Arachidonic acid is functioning as a second messenger in activating the Ca^{2+} entry process on H_1 -histaminoceptor stimulation in DDT₁ MF-2 cells

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This study was carried out to identify the cellular component activating the histamine-stimulated Ca^{2+} entry in vas-deferens-derived DDT₁ MF-2 cells. H_1 -histaminoceptor stimulation resulted in a rise in intracellular Ca^{2+} concentration, caused by Ca^{2+} release from inositol phosphate-sensitive Ca^{2+} stores and Ca^{2+} entry from the extracellular space, accompanied by a transient Ca^{2+} -activated outward K^+ current. The histamine-evoked K^+ current was still observed after preventing inositol phosphate-induced Ca^{2+} mobilization by intracellularly applied heparin. This current was activated by Ca^{2+} entry from the extracellular space, because it was abolished in the presence of the Ca^{2+} -channel blocker La^{3+} or under Ca^{2+} -free conditions. H_1 -histaminoceptor-activated Ca^{2+} entry was also observed in the presence of intracellularly applied $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, depleting their respective Ca^{2+} stores and pre-activating the inositol phosphate-regulated Ca^{2+} entry. Thus the ability of histamine to activate Ca^{2+} entry independently of Ca^{2+} mobilization and the formation of inositol phosphates suggests that another component is involved to initiate the Ca^{2+} -entry process.

It was observed that H_1 -histaminoceptor stimulation resulted in a pronounced release of arachidonic acid (AA) in DDT₁ MF-2 cells. Exogenously applied AA induced a concentration-dependent increase in internal Ca^{2+} due to activation of Ca^{2+} entry from the extracellular space. Slow inactivation of the AA-sensitive Ca^{2+} channels is suggested by the slow decline in Ca^{2+} entry. In accord, the histamine-induced Ca^{2+} entry was not observed with AA-pre-activated Ca^{2+} channels. Inhibition of the lipoxygenase and cyclo-oxygenase pathway did not affect the AA-induced Ca^{2+} entry. The histamine-induced change in internal Ca^{2+} and the concomitant K^+ current were decreased in the presence of AA and caused by Ca^{2+} mobilization from internal stores. Blocking this internal Ca^{2+} release by heparin, in the presence of AA, resulted in abolition of the histamine-induced Ca^{2+} -regulated K^+ current. These observations show that AA, released on H_1 -histaminoceptor stimulation in DDT₁ MF-2 cells, is functioning as a second messenger to activate plasma-membrane Ca^{2+} channels promoting Ca^{2+} entry from the extracellular space.

INTRODUCTION

Stimulation of smooth-muscle cells with various receptor agonists results in a contractile response by triggering an increase in intracellular Ca^{2+} . In general, this response consists of an initial transient rise, followed by a more slowly declining phase. The initial response is due to Ca^{2+} release from internal stores mediated by inositol phosphates formed upon receptor-stimulated activation of phospholipase C (Berridge, 1993). The slow phase results from Ca^{2+} entry from the extracellular space, probably through the opening of plasma-membrane Ca^{2+} channels (Putney, 1990; Irvine, 1992).

The mechanism underlying the regulation of Ca^{2+} entry by phospholipase-C-linked receptors is not yet clear, but in many cell types, including DDT₁ MF-2 smooth-muscle cells, it is distinct from the well-studied voltage-dependent Ca^{2+} channels (Von Tscherner et al., 1986; Hallam and Rink, 1989; Jacob, 1990; Molleman et al., 1991a). Ca^{2+} entry is postulated to be mediated via a receptor-operated Ca^{2+} channel, perhaps modulated via a G-protein (Benham and Tsien, 1987; Benham, 1988; Brown and Birnbauer, 1990; Graier et al., 1992). Ca^{2+} may also enter the cell via a second-messenger-operated Ca^{2+} channel, controlled by the receptor-activated formation of $\text{Ins}(1,4,5)\text{P}_3$, in addition to its activation of Ca^{2+} release from internal stores, or $\text{Ins}(1,3,4,5)\text{P}_4$ (Morris et al., 1987; Irvine, 1992; Lückhoff and

Clapham, 1992). Another proposed mechanism is the capacitative Ca^{2+} entry, where activation of Ca^{2+} influx is coupled to the filling state of the intracellular Ca^{2+} stores (Merritt and Rink, 1987; Putney, 1990).

Recently, the role of arachidonic acid (AA) in cellular signalling has been reconsidered (Axelrod, 1990; Bonventre, 1992). Stimulation of some receptors on different cell types can result in the release of AA from membrane lipids. The liberation of AA can occur through at least three mechanisms: through the direct action of phospholipase A_2 on phospholipids, through the sequential action of phospholipase C and diacylglycerol lipase, or through the action of diacylglycerol lipase on phosphatic acid generated by phospholipase D (Axelrod, 1990). It is reported that AA or its metabolites, derived through cyclo-oxygenase or lipoxygenase pathways, can have a variety of biological effects (Bonventre, 1992). AA has been shown to mobilize Ca^{2+} from internal stores (Hertelendy et al., 1992; Soliven et al., 1993) and to modulate different plasma-membrane ion channels (Vacher et al., 1989; Bonventre, 1992; Shimada and Somlyo, 1992).

In vas-deferens-derived DDT₁ MF-2 smooth-muscle cells, stimulation of the H_1 -histaminoceptor results in the formation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, a rise in intracellular Ca^{2+} , determined by Ca^{2+} mobilization from internal stores and Ca^{2+} entry, and a concomitant Ca^{2+} -regulated transmembrane K^+ current (Molleman et al., 1991b; Den Hertog et al., 1992). In the

Abbreviations used: AA, arachidonic acid; ECS, extracellular solution; DMEM, Dulbecco's Modified Eagle's Medium; NDGA, nordihydroguaiaretic acid.

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present study, we investigated the mechanism responsible for the H_1 -histaminoreceptor-activated Ca^{2+} entry in DDT₁ MF-2 cells. The observations demonstrate that the histamine-induced formation of AA plays an important role in this process.

METHODS

Cell culture

The DDT₁ MF-2 smooth-muscle cell line was a gift from Dr. J. S. Norris. The cells were cultured in monolayers at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % fetal-calf serum, 7 mM $NaHCO_3$ and 10 mM Hepes at pH 7.2 and gassed with air containing 5 % CO_2 . All culture media were obtained from Flow Laboratories, U.K.

Membrane currents

Membrane currents were measured at 22 °C by using the whole-cell patch-clamp configuration, as described by Molleman et al. (1989). The patch pipettes (Clark GC150TF-15 glass) were heat-polished and filled with intracellular solution (ICS) containing (mM): NaCl, 5; KCl, 126; $MgCl_2$, 1.2; Hepes, 10; glucose, 11. The solution had pH 7.2 and a typical resistance of 2–6 M Ω . Establishment of the whole-cell patch-clamp configuration was monitored via a CCD camera (Sony AVC-D5CE) mounted on the microscope (Olympus CK), and detected electrically by measuring the resistance and capacity. Membrane currents of the cell were recorded under voltage clamp (List EPC-7) with a holding potential of -50 mV. The cells were attached to a glass coverslip and superfused (1 ml/min) with extracellular solution (ECS) according to Hesketh et al. (1983) containing (mM): NaCl, 145; KCl, 5; $CaCl_2$, 1.0; $MgSO_4$, 0.5; Hepes, 10; glucose, 10; pH 7.4. Ca^{2+} -free conditions were obtained by addition of EGTA (0.4 mM) and $MgCl_2$ (5.7 mM) in ECS without Ca^{2+} , to remove extracellular Ca^{2+} and to prevent membrane leakage.

Intracellular Ca^{2+}

Intracellular Ca^{2+} measurements were determined by indo-1 fluorescence, as reported previously (Hoiting et al., 1990). Cell suspensions at a density of 2×10^5 cells/ml were loaded with 2 μ M indo-1 AM in DMEM + 10 % fetal-calf serum at 37 °C for 45 min. Recordings were made in ECS at an excitation wavelength of 325 nm and emission wavelengths of 400 and 480 nm at 22 °C by using a fluorescence spectrophotometer (Hitachi). Cytoplasmic free Ca^{2+} concentrations were calculated (Hesketh et al., 1983) by using 0.015 % Triton X-100 as permeabilizing agent.

[3H]AA release

Cells grown in monolayer were labelled with 0.5 μ Ci of [3H]AA/well (10^6 cells) in serum-free DMEM (1 ml) for 3 h at 37 °C. To eliminate unincorporated radioactivity, cells were washed once with ECS, twice with ECS containing 1 % BSA (essentially fatty-acid-free) and once again with ECS, before they were equilibrated for 15 min. Inhibitors were added during this period in the indicated experiments. After this, the cells were incubated with agonist for different time intervals. The experiment was ended by removing the medium, and [3H]AA release was determined by liquid-scintillation counting.

To investigate whether radioactive AA was released, the samples were analysed by reverse-phase (RP) h.p.l.c. by following the same procedure as described by Engels et al. (1986). The identification of various fatty acid metabolites was based on

co-elution with authentic standards in RP h.p.l.c. and u.v.-absorption characteristics in keeping with those of authentic compounds. In all samples measured, less than 2 % of AA metabolites were detected.

Materials

All culture media and supplements were obtained from Flow Laboratories (Zwanenburg, The Netherlands). Indo-1 AM was from Molecular Probes (U.S.A.), histamine dihydrochloride from Fluka (Switzerland), Ins(1,4,5) P_3 (sodium salt) and Ins(1,3,4,5) P_4 (sodium salt) were from Boehringer (Mannheim, Germany), heparin, arachidonic acid, indomethacin, metyrapone and nordihydroguaiaretic acid (NDGA) from Sigma (U.S.A.), and [3H]Ins(1,4,5) P_3 (3–5 Ci/mmol), [γ - ^{32}P]ATP and [3H]AA (60–100 mCi/mmol, 0.1 mCi/ml) from NEN-duPont. The other salts were of analytical grade (Merck, Germany).

RESULTS

Histamine-stimulated Ca^{2+} entry: the role of Ca^{2+} mobilization

Stimulation with histamine of indo-1-loaded DDT₁ MF-2 cells in a Ca^{2+} -containing solution resulted in an initial rise in cytoplasmic Ca^{2+} concentration, due to Ca^{2+} mobilization from internal stores, followed by a slowly declining phase, mediated by Ca^{2+} entry from the extracellular space (Molleman et al., 1991b). Enhancement of intracellular Ca^{2+} is associated with activation of Ca^{2+} -regulated K^+ channels in many cell types, including DDT₁ MF-2 cells (Molleman et al., 1991b). Histamine (100 μ M) induced a reproducible transient outward K^+ current (975 ± 65 pA; $n = 12$), as measured by using the whole-cell patch-clamp configuration (Figure 1a). The K^+ current does not show the slowly declining phase as observed in the histamine-induced change in internal Ca^{2+} concentration, possibly due to rapid inactivation of the K^+ channels. The histamine-induced change in membrane current was decreased (70.4 ± 6.9 % of control

Figure 1 The histamine-activated Ca^{2+} -regulated outward K^+ current

Changes in membrane current caused by histamine (100 μ M) were measured by using the whole-cell patch-clamp configuration at a holding potential of -50 mV. The presence of histamine is represented by a short horizontal bar. (a) Histamine evoked a reproducible outward K^+ current in the presence of extracellular Ca^{2+} . (b) The histamine-induced change in membrane current was decreased in the presence of La^{3+} (50 μ M; long horizontal bar, second response).

Figure 2 Changes in membrane current caused by histamine in the presence of heparin applied intracellularly

(a) Histamine evoked a decreased ($65.1 \pm 5.2\%$ of control response) but reproducible outward K^+ current (holding potential = -50 mV) in the presence of heparin ($100 \mu\text{g/ml}$, dot) when extracellular Ca^{2+} was available. (b) Histamine failed to induce a change in membrane current in heparin-pretreated cells when the contribution of extracellular Ca^{2+} was prevented by addition of La^{3+} ($50 \mu\text{M}$; long horizontal bar, second response). Data shown represent a typical experiment out of a series of seven.

values; $n = 6$; Figure 1b, second response) by blocking the Ca^{2+} entry with La^{3+} ($50 \mu\text{M}$) or by measuring under Ca^{2+} -free conditions (results not shown). Comparable results were obtained by measuring the histamine-activated change in internal Ca^{2+} concentration under these conditions, as shown previously (Molleman et al., 1991b). In the presence of intracellularly applied heparin ($100 \mu\text{g/ml}$), known to inhibit the $\text{Ins}(1,4,5)\text{P}_3$ -activated Ca^{2+} mobilization (Henzi and MacDermott, 1992), histamine caused a decreased but reproducible outward K^+ current ($65.1 \pm 5.2\%$ of the control value; $n = 6$; Figure 2a). This change in internal Ca^{2+} reflected by the K^+ current might be due to Ca^{2+} released from inositol phosphate-independent Ca^{2+} stores, or may be caused by Ca^{2+} entry. To discriminate between these possibilities, histamine-stimulated Ca^{2+} influx of heparin pretreated cells was prevented by addition of La^{3+} ($50 \mu\text{M}$). The histamine-induced Ca^{2+} -activated outward K^+ current was abolished under these conditions (Figure 2b, second response; $n = 7$), also indicating that heparin interacts with the intracellular $\text{Ins}(1,4,5)\text{P}_3$ receptors. The same results (not shown) were obtained with the combination of heparin and Ca^{2+} -free conditions. Thus, extracellular Ca^{2+} is exclusively responsible for the increase in cytoplasmic Ca^{2+} after blocking Ca^{2+} release from heparin-sensitive stores.

Histamine-stimulated Ca^{2+} entry: role of inositol phosphates

The role of the histamine-activated formation of inositol phosphates (Molleman et al., 1991b) in mediating Ca^{2+} entry was investigated by intracellular application of $\text{Ins}(1,4,5)\text{P}_3$ ($10 \mu\text{M}$) and $\text{Ins}(1,3,4,5)\text{P}_4$ ($20 \mu\text{M}$) to cause complete depletion of the inositol phosphate-sensitive Ca^{2+} compartments (L. van der Zee, A. Nelemans and A. den Hertog, unpublished work) and to pre-activate the inositol phosphate-regulated Ca^{2+} entry, if functional in these cells. Addition of these inositol phosphates to the cytoplasm caused an instantaneous transient K^+ current, as

Figure 3 Changes in membrane current caused by histamine in the presence of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ applied intracellularly

(a) Intracellular application of $\text{Ins}(1,4,5)\text{P}_3$ ($10 \mu\text{M}$) and $\text{Ins}(1,3,4,5)\text{P}_4$ ($20 \mu\text{M}$), caused a transient outward K^+ current (holding potential = -50 mV). Subsequent addition of histamine induced a reproducible outward transmembrane current which was decreased to $56.7 \pm 5.2\%$ of control values, comparable with the response evoked in the presence of heparin. (b) This histamine-induced current was abolished in the presence of La^{3+} (long horizontal bar, second response). Data shown represent typical experiments out of series of five.

expected on activation of their internal receptor sites (Figure 3). Subsequent addition of histamine results in a reproducible but decreased outward K^+ current ($56.7 \pm 6.1\%$ of control values; $n = 5$; Figure 3a). This response was entirely dependent on Ca^{2+} entry from the extracellular space, for it was abolished in the absence of extracellular Ca^{2+} (results not shown) or in the presence of La^{3+} ($50 \mu\text{M}$; Figure 3b, $n = 5$, second response).

In view of the results obtained so far, the transmembrane K^+ current representing receptor-stimulated Ca^{2+} entry in DDT₁ MF-2 cells is apparently independent of the state of depletion of the intracellular Ca^{2+} compartments and is not exclusively determined by $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$. Thus, another mechanism must be involved in the regulation of the H_1 -histaminoceptor-activated Ca^{2+} entry.

Histamine-stimulated Ca^{2+} entry: the role of AA

The role of AA or its metabolites in activation of the plasma-membrane Ca^{2+} channels was examined by measuring the H_1 -histaminoceptor-activated formation of AA in [^3H]AA-prelabelled DDT₁ MF-2 cells. Histamine induced a marked biphasic rise in [^3H]AA release, reached a maximum at $t = 15$ s (Figure 4). A second peak was observed after about 1 min, followed by a slowly declining release of [^3H]AA. Pretreatment of the cells with NDGA ($10 \mu\text{M}$), inhibiting both the lipoxygenase and cyclooxygenase pathways, increased the basal [^3H]AA release, but did not affect the characteristics of the histamine-induced [^3H]AA release (results not shown). These results suggest that metabolites of AA did not play an important role, which was supported by the minor quantity of [^3H]AA-labelled metabolites found (see the Methods section).

The effect of AA on the internal Ca^{2+} concentration was measured in indo-1-loaded DDT₁ MF-2 cells. External addition of AA (10 – $1000 \mu\text{M}$) resulted in an increase in intracellular Ca^{2+} concentration, reaching its maximum after about 30 s, followed

Figure 4 Time course of histamine-stimulated [^3H]AA release

[^3H]AA-pre-labelled DDT₁ MF-2 cells were stimulated with histamine (100 μM) for different time intervals. The amount of [^3H]AA released was expressed as percentage of basal value (zero time; 221.2 ± 10.4 d.p.s./ 10^6 cells). The maximum [^3H]AA release is about 5% of the total radioactivity taken up by the cells, amounting to 4123 ± 235 d.p.s./ 10^6 cells. Values are means $\pm$ S.E.M. ($n \geq 11$).

Figure 5 Changes in intracellular Ca^{2+} concentration caused by exogenous applied AA

The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of DDT₁ MF-2 cells was measured by using the fluorescent probe indo-1. (a) The change in internal Ca^{2+} evoked by AA (horizontal bar) in the presence of extracellular Ca^{2+} (left panel AA = 50 μM ; right panel, AA = 1 mM). (b) The same experimental procedure was followed as described for (a), but in the absence of extracellular Ca^{2+} . Data shown represent typical experiments out of a series of four.

by a sustained enhancement of the internal Ca^{2+} level (Figure 5a). The AA-induced rise in internal Ca^{2+} was concentration-dependent. The threshold concentration of AA required to elicit a rise in internal Ca^{2+} was about 1 μM , and a maximum Ca^{2+} release of about 240 nM was found at 1 mM AA (Figure 6). In the absence of extracellular Ca^{2+} or in the presence of La^{3+} the response induced by AA was abolished (Figure 5b, left-hand recording). Release of intracellular stored Ca^{2+} contributing to the rise in internal Ca^{2+} was observed only at high concentrations of AA ($\geq 100 \mu\text{M}$; Figure 5b, right-hand recording; Figure 6). These results show that the response elicited by AA is due to Ca^{2+} influx rather than to mobilization of intracellular stored Ca^{2+} .

Figure 6 Effect of different concentrations of AA on the internal Ca^{2+} concentration

AA induced changes in the internal Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]_i$), measured by using the fluorescent probe indo-1, in the presence (○) or absence (●) of extracellular Ca^{2+} . Values are means $\pm$ S.E.M. ($n \geq 4$).

Figure 7 Histamine-induced change in internal Ca^{2+} concentration of AA-pretreated cells

The change in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) induced by AA (50 μM , long horizontal bar) followed by histamine (100 μM , short horizontal bar) in the presence (a) or absence (b) of extracellular Ca^{2+} . Data shown represent a typical experiment out of a series of three.

Involvement of AA metabolites in raising internal Ca^{2+} was examined by exposure of the cells to different inhibitors of the AA-breakdown pathway before addition of AA. NDGA (10 μM)

Figure 8 Histamine-induced change in membrane current (I) of AA-pretreated cells

(a) In the presence of extracellular Ca^{2+} , AA (50 μM , long horizontal bar) induced an outward Ca^{2+} -regulated K^+ current (holding potential = -50 mV). Subsequent addition of histamine (100 μM , short horizontal bar) resulted in a decreased response (640 ± 55 pA; $n = 5$) compared with the current observed without AA (975 ± 65 pA; $n = 12$; see Figure 1). (b) Histamine failed to induce an outward K^+ current in the presence of both heparin (100 $\mu\text{g/ml}$, applied intracellularly; dot) and AA (50 μM , applied extracellularly). Data shown represent typical experiments out of series of at least four.

caused an increase in the internal Ca^{2+} concentration (55 ± 12 nM, $n = 3$), which was abolished in the absence of extracellular Ca^{2+} (results not shown). The NDGA-induced increment in intracellular Ca^{2+} was further enhanced by addition of AA (100 μM), showing the typical characteristics as described above (results not shown; $n = 3$). Indomethacin (10 μM , $n = 4$) and metyrapone (100 μM , $n = 3$), known to inhibit the cyclo-oxygenase and epoxigenase pathways respectively, did not change the internal Ca^{2+} concentration or the AA-induced response (results not shown). Thus, activation of transmembrane Ca^{2+} channels is caused by exogenously applied AA as well as by cellular accumulated AA, but not its metabolites.

The question whether the H_1 -histaminoceptor-activated Ca^{2+} influx is passing via AA-sensitive Ca^{2+} channels was approached by applying histamine during the AA (50 μM)-generated Ca^{2+} entry to the cells. The histamine-induced increase in internal Ca^{2+} was decreased and transient in nature under these conditions (Figure 7a). The transient histamine response is apparently caused by Ca^{2+} mobilization from internal stores, in view of a comparable change in internal Ca^{2+} under Ca^{2+} -free conditions and therefore independent of extracellular Ca^{2+} (Figure 7b). Thus the slowly declining phase of the Ca^{2+} response representing Ca^{2+} entry was not observed in the presence of AA. The remaining transient rise in internal Ca^{2+} produced by histamine is most likely due to inositol phosphate-activated Ca^{2+} release from internal stores, which process can be blocked by internal application of heparin. To determine the action of AA on the histamine-induced transmembrane Ca^{2+} flux, the Ca^{2+} -regulated K^+ current was measured in the absence and presence of intracellularly applied heparin (100 $\mu\text{g/ml}$). Addition of AA (50 μM) induced a change in membrane current ($n \geq 4$), and subsequent addition of histamine (100 μM) resulted in a decreased outward current ($65.7 \pm 9.1\%$ of values observed without AA; Figure 8a), comparable with that observed in the presence of La^{3+} (Figure 1b). Pretreatment with both AA (50 μM) and

heparin resulted in abolition of the histamine-induced change in membrane current (Figure 8b).

DISCUSSION

The rise in internal Ca^{2+} caused by receptor stimulation originates from intracellular Ca^{2+} stores and the extracellular space. The release of Ca^{2+} from internal stores is activated by $\text{Ins}(1,4,5)\text{P}_3$ (Henzi and MacDermott, 1992; Berridge, 1993), by $\text{Ins}(1,3,4,5)\text{P}_4$ (Ely et al., 1990; Ferguson et al., 1991) or by the combination of these inositol phosphates (Irvine and Moor, 1986; Cullen et al., 1990) also observed on H_1 -histaminoceptor stimulation of DDT₁ MF-2 cells (L. van der Zee, A. Nelemans and A. den Hertog, unpublished work). The regulation of the receptor-activated entry of extracellular Ca^{2+} is less understood, and different mechanisms have been suggested to explain this process, as mentioned in the Introduction. The capacitative model of cellular Ca^{2+} regulation predicts that the rate of Ca^{2+} entry into the cells is inversely related to the Ca^{2+} concentration within cellular storage pools. According to this model, no Ca^{2+} influx will be detected when the intraluminal Ca^{2+} concentration remains unchanged (Putney, 1990). The results presented here show that a Ca^{2+} -regulated K^+ current was evoked by histamine, although Ca^{2+} release from intracellular stores was inhibited by heparin. This K^+ current generated in the presence of heparin was activated by Ca^{2+} originating from the extracellular space, since it was abolished when Ca^{2+} entry was prevented. This implies that activation of a Ca^{2+} -dependent K^+ current arises not only from cytoplasmic Ca^{2+} originating from internal stores but also from influx of extracellular Ca^{2+} . Thus, the histamine-stimulated Ca^{2+} entry was activated independently of the intraluminal Ca^{2+} concentration in DDT₁ MF-2 cells. This implies that another mechanism apart from that described by the capacitative model is apparently involved in histamine-stimulated DDT₁ MF-2 cells.

Recently, a model was postulated describing the involvement of intraluminal Ca^{2+} as well as $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ in the activation of Ca^{2+} entry (Irvine, 1992). In fact, both inositol phosphates are formed on H_1 -histaminoceptor stimulation in DDT₁ MF-2 cells (Molleman et al., 1991b). Depletion of internal Ca^{2+} stores and pre-activation of a functional inositol phosphate-regulated Ca^{2+} entry by cytoplasmic addition of both inositol phosphates, however, did not abolish the histamine-induced transmembrane Ca^{2+} flux, suggesting the existence of an inositol phosphate-independent Ca^{2+} -entry mechanism in the cells studied by us. Thus the H_1 -histaminoceptor-induced activation of the plasma-membrane Ca^{2+} channels cannot be exclusively explained by the filling state of the internal Ca^{2+} compartments (Putney, 1990) or by the formation of inositol phosphates (Irvine, 1992; Lückhoff and Clapham, 1992) in DDT₁ MF-2 cells, a phenomenon also observed in bradykinin-stimulated aortic endothelial cells (Graier et al., 1991).

Receptor-induced AA release was reported to increase the local concentration of AA near the plasma membrane sufficiently to activate several biological processes. In accord, modification of the transmembrane Ca^{2+} flux was found in pituitary cells (Vacher et al., 1989), intestinal smooth-muscle cells (Shimada and Somlyo, 1992) and hippocampal CA1 pyramidal cells (Keyser and Alger, 1990). This action of AA is supposed to be due to modification of voltage-dependent Ca^{2+} channels, which are not present on DDT₁ MF-2 cell (Molleman et al., 1991a). Stimulation of the H_1 -histaminoceptors of DDT₁ MF-2 cells caused a rapid release of AA. The functionality of AA as a second messenger is suggested by the increase in internal Ca^{2+} concentration, which is mainly caused by Ca^{2+} entry from the extra-

cellular space via La^{3+} -sensitive Ca^{2+} channels observed on exogenous application. A functional relationship between the exogenous applied AA concentration and the Ca^{2+} entry is observed. Activation of the Ca^{2+} -entry process by AA is also represented by a Ca^{2+} -regulated outward K^{+} current. AA-induced Ca^{2+} mobilization from internal stores was observed at high concentrations, as also reported for rat oligodendrocytes (Soliven et al., 1993) and pancreatic islet cells (Ramanadham et al., 1992). The mechanism underlying the action of AA on Ca^{2+} influx in particular is not resolved yet, but may involve complex membrane lipid-protein interactions. The decline in the Ca^{2+} -entry process activated by AA suggests a slow inactivation of the concomitant Ca^{2+} channels. Consequently, the histamine-induced Ca^{2+} entry was not observed in the case of pre-activation of the Ca^{2+} channels by AA.

The effect of AA on Ca^{2+} entry was not due to the formation of prostaglandins or other metabolites of AA, because inhibition of epoxigenation, lipoxygenation or cyclo-oxygenation (Needleman et al., 1986) failed to inhibit the AA-induced response. It is noticed that NDGA itself was effective to some extent, which was also observed in rabbit intestinal smooth-muscle cells (Shimada and Somlyo, 1992) and human platelets (Vindlacheruvu et al., 1991). This effect is supposed to be due to accumulation of AA, as represented by the NDGA-mediated increase in basal $[\text{H}^3]\text{AA}$ release in DDT₁ MF-2 cells.

In the presence of heparin, the rise in internal Ca^{2+} , as reflected by the K^{+} current, was due to the histamine-activated Ca^{2+} entry. Pre-activation of this Ca^{2+} -entry process with AA caused complete abolition of the histamine-induced response, supporting the prominent role of AA in activation of plasma-membrane Ca^{2+} channels in DDT₁ MF-2 cells.

Several cellular mechanisms are available to activate the H_1 -histaminoceptor-induced formation of AA. One possibility is activation of phospholipase C, resulting in hydrolysis of phosphoinositides and the subsequent accumulation of inositol phosphates and diacylglycerol. Diacylglycerol can serve as a precursor for AA via activation of diacylglycerol lipase. Alternatively, AA can be released from phosphatidic acid, generated by the hydrolysis of phospholipids by phospholipase D. However, the most common pathway for the receptor-mediated generation of AA is by direct activation of phospholipase A_2 (Axelrod, 1990), which process is reported to be Ca^{2+} -dependent (Bonventre, 1990) as well as Ca^{2+} -independent (Wolf and Gross, 1985; Hazen et al., 1991). It is shown that activation of the histamine-induced Ca^{2+} -entry process in DDT₁ MF-2 cells is not dependent on a change in cytoplasmic Ca^{2+} caused by internal Ca^{2+} mobilization. In view of the biphasic pattern of the AA release observed, two routes with different time constants might be involved in DDT₁ MF-2 cells. A study is in progress to elucidate these pathways leading to the histamine-activated AA production in these cells.

In conclusion, a rise in intracellular Ca^{2+} due to mobilization from internal stores and Ca^{2+} entry from the extracellular space is caused by H_1 -histaminoceptor stimulation in DDT₁ MF-2 cells. The Ca^{2+} entry is independent of the formation of inositol

phosphates or the filling state of intracellular Ca^{2+} stores, but is related to the receptor-stimulated release of AA. These results strongly suggest that AA is functioning as a second messenger to activate plasma-membrane Ca^{2+} channels, promoting Ca^{2+} entry from the extracellular space in histamine-stimulated DDT₁ MF-2 cells.

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