Receptor activated Ca2+ signalling processes in DDT1 MF-2 smooth muscle cells

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SUMMARY

In this thesis, the Ca^{2+} signalling pathway of H_1-histaminoceptor stimulated DDT_1 MF-2 smooth muscle cells was investigated. Further, the ATP sensitive receptor on the plasma membrane was characterized. In this last chapter, the results of the previous chapters will be summarized and evaluated.

Stimulation of certain receptor sites results in a pronounced enhancement of the intracellular Ca^{2+} concentration, due to Ca^{2+} mobilization from internal stores and Ca^{2+} entry from the extracellular space. The receptor activated formation of inositol phosphates, in particular $Ins(1,4,5)P_3$, is assumed to be responsible for the release of intracellular stored Ca^{2+}. However, other inositol phosphates ($Ins(1,3,4,5)P_4$), the presence of GTP, or the Ca^{2+} activated Ca^{2+} releasing process may also contribute to the rise in internal Ca^{2+} upon receptor stimulation (chapter 1).

Activation of H_1-histaminoceptors of vas deferens derived DDT_1 MF-2 smooth muscle cells resulted in a pronounced formation of $Ins(1,3,4,5)P_4$ with respect to that of $Ins(1,4,5)P_3$, a rapid enhancement of the intracellular Ca^{2+} concentration and a Ca^{2+} dependent outward K^+ current. In chapter 2, the role of the inositol phosphates and the Ca^{2+} induced Ca^{2+} release mechanism was investigated in permeabilized as well as histamine stimulated, intact, cells. In saponin permeabilized DDT_1 MF-2 cells, it was found that both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ sensitive Ca^{2+} stores were present while a Ca^{2+} activated Ca^{2+} releasing mechanism could not be detected in this permeabilized cell system. The Ca^{2+} releasing capacity of $Ins(1,3,4,5)P_4$ was dependent on the simultaneous presence of $Ins(1,4,5)P_3$, and the effect of both inositol phosphates was inhibited by the $Ins(1,4,5)P_3$ receptor antagonist heparin. Thus, the additional Ca^{2+} released by $Ins(1,3,4,5)P_4$ was mediated via $Ins(1,4,5)P_3$ sensitive Ca^{2+} channels and it was concluded that the effect of $Ins(1,3,4,5)P_4$ was mediated via connection of an $Ins(1,4,5)P_3$ insensitive Ca^{2+} store to the $Ins(1,4,5)P_3$ sensitive Ca^{2+} compartment.

H_1-histaminoceptor stimulation in intact cells induced a Ca^{2+} dependent outward K^+ current, representing the internal Ca^{2+} release if Ca^{2+} entry from the extracellular space was prevented. The histamine activated current was not detected if the inositol phosphate induced Ca^{2+} release was inhibited by heparin. This result indicated that both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ sensitive Ca^{2+} stores could play a role in the rise in internal Ca^{2+} upon stimulation with histamine and excluded the contribution of the Ca^{2+} induced Ca^{2+} release mechanism to this process. Depletion of $Ins(1,4,5)P_3$ sensitive Ca^{2+} stores in advance reduced the K^+ current only to some extent. However, when both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ sensitive Ca^{2+} stores were depleted prior to histamine stimulation, the Ca^{2+} sensitive K^+ current could not be detected anymore. These results demonstrated that $Ins(1,3,4,5)P_4$ sensitive Ca^{2+} compartments were mainly responsible for the histamine activated Ca^{2+} release

from internal stores thereby showing the importance of $Ins(1,3,4,5)P_4$ in the Ca^{2+} releasing process of H_1-histaminoceptor activated DDT_1 MF-2 cells, as depicted in Figure 1.

Apart from the mobilization of intracellular stored Ca^{2+}, the receptor activated Ca^{2+} entry process is also of considerable importance upon histamine stimulation. As mentioned in the introduction (chapter 1.2), the mechanism underlying the receptor activated Ca^{2+} influx is not established yet, but in most cells it is different from the well documented voltage operated Ca^{2+} channels. Although activation of receptor stimulated Ca^{2+} entry may be mediated via different mechanisms, the capacitative Ca^{2+} entry, in which Ca^{2+} entry is related to the filling state of the intracellular Ca^{2+} stores and the formation of inositol phosphates, in particular $Ins(1,3,4,5)P_4$, are considered to be the most important mechanisms for activation of receptor operated Ca^{2+} entry. The study presented in chapter 3 was carried out to identify the cellular component activating the histamine stimulated Ca^{2+} entry process in DDT_1 MF-2 cells. The results demonstrated that the Ca^{2+} entry process was independent of the histamine activated formation of the second messengers $Ins(1,4,5)P_3$ or $Ins(1,3,4,5)P_4$ and was still detected when depletion of intracellular Ca^{2+} stores was prevented by heparin, thereby excluding activation of the capacitative or inositol phosphate induced Ca^{2+} entry. These results were therefore suggestive for another component involved in initiation of the H_1-histaminoceptor activated Ca^{2+} entry process. It was observed that stimulation with histamine resulted in a marked release of AA in DDT_1 MF-2 cells. This AA formation was biphasic in nature, consisting of a fast rise followed by a more sustained second phase. Exogeneously applied AA to these cells induced a concentration dependent increase in internal Ca^{2+} concentration, due to activation of Ca^{2+} entry from the extracellular space. This effect of AA was not mediated by its metabolites, since inhibition of lipoxygenase, cyclo-oxygenase or epoxygenase pathways did not affect the AA induced Ca^{2+} entry. Pre-activation of the Ca^{2+} channels involved by AA resulted in abolition of the histamine activated Ca^{2+} entry, but not the internal Ca^{2+} mobilization. These observations showed that AA is functioning as a second messenger to activate plasmambrane Ca^{2+} channels, thereby promoting Ca^{2+} entry from the extracellular space in DDT_1 MF-2 cells, as is represented in Figure 1.

Although several enzymatic pathways are reported to be responsible for the mobilization of AA from membrane lipids, the most common pathway to release receptor activated AA is via the action of phospholipase A_2. Alternatively, PLC derived DAG can serve as a precursor for AA via activation of diglyceride lipase. Further, AA can be released from phosphatidic acid, derived from the hydrolysis of membrane lipids by PLD (chapter 1). In chapter 4 the biochemical pathway involved in H_1-histaminoceptor activated AA release of DDT_1 MF-2 cells was studied, to understand the histamine activated Ca^{2+} entry process. For DDT_1 MF-2 smooth muscle cells it was demonstrated that the histamine stimulated formation of AA was independent of the PLA_2 or PLD

activity, respectively, but could be blocked by the PLC inhibitor neomycine. These results suggested that AA release occured through the action of PLC and diglyceride lipase. In accord, it was found that inhibition of the diglyceride lipase activity resulted in a marked accumulation of DAG, resulting in reduction of the AA release. Therefore, in contrast to receptor activation in many other cell types, the H_1-histaminoceptor stimulated release of AA in DDT_1 MF-2 smooth muscle cells is predominantly mediated via the sequential action of PLC and diglyceride lipase (Figure 1).

Receptor activated Ca^{2+} signalling may be regulated by activation of PKC; a large family of ubiquitously distributed isozymes. It is reported that PKC modulates channel activity and PLC activity, resulting in the reduction of the inositol phosphate formation and subsequent Ca^{2+} release from internal stores and may affect the receptor activated release of AA (chapter 1). In chapter 5, the role of PKC in the histamine stimulated Ca^{2+} signalling process, including the formation of inositol phosphates and AA release, was studied. When the possible role of PKC regulating cellular processes is considered, on has to separate two types of experimental design. First, exogeneous activation of PKC by the use of phorbol esters like PMA, provides evidence about the existence of a PKC regulated feedback loop. However, to determine whether such a loop is physiologically activated upon receptor stimulation, one has to look at the consequences of interruption of the loop, which can be achieved by the use of PKC inhibitors like staurosporine or via down regulation of PKC activity by long term pretreatment with PMA. For DDT_1 MF-2 cells it was shown that PKC activation with PMA reduced the histamine stimulated formation of $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$ and the concomitant Ca^{2+} mobilization from intenal stores. Moreover, the receptor activated Ca^{2+} entry was abolished, while the release of AA was strongly reduced. Thus, these results show the existence of a negative feedback mechanism between exogeneously activated PKC and the formation of inositol phosphates, the subsequent mobilization of Ca^{2+}, the release of AA and the Ca^{2+} entry. In contrast, inhibition of PKC-activity by staurosporine or by long-term pretreatment with PMA, only affected the histamine activated Ca^{2+} entry, while the formation of inositol phosphates, the Ca^{2+} mobilization or the release of AA were not changed. These observations demonstrated that only the feedback circuit between PKC and Ca^{2+} entry was functionally activated upon stimulation with histamine, resulting in a PKC activated inhibition of Ca^{2+} entry in DDT_1 MF-2 cells.
Apparently different isoforms of PKC have to considered in these cells, coupled to PLC activity, the release of AA and the receptor activated Ca^{2+} entry, respectively. The exact nature of the PKC-isozymes remained to be identified.

Thus, the results obtained from this and the previous studies, as represented by Figure 1, indicate that stimulation of H_1-histaminoceptors on these cells causes activation of PLC, resulting in hydrolysis of phosphatidyl inositol 4,5 bisphosphate into AA-containing DAG and the inositol phosphates $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$. The inositol phosphates bind to their receptors to

mobilize Ca^{2+} from intracellular stores and DAG is metabolized by diglyceride lipase to produce AA. In turn, AA activates Ca^{2+} channels in the plasmamembrane to allow Ca^{2+} entry from the extracellular space. Besides its functioning as a precursor for AA, the formation of DAG may serve to activate protein kinase C (PKC). Since a functional inhibitory feedback loop between histamine stimulated PKC and Ca^{2+} entry, but not the formation of inositol phosphates and the release of AA was observed, an attractive hypothesis is that receptor stimulated DAG is first rapidly metabolized to AA, thereby promoting Ca^{2+} entry, which process is limited by a Ca^{2+} sensitive PKC, activated by (a subsequent) release of DAG.

In chapter 6, the effect of different nucleotides on the $Ins(1,4,5)P_3$ activated Ca^{2+} release of saponin permeabilized cells was investigated. Although the presence of ATP was not required to activate the $Ins(1,4,5)P_3$ induced Ca^{2+} releasing process, the amount of Ca^{2+} released by different concentrations $Ins(1,4,5)P_3$ was considerably enhanced upon addition of this nucleotide. The amount of additional Ca^{2+} released at a certain concentration of ATP was independent of the $Ins(1,4,5)P_3$ concentration used and an $Ins(1,4,5)P_3$ concentration-response curve with similar EC_{50} values and other characteristics as observed without ATP was obtained in the presence of ATP. Thus, even at maximal effective $Ins(1,4,5)P_3$, assumed to fully occupy the $Ins(1,4,5)P_3$ sensitive receptors and completely depleting the $Ins(1,4,5)P_3$ sensitive Ca^{2+} store, the presence of ATP induced an additional release of Ca^{2+}. The P_{2Y}-purinoceptor agonist 2-methylthio-ATP and the non-hydrolysable ATP analogue ATPγS enhanced the $Ins(1,4,5)P_3$ activated Ca^{2+} release to the same extent as ATP. Less effective were GTP, ADP and AMP, while UTP and α,β-methylene ATP appeared to be ineffective. The rank order of potency was 2-methylthio-ATP = ATPγS = ATP > GTP $\geq$ ADP > AMP > α,β methylene ATP = UTP. These results demonstrated that the presence of these nucleotides enhanced the total releasing capacity of the $Ins(1,4,5)P_3$ sensitive Ca^{2+} store via an ATP sensitive binding site, without changing the affinity of the $Ins(1,4,5)P_3$ receptor for $Ins(1,4,5)P_3$. The mechanism by which this ATP activated binding site exerts its effect may be due by increasing the efficacy of the $Ins(1,4,5)P_3$ receptor. In view of the sensitivity for ATP, ATPγS and 2-methylthio ATP, it was concluded that the effect observed is mediated by an internal binding site with comparable characteristics as an external P_{2Y}-purinoceptor.

Apart from the presence of an internal ATP sensitive binding site, intact DDT_1 MF-2 cells were previously described to possess external P_2-purinoceptors located on the plasma membrane. Stimulation of these receptors with ATP caused responses comparable to that obtained upon H_1-histaminoceptor stimulation: a relative small increase in $Ins1,4,5)P_3$ concentration and a pronounced formation of $Ins(1,3,4,5)P_4$ were accompanied by a rise in internal Ca^{2+} due to Ca^{2+} mobilization from internal stores and Ca^{2+} influx from the extracellular space. Furthermore, stimulation of these P_2-purinoceptors resulted in a change in membrane current, consisting of a Ca^{2+} dependent outward K^+

current followed by a sustained, non-specific inward current. The aim of the study presented in chapter 7 was to characterize the ATP sensitive receptors of DDT_1 MF-2 cells by investigating the cellular response to different nucleotides, as represented by the membrane current. It was found that these receptors could not be classified as P_1-purinoceptors, since adenosine appeared to be inactive in mediating a change in membrane current. The cellular response was also not mediated via P_{2T}-, P_{2X}-, and P_{2Y}-purinoceptors, since ADP, α,ß-methylene ATP and 2-methylthio-ATP, known to stimulate these receptors, respectively, did not change the membrane current either. In contrast, it was observed that pyrimidine containing nucleotides, in particular UTP, caused a pronounced change in membrane current with similar characteristics as observed with ATP. The rank order of potency to evoke the response was ATP > UTP > TTP > CTP = GTP. The responses mediated by ATP and UTP were not additive, and could be blocked by the reversible P_2-purinoceptor antagonist suramin. In view of the sensitivity for purine as well as pyrimidine containing nucleotides, the external ATP sensitive receptor of DDT_1 MF-2 cells was characterized as a nucleotide receptor (P_{2U}-purinoceptor).

In summary, this thesis described the receptor stimulated Ca^{2+} signalling process of DDT_1 MF-2 cells. It was shown that although Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 but no Ca^{2+} sensitive Ca^{2+} stores were present in these cells, the H_1-histaminoceptor stimulated rise in internal Ca^{2+} was predominantly derived from Ins(1,3,4,5)P_4 sensitive Ca^{2+} stores.

Furthermore, it was found that the histamine activated Ca^{2+} entry process was independent of the state of depletion of internal stores or the formation of inositol phosphates, but was activated by the release of AA generated by the ordered action of PLC and diglyceride lipase. Inhibition of this Ca^{2+} entry process was shown to be mediated by a histamine activated PKC isozyme. Evidence about the presence of PKC isozymes regulating PLC activity and AA release was also obtained, although involvement of these isozymes was not observed on histamine stimulation.

The Ins(1,4,5)P_3 activated Ca^{2+} release was enhanced by the presence of adenine nucleotides via activation of an internal binding site. In particular ATP, ATPγS and the P_{2Y}-purinoceptor agonist 2-methylthio-ATP considerably augmented the Ca^{2+} release induced by Ins(1,4,5)P_3, without changing the affinity of the Ins(1,4,5)P_3 receptor for Ins(1,4,5)P_3. On the other hamd, extracellular ATP was shown to activate Ca^{2+} signalling by interaction with a plasmamembrane located nucleotide receptor, which was also sensitive for the pyrimidine containing nucleotide UTP. A model representing these receptor stimulated Ca^{2+} signalling processes in DDT_1 MF-2 cells is presented in Figure 1.