optimal, but there was no alternative at the time. The chickens were derived from the hybrid line „Medes white“ (Deersheim, 13, inbred line). Rabbits (HsdPoc:NZW, breeding Harlan Winkelmann, Borchen) were sampled by bleeding (rabbit).

The keeping parameters were

Table 1: Comparison of the log IC<sub>50</sub> values obtained testing modified CCK-sequences in an „avian“ and „mammalian“ RIA, respectively.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Ch</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK-8SE</td>
<td>H-Asp-Tyr(MET-Gly-TRP-MET-ASP-PHE-NH₂)</td>
<td>0.77</td>
<td>0.61</td>
</tr>
<tr>
<td>CCK-8NS</td>
<td>H-Asp-Tyr(MET-Gly-TRP-MET-ASP-PHE-NH₂)</td>
<td>0.67</td>
<td>0.83</td>
</tr>
<tr>
<td>Succ-CCK-8SE</td>
<td>Succ-Tyr(MET-Gly-TRP-MET-ASP-PHE-NH₂)</td>
<td>0.80</td>
<td>0.47</td>
</tr>
<tr>
<td>Turtle CCK-8SE</td>
<td>H-Asp-Tyr(MET-PRO-Gly-TRP-MET-ASP-PHE-NH₂)</td>
<td>1.05</td>
<td>1.80</td>
</tr>
<tr>
<td>Chicken CCK-8SE</td>
<td>H-Ph-Asp-(Tyr(PRO-ASP)-TRP-MET-ASP-PHE-NH₂)</td>
<td>1.55</td>
<td>1.51</td>
</tr>
<tr>
<td>Gastrin 13SE</td>
<td>Glu-Ala-(Tyr(MET-Gly-TRP-MET-ASP-PHE-NH₂))</td>
<td>0.52</td>
<td>1.36</td>
</tr>
<tr>
<td>CCK-8 TyrSE</td>
<td>H-Asp-(Tyr(MET-Gly-TRP-MET-ASP-PHE-Tyr(SE)-NH₂))</td>
<td>0.71</td>
<td>-0.61</td>
</tr>
<tr>
<td>GE-410</td>
<td>Succ-Tyr(MET-Gly-TRP-MET-ASP-PHE-PEA)</td>
<td>2.52</td>
<td>3.12</td>
</tr>
<tr>
<td>Ki-1001</td>
<td>Succ-Tyr(MET-Gly-TRP-MET-ASP-PHE-PEA)</td>
<td>6.29</td>
<td>6.29</td>
</tr>
<tr>
<td>CCK-5</td>
<td>H-Gly-TRP-MET-ASP-PHE-NH₂</td>
<td>1.15</td>
<td>2.37</td>
</tr>
<tr>
<td>Boc-CCK-5</td>
<td>Boc-Gly-TRP-MET-ASP-PHE-NH₂</td>
<td>1.07</td>
<td>2.15</td>
</tr>
<tr>
<td>CCK-5-Tyr</td>
<td>H-Gly-TRP-MET-ASP-PHE-Tyr(NH₂)</td>
<td>5.51</td>
<td>6.29</td>
</tr>
<tr>
<td>CCK-4</td>
<td>H-TRP-MET-ASP-PHE-NH₂</td>
<td>2.46</td>
<td>2.96</td>
</tr>
<tr>
<td>CCK-2-6</td>
<td>Succ-Tyr(MET-Gly-TRP-MET-ASP-PHE-PEA)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cycl. CCK-5</td>
<td>cycl(Asp-Tyr-MET-Gly-TRP-MET-ASP-PHE)</td>
<td>5.28</td>
<td>3.43</td>
</tr>
<tr>
<td>Cycl. CCK-8</td>
<td>cycl(Asp-Tyr-MET-Gly-TRP-MET-ASP-PHE)</td>
<td>3.45</td>
<td>3.45</td>
</tr>
</tbody>
</table>

The marked amino acids indicate the modifications compared to the original molecule. Marked IC<sub>50</sub> values indicate major differences between them. Boc = tert. butyloxy carbonyl, PEA = β-phenethylamid.
thylamino-carbodiimide (EDC, SuccC-CK-8 SE, KL 1001) according to usual coupling procedures. Aliquots of the freeze-dried conjugates were dissolved in 1μl DMSO and diluted with 0.1 M phosphate buffer (pH 7.4). Serial dilutions of the conjugate were prepared at constant KLH concentration (0.25 mg/ml). Subsequently, 2 μl, containing 0.5μg – 0.005 μg of a conjugate, were spotted on nitrocellulose paper. As blanks, glutaraldehyde/EDC treated KLH were spotted. After blocking the protein binding capacity of the nitrocellulose paper with gelatin (0.1 %) and subsequent washing steps, the paper was incubated overnight with the rabbit anti-CCK antibody (1:1.200 diluted in Tris-buffered saline, TBS, pH 7.4) or with chicken anti-CCK antibodies (1:200 diluted in TBS). The primary antibodies were visualised by means of the PAP/DAB staining.

Additionally, by means of a sandwich enzyme immunoassay (EIA, Porstmann et al., 1988) the binding of anti-CCK antibodies from different chickens with coupled CCK in comparison with unchanged CCK (RIA) was studied. For this test, microplates (Nunc) were coated with an Ovalbumin-CCK-8 Tyr SE complex (3 μg/well). The antibody samples diluted to 1:100 with phosphate buffered saline (PBS, pH 7.2) were used.

3 Results

Table 1 shows the peptide sequences used for investigation of antibody-specificity and the corresponding IC₅₀ values obtained using the „mammalian“ or „avian“ RIA. In fig. 1, a comparison is shown between several binding curves achieved with chicken or rabbit anti-CCK antibody. It can be seen that the avian antibody do not differentiate between the larger CCK-
sequences (8 amino acids) in contrast to the mammalian antibody. Furthermore, there are partly remarkable differences between the corresponding IC50 values e.g. for gastrin and particularly for CCK-8 TyrSE. That holds also true for some of the short sequences as e.g. CCK-5 and Boc-CCK-5 which are more sensitively recognised by the avian antibody. This result is supported by data from the spot blot assay (table 2) which indicate a higher sensitivity of the IgY-antibody against CCK-4 in contrast to CCK-8 SE. However, a direct comparison between RIA-results and SBA-results is hardly possible since in the first case unchanged molecules are used in contrast to coupled molecules in SBA. These results strongly support the suggestion that the coupling procedure may change the molecule’s conformation and consequently, leads to a changed binding behaviour (see the data for Succ-CCK 8SE). Fig. 2 shows a comparison between the binding activity of several avian anti-CCK-8 TyrSE antibody samples in RIA and EIA. As can be seen, there are only a few antibody samples working identically in both test systems. Most of the antibody samples work either in RIA or in EIA which indicates that at least two different specificities exist.

The CCK content of rat brain tissue samples (striatum) was determined using an avian antibody in comparison to two different rabbit antibodies (raised against CCK-8 SE, antibody 4 and against CCK-8 Tyr SE, antibody 5). A statistical analysis yielded a significant correlation between the data obtained with the avian and the mammalian antibodies (fig. 3).

4 Discussion

The most crucial point in immunocytochemistry is the characterisation of specificity of the antibody used to visualise a certain substance. That is of particular importance, if tissue fixation is involved in staining procedures since the molecular structure of the substance of interest may be changed in non-defined ways. A simple control is the preabsorption of the antibody with the presumably same epitope of a polyclonal avian antibody bound with the presumably same epitope of a human derived procollagen type III molecule. However, only the avian antibody showed reactivity with both, rat and human derived procollagen. The assumption that conformational events may influence the binding behaviour is further supported by the results from SBA. In this assay the avian antibody preferentially reacts with KLH-coupled CCK-4 in contrast to the mammalian antibody.

A quantitative RIA for CCK content in striatal tissue of rat brain revealed a good correlation between the CCK-values obtained by the “avian” and the “mammalian” assays. There are two
Comparing of binding behaviour of different samples of avian anti CCK-antibodies in two different assays

Figure 2: Shown is the binding of antibody-samples in a RIA in comparison to binding in EIA. Each point represents an antibody sample extracted from one egg. The eggs originated from a group of five hens kept together. Thus, the eggs could not identified and, consequently, a titre development could not registered.

possibilities to discuss this result. 1.: It is thinkable, that the rabbit and chicken antibody each bind with several CCK-sequences but in a different ratio which finally results in a corresponding total CCK content. 2.: More simply, following the tissue preparation for CCK determination only one CCK-species is present in the probes to be measured. The differences between avian and mammalian antibody in recognising certain epitopes become important if the molecule is processed by fixation.

In conclusion, substantial differences between the binding behaviour of avian and mammalian anti CCK-antibodies in connection with the assay used have been observed. The reactivity of the avian antibody additionally seems to be influenced if coupled CCK-sequences are used instead of „native“ molecules.

A prerequisite for the visualisation of neuronal CCK is the perfusion of the brain with a fixation solution. This procedure may result in a conformational change of neuronal CCK. Since CCK is synthesized in neurones as pre-pro peptide and subsequently processed during axonal transport, several CCK-species may be present in mammalian brain (Rehfeld, 1978). Thus, the different staining patterns obtained by using the avian antibody probably reflect the differences in reactivity as

Figure 3: Shown is the comparison of striatal CCK-content measured by means of a RIA based on avian or mammalian antibody. Rabbit antibody 4 was raised against CCK-8SE, whereas rabbit antibody 5 was raised against CCK-8TyrSE. The statistical analysis revealed correlation coefficients of \( r = 0.86 \) (rabbit antibody 4/rabbit antibody 5), \( r = 0.90 \) (rabbit antibody 4/chicken antibody), and \( r = 0.95 \) (rabbit antibody 5/chicken antibody).
discussed. In addition, there is a great phylogenetic distance between the mammalia and aves and structural characteristics of avian antibody differ significantly from mammalian antibody (Shimizu et al., 1992). Thus, the idea that a different reactivity of avian antibody may result in a different distribution pattern of neuronal CCK is in agreement with the results presented. Finally, the preparation of brain tissue for visualisation of small molecules like peptides causes conformational changes of many substances which may be recognised by avian but not by mammalian antibodies (and vice versa). Therefore, the introduction of avian antibodies in immunohistochemistry may be a valuable addition for studies on neuronal transmission.

References


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