SUMMARY

The dynamic assembly/disassembly of tight junction is critical for the regulation of permeability of blood-tissue barriers in multicellular organisms. Tight junction formation is thought to be regulated by protein kinase C, although the molecular mechanisms involved are poorly understood. Occludin is an integral membrane phosphoprotein specifically associated with tight junctions, contributing to the structure and function of this intercellular seal. Previous studies indicate that the rearrangement of tight junctions is paralleled by the changes in occludin phosphorylation state, suggesting that occludin may play a regulatory role in tight junction biogenesis. However, the occludin kinase and its phosphorylation sites have not been identified. Therefore, the aims of this work were:

- to evaluate the involvement of protein kinase C pathways in the regulation of the occludin phosphorylation and cellular distribution.
- to investigate how novel and conventional PKC, the two diacylglycerol-sensitive PKC subfamilies, are involved in tight junction assembly and disassembly.
- to identify *in vivo* sites of occludin phosphorylation.

Results: To study the effects of protein kinase C activators and inhibitors on occludin phosphorylation and tight junction assembly, the calcium switch model has been applied. MDCK cells cultured in low-calcium medium completely lost intercellular contacts. Upon switching back to normal calcium medium, cell monolayers formed functional tight junctions within few hours. Phorbol 12-myristate 13-acetate and 1,2-dioctanoylglycerol induced the rapid phosphorylation of occludin in MDCK cells cultured in low extracellular calcium medium with a concomitant translocation of occludin to the regions of cell-cell contact. The extent of occludin phosphorylation, as well as the incorporation of occludin and ZO-1 into tight junctions, induced by protein kinase C activators or calcium switch was markedly decreased by the *classical* and *novel* protein kinase C inhibitor GF-109203X. Similarly, rottlerin, the inhibitor of *novel* δ and θ protein kinase C isotypes, blocked both the phosphorylation of occludin and tight junction formation induced by the switch to normal calcium medium. In addition, i*n vitro* experiments showed that recombinant COOH-terminal domain of murine occludin could be directly phosphorylated by purified PKCδ. The application of *classical* PKC inhibitor Gö6976 during calcium switch unexpectedly accelerated the phosphorylation of tight junction protein occludin. The phosphorylation was accompanied by the accelerated incorporation of occludin into newly forming tight junction. Furthermore, Gö6976 caused the assembly of tight junctional complex and occludin phosphorylation in cells cultivated in low calcium medium and strongly attenuated the disruption of tight junction complex induced by switch to low calcium medium.

A simple procedure for the isolation of highly-phosphorylated occludin forms from rat liver was developed for subsequent identification of phosphorylation sites. Ser371 of rat occludin was identified as an *in vivo* occludin phosphorylation site by electrospray ionization tandem mass-spectroscopy.

Conclusions:

TJ assembly is regulated by antagonistic signalling of novel and classical PKC isozymes: the formation of TJ complex is favoured by simultaneous activation of novel PKCs (presumably PKCδ) and inhibition of classical PKC/PKCμ signalling.

Occludin was shown to be a target for PKC-mediated signalling: activation of novel PKCs induces the rapid phosphorylation of occludin with a concomitant translocation to the regions of cell-cell contact. Both occludin phosphorylation and incorporation into the newly forming tight junctions was counteracted by the classical PKC signalling. *In vitro* experiments confirmed that protein kinase Cδ may directly phosphorylate occludin.

Ser371 was for the first time identified as an *in vivo* phosphorylation site of *Rattus norvegicus* liver occludin isolated by a newly developed procedure.