Journal of Physiology (1992), **454**, *pp.* 591–607
With 9 figures
Printed in Great Britain

CALCIUM RELEASE FROM SEPARATE RECEPTOR-SPECIFIC INTRACELLULAR STORES INDUCED BY HISTAMINE AND ATP IN A HAMSTER CELL LINE

BY ADRIAAN DEN HERTOG, BEN HOITING, ARELES MOLLEMAN, JAN VAN DEN AKKER, MARRY DUIN AND ADRIAAN NELEMANS

From the Department of Pharmacology/Clinical Pharmacology, University of Groningen, Bloemsingel, 1, 9713 BZ Groningen, The Netherlands

(*Received 4 September 1991*)

SUMMARY

1. The specificity of intracellular Ca^{2+} stores to Ca^{2+}-mobilizing agonists was studied in DDT_1 MF-2 vas deferens cells of the Syrian hamster.

2. Application of histamine (100 μM) or ATP (100 μM) to the DDT_1 MF-2 cells caused an initial increase of intracellular Ca^{2+} followed by a lower phase as measured by using Indo-1 as fluorescent probe at 22 °C. The basal Ca^{2+} level (146 nM) was enhanced to 309 nM by histamine and to 379 nM by ATP.

3. A transient rise in intracellular Ca^{2+} lasting for about 2 min was measured in the presence of histamine or ATP in the absence of extracellular Ca^{2+}. The basal Ca^{2+} level (78 nM) was increased to 128 nM by histamine and to 145 nM by ATP.

4. A transient hyperpolarization was elicited in single cells as measured with microelectrodes by both agonists under Ca^{2+}-free conditions with a similar time course as the change in internal Ca^{2+}. The hyperpolarization observed in the presence of histamine amounted to 23 mV and 31 mV with ATP. The histamine-induced responses were abolished by the H_1 histaminoceptor antagonist mepyramine (10 μM) and the responses evoked by ATP were blocked by the P_2 purinoceptor antagonist suramin (300 μM).

5. A second internal Ca^{2+} response could only be evoked under Ca^{2+}-free conditions by applying a higher agonist concentration or after replenishing the intracellular stores with Ca^{2+} from the extracellular space.

6. A second addition of an optimal concentration (100 μM) of the agonist to the cells under Ca^{2+}-free conditions did not evoke mobilization of internal Ca^{2+} or hyperpolarization, but resulted in a rise of the cellular inositol (1,4,5)-trisphosphate content (Ins(1,4,5)P_3) as determined by a radioligand binding assay.

7. The cells responded to both agonists (100 μM) with a transient Ca^{2+} response if successively applied at a maximal effective concentration (100 μM) under Ca^{2+}-free conditions.

8. Simultaneous stimulation of H_1 histaminoceptors and P_2 purinoceptors resulted in the absence of external Ca^{2+} in an additional increase in internal Ca^{2+} represented by the amplitude and area of the response and in an increased response area of the hyperpolarization.

MS 9706

9. A transient increase in cytoplasmic Ca^{2+} was evoked by ATP or histamine (100 μM) in the presence of both external Ca^{2+} (1·2 mM) and La^{3+} (50 mM, 10 min). A second addition of the same agonist failed to evoke a Ca^{2+} response if preceded by a recovery period (15 min) in the presence of Ca^{2+} and La^{3+}. Using the other agent as second agonist resulted again in a transient rise in internal Ca^{2+}.

10. Subsequent application of the agonists to the cell population under Ca^{2+}-free conditions, after removing the first agonist in between both responses, resulted in a rise in internal Ca^{2+} and Ins(1,4,5)P_3. Hyperpolarization was observed in single cells following this experimental protocol.

11. The results imply that stimulation of H_1 histaminoceptors or P_2 purinoceptors on DDT_1 MF-2 vas deferens cells activate a process resulting in calcium release from separate receptor-specific intracellular stores.

INTRODUCTION

Intracellular calcium changes induced by a variety of neurotransmitters and hormones are caused by activation of phosphatidylinositol metabolism and the formation of inositol phosphates (Berridge, 1987). Inositol, 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) are believed to function as second messengers to release Ca^{2+} from intracellular organelles and to promote Ca^{2+} entry from the extracellular compartment (Merritt & Rink, 1987; Morris, Gallacher, Irvine & Petersen, 1987; Changya, Gallacher, Irvine & Petersen, 1989). Formation of these inositol phosphates are also responsible for the changes in internal Ca^{2+} observed in DDT_1 MF-2 vas deferens cells of the Syrian hamster on stimulation by ATP or histamine (Hoiting, Molleman, Duin, Den Hertog & Nelemans, 1990*a*; Molleman, Hoiting, Duin, Van den Akker, Nelemans & Den Hertog, 1991*a*). A short-lasting transient increase in Ins(1,4,5)P_3 and a sustained increase in Ins(1,3,4,5)P_4 was found in DDT_1 MF-2 cells. The time-related formation of Ins(1,4,5)P_3 was similar to that in airway smooth muscle observed on receptor stimulation (Chilvers, Challiss, Barnes & Nahorski, 1989). Besides enhancement of internal Ca^{2+}, stimulation of the ATP-sensitive receptors (Molleman, Nelemans & Den Hertog, 1989), or histamine-sensitive receptors (Molleman *et al.* 1991*a*) also caused an outward K^+ current in DDT_1 MF-2 cells. Thus, the cellular responses evoked via P_2 purinoceptors and H_1 histaminoceptors are similar referring to the formation of inositol phosphates, internal Ca^{2+} mobilization and the transmembrane K^+ current.

Previous reports suggest that agonist–receptor interaction caused Ca^{2+} release from structures in the vicinity of the occupied receptor (Loutzenhiser & Van Breemen, 1981; Den Hertog, 1982) and that Ca^{2+} release was initiated in a limited area near the plasma membrane (Berridge, Cobbold & Cuthbertson, 1988). The question of whether a common Ca^{2+} compartment or separate stores are involved in stimulation of different receptors was addressed by studying the formation of Ins(1,4,5)P_3, the internal Ca^{2+} concentration and the membrane potential on stimulation of H_1 histaminoceptors and P_2 purinoceptors of DDT_1 MF-2 cells under selected experimental conditions. The independent cellular responses to histamine and ATP observed by us are suggestive of the existence of separate receptor-specific calcium stores.

METHODS

Cell culture. The DDT_1 MF-2 cells, derived from a Syrian hamster vas deferens (a gift from Dr J. S. Norris, Medical University of South Carolina, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with $NaHCO_3$ (7 mM), 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES; 10 mM) and 10% fetal calf serum at pH 7·2, 37 °C in 95% O_2–5% CO_2 (Nelemans, Hoiting, Molleman, Duin & Den Hertog, 1990). In electrophysiological measurements the cells were plated on glass cover slips, in inositol phosphate mass measurement in dishes and in Ca^{2+} measurements the cells were brought into suspension.

Membrane potential. Conventional microelectrode measurements were performed at 22 °C. Microelectrodes (Clark GC150G-15 glass, UK) were filled with 1 M-KCl and had a typical resistance of 50–80 MΩ. Cells, superfused at a constant flow rate (1 ml/min), were penetrated with a microelectrode using a nano-stepper (Model B, WPI, USA) and membrane potentials were recorded by a high-resistance amplifier (Model M4, WPI, USA) on a paper writer (Kipp BD-8, The Netherlands).

Intracellular calcium. Cytoplasmic free Ca^{2+} levels were determined by Indo-1 fluorescence. Cell suspensions at a density of 2×10^5 cells/ml were loaded with Indo-1 AM (2 μM) for 45 min at 37 °C. Recordings were made at an excitation wavelength of 325 nm and emission wavelengths of 400 and 480 nm at 22 °C using a fluorescence spectrophotometer (Hitachi). Cytoplasmic free Ca^{2+} concentrations were determined (Hesketh, Smith, Moore, Taylor & Metcalfe, 1983), using 0·015% of Triton X-100 as permeabilizing agent.

Inositol 1,4,5-trisphosphate. Cells grown in monolayers were used in experiments for mass measurements using a radioligand binding assay. Fifteen minutes before starting the experiment, the medium in the dishes was replaced by DMEM (1 ml) at 22 °C. The reaction was started by the addition of ATP (100 μM) or histamine (100 μM) and stopped by addition of trichloroacetic acid (TCA: 15%, 1 ml) after removing the medium. The TCA was extracted (four times) with water-saturated diethylether, the samples were neutralized with KOH and stored (−20 °C). The samples were assayed in 25 mM-Tris/HCl (pH 9·0), 1 mM-EDTA, 1 mg of bovine serum albumin [^{3}H]Ins(1,4,5)P_3 (2000 c.p.m./assay), and about 1 mg of binding protein (Chilvers *et al.* 1989). The binding protein was isolated from fresh beef liver (Donié & Reiser, 1989). Bound and free radioactivity were separated by centrifugation. The radioactivity in the pellet was determined by scintillation counting.

Solutions. Electrophysiological measurements were performed in extracellular solution containing (mM): NaCl, 125; KCl, 6; $CaCl_2$, 1·2; $MgCl_2$, 2·5; NaH_2PO_4, 1·2; HEPES, 10; glucose, 11; pH = 7·4. Fluorescence experiments were performed in a solution containing (mM): NaCl, 145; KCl, 5; $MgSO_4$, 0·5; $CaCl_2$, 1; HEPES, 10; glucose, 10; pH = 7·4. Calcium-free conditions were accomplished by addition of EGTA (0·77 mM) and $MgCl_2$ (3·7 mM) to Ca^{2+}-free solution (microelectrode experiments) or EGTA (0·4 mM) and $MgCl_2$ (6·0 mM) to Ca^{2+}-free solution (fluorescence experiments) to remove extracellular Ca^{2+} and to prevent membrane leakage (Den Hertog, 1981).

Materials. All culture media and supplements were obtained from Flow Laboratories, UK, Indo-1 AM from Molecular Probes (USA), histamine dihydrochloride from Fluka (Switzerland), mepyramine from Sigma (USA), adenosine-5′-trisphosphate sodium salt from Serva (FRG), suramin from Bayer (FRG) and D-[2-^{3}H]inositol 1,4,5-trisphosphate from Du Pont-New England Nuclear (USA). The other salts were of analytical grade (Merck, FRG). Data are presented as means ± S.E.M. and considered to be significantly different ($P < 0·005$) from control values using Student's t test.

RESULTS

Effect of histamine or ATP on intracellular Ca^{2+}

The DDT_1 MF-2 vas deferens cells responded to application of ATP or histamine with an initial increase in intracellular Ca^{2+} followed by a lower slowly declining phase (Fig. 1*A*). The basal intracellular Ca^{2+} concentration was 146·0 ± 5·3 nM ($n = 27$). The increase in internal Ca^{2+} produced by a maximal effective concentration of the agonists (100 μM) (Fig. 1*A*, bars) was reached after about 30 s, in agreement

with previous reports (Mitsuhashi & Payan, 1989; Molleman *et al.* 1989, 1991*a*). Reproducible responses could be evoked by the agonists in DDT_1 MF-2 cells after a recovery period of 15 min. The change in internal Ca^{2+} on receptor stimulation was transient in the absence of extracellular Ca^{2+} (15 min). This response reached its

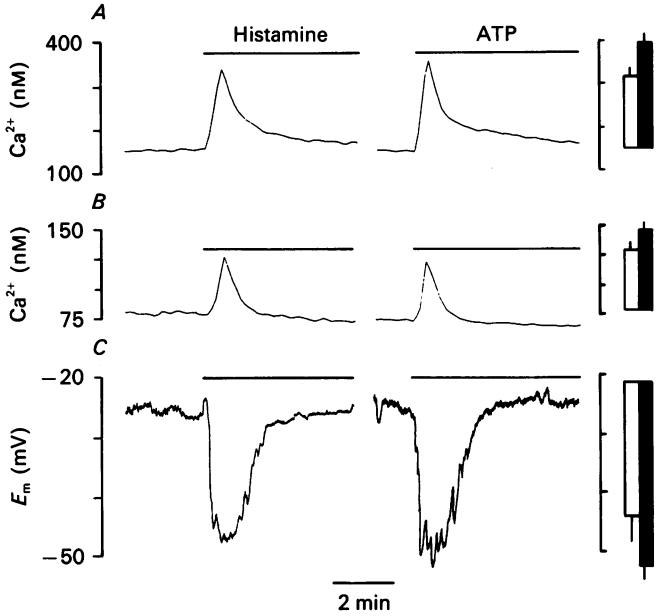

Fig. 1. The traces show the individual effect of histamine (100 μM) and ATP (100 μM) on intracellular Ca^{2+} and on the membrane potential. The data presented in this and subsequent figures were obtained from Ca^{2+} measurements on a population of cells (2×10^5 cells/ml) and from electrophysiological measurements on single cells. The bars on the right side of the traces represent the maximum amplitude of the responses ($n = 24$; histamine, open bars; ATP, filled bars). *A*, the initial increase in cytosolic Ca^{2+} (nM) followed by a slowly declining phase evoked by histamine and ATP. *B*, the transient rise in internal Ca^{2+} (nM) elicited by the agonists in the absence of extracellular Ca^{2+} (15 min). *C*, the transient hyperpolarization (mV) evoked by the agonists under Ca^{2+}-free conditions.

maximum also after about 30 s and lasted for about 2 min (Fig. 1*B*, bars). The internal basal Ca^{2+} concentration was $77{\cdot}9 \pm 2{\cdot}3$ nM ($n = 27$) under Ca^{2+}-free conditions (15 min). These results show that the agonist-induced Ca^{2+} response evoked in the presence of external Ca^{2+} is higher in amplitude than under Ca^{2+}-free conditions and has a slowly declining tail, not observed in the absence of Ca^{2+}.

Enhancement in cytoplasmic Ca^{2+} is associated with the opening of Ca^{2+}-sensitive K^+ channels (Marty, 1981) in many cell types and also in DDT_1 MF-2 cells (Molleman *et al.* 1989). The membrane potential ($-48{\cdot}5 \pm 1{\cdot}8$ mV, $n = 23$) was decreased in the absence of extracellular Ca^{2+} reaching a value of about 25 mV ($-24{\cdot}7 \pm 2{\cdot}6$ mV, $n = 23$). Stimulation of single cells with histamine or ATP caused a hyperpolarization of about 30 mV under Ca^{2+}-free conditions (Fig. 1*C*, bars). The response did not show a pronounced depolarizing phase caused by a non-specific inward current as observed

at higher ATP concentrations in arterial smooth muscle (Benham, Bolton, Byrne & Large, 1987) and DDT_1 MF-2 cells (Molleman *et al.* 1989). It is noticed that the agonists also caused fluctuations in membrane potential occurring during the transient hyperpolarization, as reported for other cells (Berridge, 1990; Petersen &

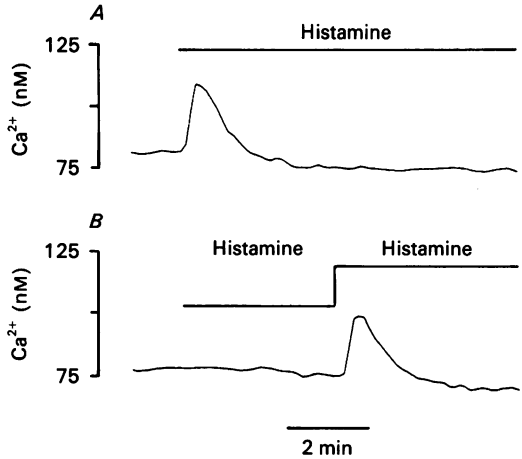

Fig. 2. Changes in the intracellular Ca^{2+} concentration are shown on stimulation of the cells with histamine in the absence of external Ca^{2+}. *A*, the rise in Ca^{2+} evoked by a submaximal concentration of histamine (3 μM). *B*, continuing the experiment under Ca^{2+}-free conditions, second application of the same concentration of histamine failed to evoke a Ca^{2+} response, but stimulation of the same cells with a higher histamine concentration (100 μM) again caused a rise in internal Ca^{2+}. In both panels typical tracings are shown out of four experiments.

Wakui, 1990). The rise in internal Ca^{2+} and hyperpolarization caused by ATP (100 μM) could be blocked by the P_2 purinoceptor antagonist suramin (300 μM) (Dunn & Blakeley, 1988; Den Hertog, Van den Akker & Nelemans, 1989; Nakazawa, Fujimori, Takanaka & Inoue, 1990; Hoyle, Knight & Burnstock, 1990) as shown previously (Hoiting, Molleman, Nelemans & Den Hertog, 1990*b*). The histamine (100 μM)-induced response was abolished by the H_1 histaminoceptor antagonist mepyramine (10 μM) as shown previously (Mitsuhashi & Payan, 1989; Hoiting *et al.* 1990*b*).

The experiments described below are carried out under Ca^{2+}-free conditions to eliminate the contribution of extracellular Ca^{2+} to the agonist-induced responses in order to investigate Ca^{2+} mobilization from internal stores. Repeated stimulation of the receptors by using the same submaximal agonist concentration after recovery in Ca^{2+}-free solution (15 min) did not produce a second rise in Ca^{2+} unless a higher agonist concentration was used (Fig. 2). Accordingly, it was found that repeated stimulation of the cells with a maximal agonist concentration (100 μM) was not effective either (Fig. 3). A second reproducible response could also be elicited by exposure of the cells to extracellular Ca^{2+} (10 min) in advance (Fig. 3). These changes in internal Ca^{2+} were accompanied by hyperpolarization of the cells with similar time-related characteristics ($n = 6$; not shown).

Effect of ATP and histamine on Ins(1,4,5)P_3

The basal Ins(1,4,5)P_3 content of the cells under Ca^{2+}-free conditions (15 min) determined by using a radioligand binding assay was $18{\cdot}2 \pm 2{\cdot}2$ pmol/10^6 cells (n = 12). Addition of histamine (100 μM) to the cells caused an increase of Ins(1,4,5)P_3

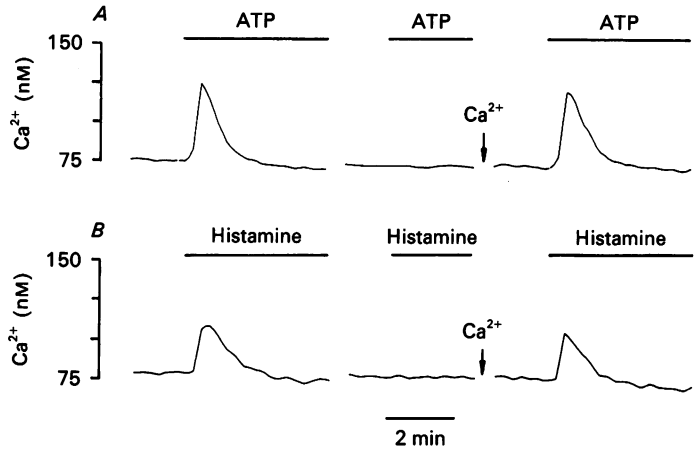

Fig. 3. Changes in internal Ca^{2+} evoked by stimulation of the DDT_1 MF-2 cell under Ca^{2+}-free conditions are shown. *A*, application of a maximal effective concentration of ATP (100 μM) was followed by a recovery period (15 min) still in the absence of Ca^{2+} and second stimulation with ATP (100 μM) did not result in a Ca^{2+} response (middle trace). Continuing the experiment by subsequent exposure to Ca^{2+}-containing solution (1 mM, 10 min) and Ca^{2+}-free solution (15 min) respectively before third addition of ATP (100 μM) to the cells again produced a rise in internal Ca^{2+} (right-hand tracing). *B*, the same protocol as in *A* was used with a maximal effective concentration of histamine (100 μM), resulting in similar characteristics as with ATP. Typical tracings are shown out of six experiments.

reaching an initial maximum after 15 s followed by a second rise (Fig. 4*A*). Following the same experimental protocol as in the internal Ca^{2+} measurement, the agonist was removed and the cells were allowed to recover (15 min) in Ca^{2+}-free solution, resulting in a further decrease of the basal Ins(1,4,5)P_3 content of the cells reaching a value of $10{\cdot}5 \pm 1{\cdot}7$ pmol/10^6 cells. Second addition of the agonist again caused an initial rise in Ins(1,4,5)P_3 followed by a second enhancement of this inositol phosphate after about 60 s (Fig. 4*A*; see Table 2), in contrast to the Ca^{2+} mobilization experiments in which another response was not detectable after repeated stimulation of the cell (Fig. 3). The cellular Ins(1,4,5)P_3 content also showed a rise on stimulation of the cells with ATP (100 μM) under Ca^{2+}-free conditions (Fig. 4*B*). An initial rise in Ins(1,4,5)P_3 content as elicited with histamine was also observed on second addition of ATP (Fig. 4*B*; see Table 2) although an early rise on first addition of the agonist was not detectable, a feature also seen in the presence of external Ca^{2+} (Hoiting *et al.* 1990*a*). These characteristics of the Ins(1,4,5)P_3 level on stimulation of H_1 histaminoceptors or P_2 purinoceptors under Ca^{2+}-free conditions are essentially similar to those obtained in the presence of external Ca^{2+} (Hoiting *et al.* 1990*a*; Molleman *et al.* 1991*a*).

Effect of the combination of histamine and ATP

The reactivity of the cells to simultaneous stimulation of H_1 histaminoceptors and P_2 purinoceptors was investigated to obtain evidence for the specificity of the Ca^{2+} stores. The agonists were applied at a maximal effective concentration (100 μM) to

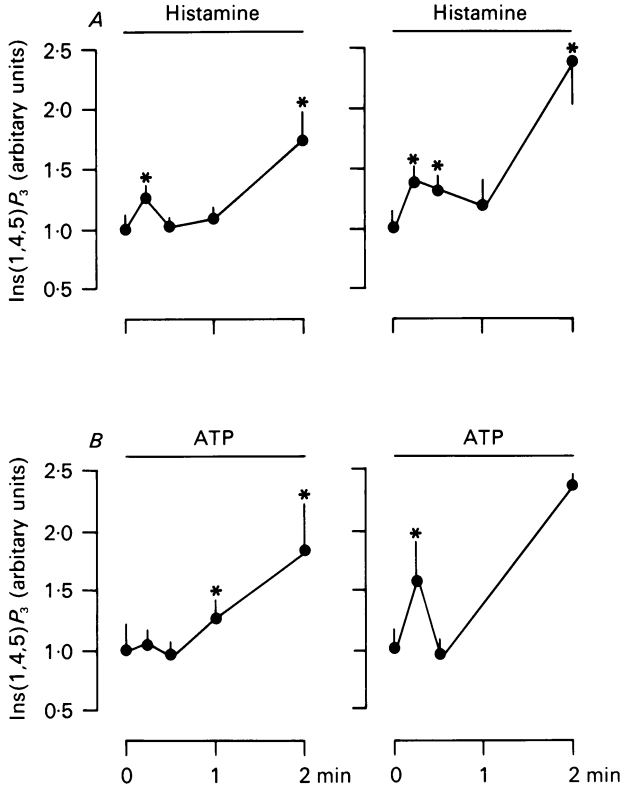

Fig. 4. Changes in Ins(1,4,5)P_3 content of the cells evoked by the agonists on first addition under Ca^{2+}-free conditions (15 min) and on second exposure of the cells after a recovery period (15 min) also in the absence of external Ca^{2+}. *A*, the Ins(1,4,5)P_3 content on stimulation of the cells with histamine (100 μM) for the first (left-hand panel) and second time (right-hand panel). *B*, the Ins(1,4,5)P_3 content in the presence of ATP (100 μM; first addition, left-hand panel; second addition, right-hand panel). Significantly different values from basal levels are indicated by the asterisks ($n = 6$). Values of Ins(1,4,5)P_3 before stimulation were taken as unity (before first stimulation, Ins(1,4,5)P_3 = 18·2±2·2 pmol/10^6 cells; before second stimulation, Ins(1,4,5)P_3 = 10·5±1·7 pmol/10^6 cells).

release all the available receptor-specific stored Ca^{2+}. Stimulation of H_1 histaminoceptors caused a change in Ca^{2+} and after reaching the basal level also the P_2 purinoceptors were stimulated by ATP resulting again in a transient rise in Ca^{2+} (Fig. 5). The amplitude of the Ca^{2+} responses are presented by the bars (Fig. 5*A*), showing that the ATP-induced response is not significantly affected by the preceding histamine response. Following the reversed protocol it is demonstrated that the cells

are also capable of responding to stimulation of H_1 histaminoceptors if preceded by the ATP response (Fig. 5*B*) although the amplitude of the response to histamine is diminished to about 65% (Fig. 5*B*, bars). The question of whether the cells had the ability to respond to simultaneous addition of both agonists with a cumulative rise

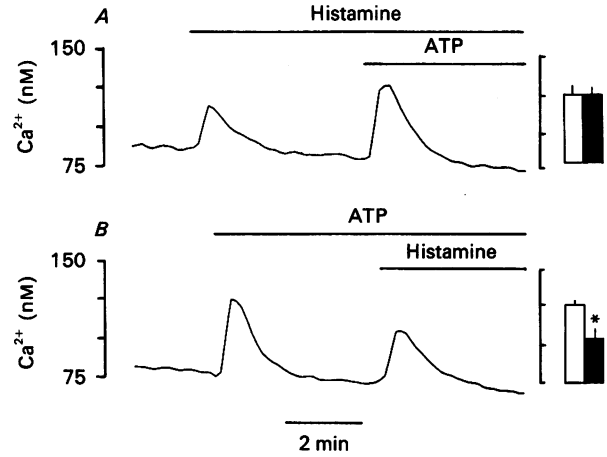

Fig. 5. The transient rise in internal Ca^{2+} evoked by one of the agonists was measured. After reaching the original Ca^{2+} level the response of the cells to the second agonist was recorded. This experiment was carried out under Ca^{2+}-free conditions (15 min or more) using maximal effective agonist concentrations (100 μM). *A*, the Ca^{2+} response evoked by histamine followed by the change in internal Ca^{2+} induced by ATP, still in the presence of histamine. The amplitude of the responses (nM; $n = 4$) is presented by the bars (open bar, histamine; filled bar, histamine and ATP) on the right hand side. *B*, similar results are obtained by applying ATP as first agonist and histamine as second, following the same experimental protocol as in *A*. *The amplitude of the histamine response evoked in the presence of ATP is significantly different from the histamine response in the absence of ATP.

in internal Ca^{2+} was addressed. During the decline of the ATP-induced Ca^{2+} response histamine was added again causing a rise in internal Ca^{2+} exceeding the maximum value obtained by stimulation of the P_2 purinoceptors alone (Fig. 6). The area of the response reflecting the amount of Ca^{2+} released is also increased (Table 1). These changes in internal Ca^{2+} were accompanied by hyperpolarization measured on single cells. The amplitude of the hyperpolarization did not show summation after addition of both agonists with a short time interval, but the response area was increased similarly to the internal Ca^{2+} concentration (Fig. 6, Table 1). Following the opposite procedure with respect to the sequence of the agonists similar cellular responses were observed (Table 2).

Effect of subsequent additions of histamine and ATP

Repeated stimulation of the cells with the same agonist after wash-out (15 min) under Ca^{2+}-free conditions to remove the formed cellular components did not evoke a second response (Fig. 2). Stimulation of the cells with ATP, removing this agonist and allowing the cells to recover (15 min) and continuing the experiment by

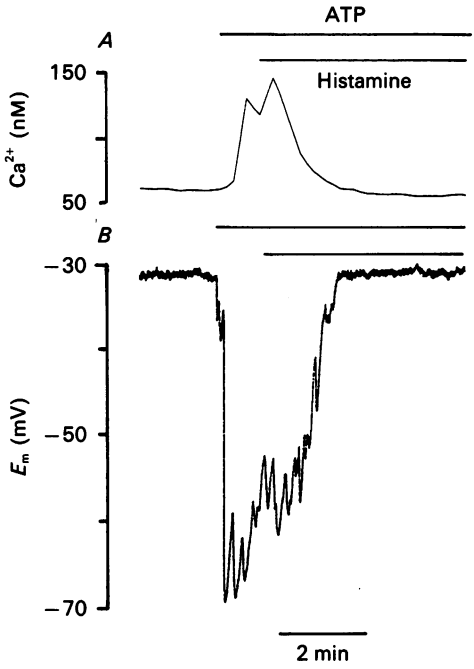

Fig. 6. The ability of the cells to cause an additional increase in response amplitude to simultaneous application of both agonists (100 μM) was tested under Ca^{2+}-free conditions. This experiment was carried out by applying the agonists within a time interval of about 1 min as shown. *A*, the increase in internal Ca^{2+} evoked by stimulation of the H_1 histaminoceptors by histamine is superimposed on the decline of the Ca^{2+} response caused by ATP. *B*, stimulation of the P_2 purinoceptors by ATP followed by application of histamine caused a superimposed hyperpolarization. Typical tracings are shown out of four experiments.

TABLE 1. The transient increase in cytoplasmic Ca^{2+} and in the membrane potential, expressed as the area of the response and as a percentage of the first concomitant response taken as 100 % of that evoked by histamine (100 μM), ATP (100 μM) or by the combination of both agonists applied after a short time interval (30–60 s) under Ca^{2+}-free conditions (15 min) determined in four different experiments

Agonist	Ca^{2+} response (nM min)	(%)	Membrane potential (mV min)	(%)
Histamine	59±6	100	53±9	100
Histamine–ATP	100±8	172±11	83±6	177±35
ATP	74±13	100	65±3	100
ATP–histamine	120±16	167±11	97±7	150±13

stimulation of the H_1 histaminoceptors again caused an increase in internal Ca^{2+} and hyperpolarization of the cells (Fig. 7). The amplitude of the histamine-induced responses are significantly lower than the changes in internal Ca^{2+} and in membrane potential not preceded by stimulation of the cells with ATP, as represented by the bars (Fig. 7). The rise in internal Ca^{2+} evoked by histamine following this experimental protocol was about 55 % of the increase in internal Ca^{2+} found without

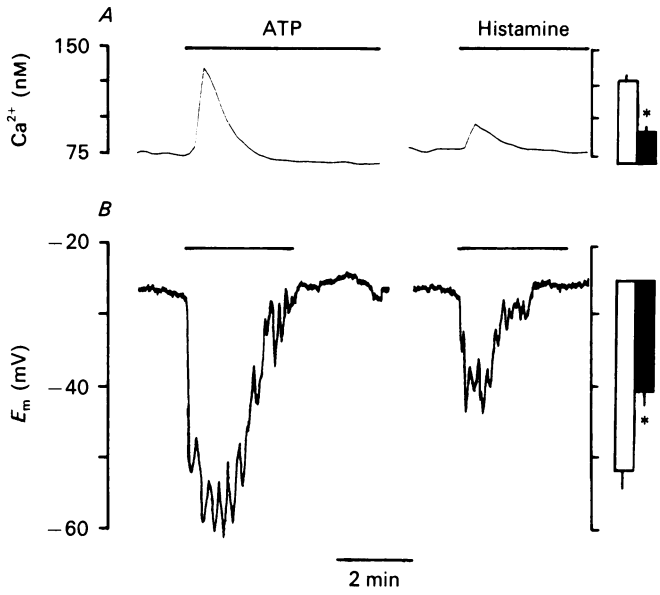

Fig. 7. The cellular response to H_1 histaminoceptor stimulation (100 μM) preceded by activation of the P_2 purinoceptor-sensitive process by ATP (100 μM) and a recovery period (15 min) under Ca^{2+}-free conditions. *A* and *B*, the Ca^{2+} response and hyperpolarization. The maximum amplitudes of these responses are presented by the bars on the right-hand side (open bar, ATP; filled bar, histamine; $n = 4$). * The histamine response evoked after the response to ATP is significantly different from the histamine response not preceded by the ATP response.

TABLE 2. The maximal value of the initial rise in Ins(1,4,5)P_3 content under Ca^{2+}-free conditions

Pretreatment	Treatment	Ins(1,4,5)P_3 (pmol/10^6 cells)
Histamine	Histamine	4·2 ± 1·6
ATP	ATP	6·5 ± 3·2
ATP	Histamine	4·0 ± 2·7
Histamine	ATP	9·8 ± 4·3

The formation of Ins(1,4,5)P_3 was evoked by addition of the second agonist (100 μM) after a recovery period (15 min) from the pretreatment of the cells with the first agonist (3 min) in the absence of external Ca^{2+} as measured in six different experiments. The basal Ins(1,4,5)P_3 content before second addition of the agonist was 10·5 ± 1·7 pmol/10^6 cells ($n = 12$).

a preceding stimulation of the cells by ATP, while the hyperpolarization was about 75% of the control values. Stimulation of the receptors by using the reversed application sequence of the agonists, showed that the ATP-evoked changes in internal Ca^{2+} and in membrane potential were not altered significantly by prior stimulation of the cells with histamine as represented by the bars (Fig. 8). The presence of external Ca^{2+} might be essential in receptor interaction and the concomitant activation of the cellular processes leading to Ca^{2+} mobilization. Therefore, the internal Ca^{2+} concentration was also measured on subsequent addition of the agonists to the cells in the presence of external Ca^{2+} (1·2 mM) and La^{3+} (50 μM),

which did not change the basal internal Ca^{2+} level ($158 \pm 5{\cdot}9$ nM; $n = 4$). The cells were pre-incubated with La^{3+} for 10 min before histamine (100 μM) was added as first agonist, resulting in a transient increase of cytoplasmic Ca^{2+} as observed in the absence of external Ca^{2+}. After washing the cells in the presence of external Ca^{2+} and

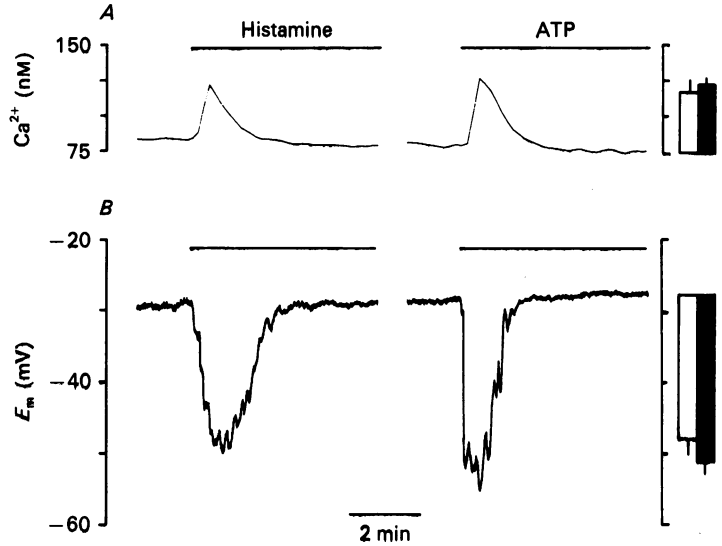

Fig. 8. The cellular response to P_2 purinoceptor stimulation (100 μM) preceded by stimulation of H_1 histaminoceptor by histamine (100 μM) and a recovery period (15 min) under Ca^{2+}-free conditions. *A* and *B*, the Ca^{2+} response and hyperpolarization. The maximum amplitudes of these responses are presented by the bars on the right-hand side (open bar, histamine; filled bar, ATP; $n = 4$).

La^{3+} (15 min), a second addition of histamine did not change the internal Ca^{2+} concentration, a feature also seen under Ca^{2+}-free conditions (Fig. 9*A*). Replenishment of the histamine-sensitive Ca^{2+} store is apparently prevented by La^{3+}. Application of ATP (100 μM) instead of histamine as second agonist, resulted again in a transient rise of internal Ca^{2+} (Fig. 9*B*). A similar responsiveness of the cells to that under Ca^{2+}-free conditions (Figs 7 and 8) was observed by using ATP as first and histamine as second agonist (Fig. 9*C*). It is noticed that repeated addition of ATP to the cells in the presence of La^{3+} following the same protocol as with histamine did not evoke a rise in internal Ca^{2+} ($n = 4$; not shown).

The Ins(1,4,5)P_3 content of the cells was also measured in the presence of one of the agonists followed by wash-out (15 min) and addition of the other agonist, using the same experimental protocol as in the Ca^{2+} and membrane potential measurements. These data show that histamine applied as first agonist caused an increase in Ins(1,4,5)P_3 content of the cells as demonstrated in Fig. 4. Addition of ATP as second agonist added to the same cells after recovery under Ca^{2+}-free conditions (15 min) also activated the formation of Ins(1,4,5)P_3 significantly (Table 2). Following the opposite experimental sequence with respect to the first and second agonist also resulted in a change of the Ins(1,4,5)P_3 content (Table 2).

DISCUSSION

The contribution of Ca^{2+} from the extracellular compartment to the rise in cytoplasmic Ca^{2+} on stimulation of H_1 histaminoceptors by histamine or of P_2 purinoceptors by ATP in DDT_1 MF-2 vas deferens cells is represented by a more

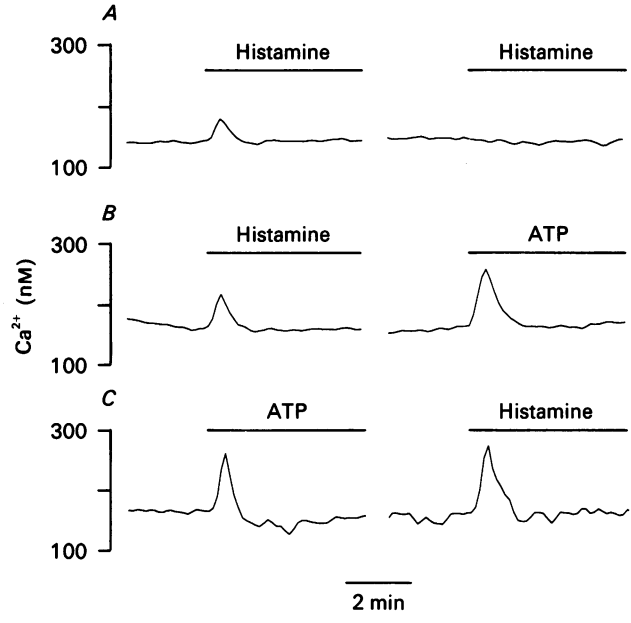

Fig. 9. Changes in internal Ca^{2+} evoked by the agonists in the presence of external Ca^{2+} (1·2 mM) and $LaCl_3$ (50 μM). *A*, the internal Ca^{2+} concentration in the presence of histamine (100 μM) after 10 min pre-incubation of the cells with La^{3+} (left-hand recording). The lack of responsiveness of the cells to second addition of histamine (100 μM) after washing the cells (15 min) in the presence of Ca^{2+} and La^{3+} (right-hand recording). *B*, following the same protocol as in *A*, ATP (100 μM) added as second agonist again caused a transient rise in internal Ca^{2+}. *C*, same procedure as in *A*, using ATP as first agonist and histamine as second agonist. Typical recordings are shown out of four experiments.

pronounced elevation followed by a slowly declining phase not observed under Ca^{2+}-free conditions as reported previously (Hoiting *et al.* 1990*a*; Molleman *et al.* 1991*a*). The transient rise in internal Ca^{2+} evoked by both agonists in DDT_1 MF-2 cells under Ca^{2+}-free conditions is apparently due to release from intracellular organelles as observed in a variety of cell types (Merritt & Rink, 1987; Irvine, 1990). It was shown that the agonist-sensitive Ca^{2+} stores could be refilled with Ca^{2+} from the extracellular space. The pathway used by Ca^{2+} to enter the cells is still under discussion (Casteels & Droogmans, 1981; Den Hertog, 1981; Putney, 1986; Irvine, 1990; Missiaen, Declerck, Droogmans, Plesser, De Smedt, Raeymakers & Casteels, 1990). It is likely that the contribution of this process of Ca^{2+} store replenishment to the internal Ca^{2+} release during receptor stimulation is reflected by the difference in Ca^{2+} response area recorded in the presence of external Ca^{2+} and to the area of the transient response in the absence of Ca^{2+}. This refilling process might even be accelerated in the presence

of the receptor agonist due to the availability of Ins(1,3,4,5)P_4, thought to promote Ca^{2+} translocation (Irvine, 1990; Cullen, Irvine & Dawson, 1990). We demonstrated that the Ca^{2+} release evoked by the agonists in the presence of external Ca^{2+} and La^{3+} showed similar characteristics to those under Ca^{2+}-free conditions. The Ca^{2+} release was transient and could not be repeated by a second addition of the agonist after a recovery period, while the basal internal Ca^{2+} concentration was not changed in the presence of La^{3+}. These observations can be readily explained by assuming that Ca^{2+} store replenishment from the extracellular space is inhibited by La^{3+} and subsequently preventing the receptor-activated Ca^{2+} translocation. Thus, both Ca^{2+} pathways used to refill the histaminoceptor-activated store and the purinoceptor-activated store, respectively, are sensitive to La^{3+}. These results also exclude the possibility that the failure of readmission of the same agonist to the cells to evoke a second Ca^{2+} release is due to inactivation of the receptor-stimulated process caused by external Ca^{2+} not being available or to a lower basal internal Ca^{2+} concentration.

Inositol phosphates, in particular Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are considered to play a role in mobilization of internal Ca^{2+} (Irvine & Moor, 1986; Irvine, 1990). Inositol phosphate formation was activated also in DDT_1 MF-2 cells in the presence of ATP (Hoiting *et al.* 1990*a*) or histamine (Molleman *et al.* 1991*a*). It was reported by us that both the Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 cellular content were increased by the agonists in the presence of external Ca^{2+}, a feature also observed in ileal smooth muscle on stimulation of histamine- or carbachol-sensitive receptors (Bielkiewicz-Vollrath, Carpenter, Schultz & Cook, 1987). The basal value of Ins(1,4,5)P_3 appeared to be high in contrast to that of Ins(1,3,4,5)P_4. Both the Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 formation, however, were enhanced by stimulation of the H_1 histaminoceptors or the P_2 purinoceptors in the presence of extracellular Ca^{2+}. The function of these inositol phosphates, in particular the contribution of Ins(1,3,4,5)P_4 in raising the cytoplasmic Ca^{2+} concentration, is still under discussion (Petersen, 1989). The first initial rise in Ins(1,4,5)P_3 detected upon receptor stimulation in smooth muscle DDT_1 MF-2 cells are small compared to those found in other cells including aorta smooth muscle (Manolopoulos, Pipili-Synetos, Den Hertog & Nelemans, 1991). This might be due to a high activity of the 3-kinase causing a rapid phosphorylation of Ins(1,4,5)P_3 into Ins(1,3,4,5)P_4 (inositol 1,3,4,5-tetrakisphosphate). This process is thought to be accelerated by internal Ca^{2+} (Biden & Wollheim, 1986), which concentration is enhanced in the presence of the agonist. The results presented here show that changes in Ins(1,4,5)P_3 content of the cells were also induced by the agonists in the absence of external Ca^{2+}. Thus, a coupling between Ins(1,4,5)P_3 formation and internal Ca^{2+} mobilization can also be appropriate to Ca^{2+}-free conditions. The transient rise in internal Ca^{2+} might be associated with the time course of the Ins(1,4,5)P_3 formation, but might also be explained by assuming a quantal release process (Muallem, Pandol & Beeker, 1989) or a Ca^{2+}-regulated Ins(1,4,5)P_3 receptor affinity (Irvine, 1990). These models predict that repeated receptor stimulation with the same agonist concentration under Ca^{2+}-free conditions does not result in Ca^{2+} mobilization unless a higher agonist concentration is used to enhance the inositol phosphate formation or unless the Ca^{2+} stores are replenished with Ca^{2+} from the extracellular space. Both phenomena were observed by us in DDT_1 MF-2 cells.

It was mentioned that second application of an optimal concentration of the same

agonist did not evoke a Ca^{2+} response in the absence of external Ca^{2+}. The Ins(1,4,5)P_3 content, however, was activated again after second exposure of the cells to the agonist showing the characteristic initial increase followed by a second rise. These results imply, that the different steps in the process activated on receptor stimulation and leading to Ins(1,4,5)P_3 formation are functional and accordingly not desensitized or inhibited at the time of second agonist stimulation. Accordingly, the lack of effect on internal Ca^{2+} release of repeated stimulation of the cells with the same agonist is most probably due to depletion on this agonist-sensitive Ca^{2+} store during preceding stimulation.

The changes in internal Ca^{2+} in the presence of the agonist were accompanied by hyperpolarization of the cells also observed in the absence of external Ca^{2+}. Hyperpolarization of DDT_1 MF-2 cells evoked by the agonists can be explained by activation of Ca^{2+}-regulated K^+ channels (Hoiting *et al.* 1990*a*; Molleman *et al.* 1991*a*) as reported for other cell types (Marty, 1981; Singer & Walsh, 1987). Besides promotion of Ca^{2+} translocation towards the internal Ins(1,4,5)P_3-sensitive store (Irvine, 1990), Ins(1,3,4,5)P_4 may also be essential to activate the Ca^{2+}-regulated K^+ channels involved in the histamine- and ATP-induced hyperpolarization (Molleman *et al.* 1991*a*).

It is shown here that Ca^{2+} release from internal stores is limited on stimulation of a specific population of receptors in DDT_1 MF-2 cells. The ability of the cells to respond to H_1 histaminoceptor stimulation after depletion of the P_2 purinoceptor-sensitive Ca^{2+} store suggests that the amount of releasable Ca^{2+} from a common store is not limited or that different receptor-specific Ca^{2+} stores are involved. The number of receptors coupled to the Ca^{2+} stores is comparable in view of the maximal amount of mobilized Ca^{2+} activated via H_1 histaminoceptor or P_2 purinoceptor stimulation. An excess increase in internal Ca^{2+}, reflected by the amplitude and response area, was observed on simultaneous stimulation of the receptors exceeding the maximum value reached with one of the agonists. The hyperpolarization evoked in single cells in the presence of the agonists is carried by K^+ ions and accordingly limited by the equilibrium potential. Stimulation of both the histamine and ATP-sensitive receptors, however, resulted also in accumulation of the response area represented by the hyperpolarization. This implies that H_1 histaminoceptors and P_2 purinoceptors are both present on the same cell and that simultaneous activation of the agonist-sensitive processes can explain the additional response.

So far, accumulation of the cellular components on application of both agonists involved in the Ca^{2+} release process or in activation of K^+ channels can account for the events observed in DDT_1 MF-2 cells, referring to the proposed quantal release (Muallem *et al.* 1989) or the Ca^{2+}-regulated inositol phosphate receptor affinity (Irvine, 1990). Subsequent addition of the agonists, however, after removing the cellular products before application of the second agonist to prevent, in particular, accumulation of Ins(1,4,5)P_3, also resulted in release of internal Ca^{2+}. A second transient rise in internal Ca^{2+} evoked by one of the agonists if preceded by Ca^{2+} mobilization caused by the other agonist was not only observed under Ca^{2+}-free conditions, but also in the presence of external Ca^{2+} and La^{3+}. Accordingly, it is shown that La^{3+} prevents the contribution of extracellular Ca^{2+} to the receptor-activated rise in internal Ca^{2+} in these cells (Reynolds & Dubyak, 1986). In

agreement with the enhancement of internal Ca^{2+} and hyperpolarization observed on subsequent stimulation of the cells by the agonists, it was also shown by us that the cellular Ins(1,4,5)P_3 content was increased. This implies that the cellular response to stimulation of H_1 histaminoceptors or P_2 purinoceptors is most probably due to Ca^{2+} release from separate receptor-coupled stores. Activation of these processes by the agonists as represented by the hyperpolarization demonstrates that these separate Ca^{2+} stores are present in the same cell.

Release of Ca^{2+} from structures in the vicinity of the specific receptor was also suggested to exist in aorta (Loutzenhiser & Van Breemen, 1981) and taenia caeci smooth muscle cells (Den Hertog, 1982). Cross-talk between these receptor-specific Ca^{2+} compartments may occur depending on the spatial distribution and density of the receptor sites on the plasma membrane. The results presented by us, in particular the diminished rise in internal Ca^{2+} and in hyperpolarization of the cells elicited by histamine, if preceded by stimulation of the cells with ATP, suggest a limited interaction between the processes activated via both receptors. Thus, cross-talk may occur depending on the cellular organization of the processes involved in the H_1 histaminoceptor- and P_2 purinoceptor-mediated responses in DDT_1 MF-2 cells. Whether 'calciosomes' or other subcellular membranes in the vicinity of the plasma membrane (Volpe, Krause, Hashimoto, Zorzato, Pozzan, Meldolesi & Lew, 1988) are part of this cellular organization needs to be established.

The main conclusion from this study is that stimulation of H_1 histaminoceptors and P_2 purinoceptors in DDT_1 MF-2 cells caused Ca^{2+} release from separate receptor-specific stores. The relevance of this observation to smooth muscle Ca^{2+} handling in general is unknown, since DDT_1 MF-2 cells have lost smooth muscle characteristics such as morphology and the ability to gate Ca^{2+} through voltage-sensitive Ca^{2+} channels (Molleman, Nelemans, Van den Akker, Duin & Den Hertog, 1991*b*). It would be of great interest to test whether the crucial localization of receptors and Ca^{2+} stores as described for DDT_1 MF-2 cells is a common feature in other morphologically intact cell systems.

This study was partly supported by grant 900-549-084 from The Netherlands Foundation for Medical Research (MEDIGON).

REFERENCES

BENHAM, C. D., BOLTON, T. B., BYRNE, N. G. & LARGE, W. A. (1987). Action of extracellular adenosine trisphosphate in single smooth muscle cells dispersed from the rabbit ear artery. *Journal of Physiology* **387**, 473–488.

BERRIDGE, M. J. (1987). Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annual Review of Biochemistry* **56**, 159–193.

BERRIDGE, M. J. (1990). Calcium oscillations. *Journal of Biological Chemistry* **265**, 9583–9586.

BERRIDGE, M. J., COBBOLD, P. H. & CUTHBERTSON, K. S. R. (1988). Spatial and temporal aspects of cell signalling. *Philosophical Transactions of the Royal Society* B **320**, 325–343.

BIDEN, T. J. & WOLLHEIM, C. B. (1986). Ca^{++} regulates the inositol tris/tetrakisphosphate pathway in intact and broken preparations of insulin secreting RINm5F cells. *Journal of Biological Chemistry* **261**, 11931–11934.

BIELKIEWICZ-VOLLRATH, B., CARPENTER, J. R., SCHULTZ, R. & COOK, D.A. (1987). Early production of 1,4,5-inositol trisphosphate and 1,3,4,5-inositol tetrakisphosphate by histamine and carbachol in ileal smooth muscle. *Molecular Pharmacology* **31**, 513–522.

CASTEELS, R. & DROOGMANS, G. (1981). Exchange characteristics of the noradrenaline-sensitive calcium store in vascular smooth muscle cells of rabbit ear artery. *Journal of Physiology* **317**, 263–279.

CHANGYA, L., GALLACHER, D. V., IRVINE, R. F. & PETERSEN, O. H. (1989). Inositol 1,3,4,5 tetrakisphosphate and inositol 1,4,5 trisphosphate act by different mechanisms when controlling Ca^{2+} in mouse lacrimal acrinal cells. *FEBS Letters* **251**, 43–48.

CHILVERS, E. R., CHALLISS, R. A., BARNES, P. J. & NAHORSKI, S. R. (1989). Mass changes of inositol (1,4,5) trisphosphate in trachealis muscle following agonist stimulation. *European Journal of Pharmacology* **164**, 587–590.

CULLEN, P. J., IRVINE, R. F. & DAWSON, A. O. (1990). Synergistic control of Ca^{2+} mobilization in permeabilized mouse L1210 lymphoma cells by inositol 2,4,5-trisphosphate and inositol 1,3,4,5 tetrakisphosphate. *Biochemical Journal* **271**, 549–553.

DEN HERTOG, A. (1981). Calcium and the alpha-action of catecholamines on guinea-pig taenia caeci. *Journal of Physiology* **316**, 109–125.

DEN HERTOG, A. (1982). Calcium and the action of adrenaline, adenosine trisphosphate and carbachol on guinea-pig taenia caeci. *Journal of Physiology* **325**, 423–439.

DEN HERTOG, A., VAN DEN AKKER, J. & NELEMANS, A. (1989). Suramin and the inhibitory junction potential in taenia caeci of the guinea-pig. *European Journal of Pharmacology* **173**, 207–209.

DONIÉ, F. & REISER, G. (1989). A novel specific binding protein assay for quantitation of intracellular inositol 1,3,4,5 tetrakisphosphate ($InsP_4$) using a high-affinity $InsP_4$ receptor from cerebellum. *FEBS Letters* **254**, 155–158.

DUNN, P. M. & BLAKELEY, A. G. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *British Journal of Pharmacology* **93**, 243–245.

HESKETH, T. R., SMITH, G. A., MOORE, J. P., TAYLOR, M. V. & METCALFE, J. C. (1983). Free cytoplasmic calcium concentration and mitogenic stimulation of lymphocytes. *Journal of Biological Chemistry* **258**, 4876–4882.

HOITING, B., MOLLEMAN, A., DUIN, M., DEN HERTOG, A. & NELEMANS, A. (1990*a*). P_2 purinoceptor-mediated inositol phosphate formation in relation to cytoplasmic calcium in DDT_1 MF-2 smooth muscle cells. *European Journal of Pharmacology* **189**, 31–39.

HOITING, B., MOLLEMAN, A., NELEMANS, A. & DEN HERTOG, A. (1990*b*). P_2-purinoceptor-activated membrane currents and inositol tetrakisphosphate formation are blocked by suramin. *European Journal of Pharmacology* **181**, 127–131.

HOYLE, C. H. V., KNIGHT, G. E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *British Journal of Pharmacology* **99**, 617–621.

IRVINE, R. F. (1990). 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates – a possible mechanism. *FEBS Letters* **263**, 5–9.

IRVINE, R. F. & MOOR, R. M. (1986). Microinjection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochemical Journal* **240**, 917–920.

LOUTZENHISER, R. & VAN BREEMEN, C. (1981). Mechanism of activation of isolated rabbit aorta by PGH_2 analogue U-44069. *American Journal of Physiology* **241**, C243–248.

MANOLOPOULOS, V. G., PIPILI-SYNETOS, E., DEN HERTOG, A. & NELEMANS, A. (1991). Inositol phosphates formed in rat aorta after $alpha_1$-adrenoceptor stimulation are inhibited by forskolin. *European Journal of Pharmacology* **207**, 29–36.

MARTY, A. (1981). Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* **291**, 497–500.

MERRITT, J. E. & RINK, T. J. (1987). Regulation of cytosolic free calcium in fura-2 loaded rat parotid acinar cells. *Journal of Biological Chemistry* **262**, 17362–17369.

MISSIAEN, L., DECLERCK, I., DROOGMANS, G., PLESSERS, L., DE SMEDT, H., RAEYMAEKERS, L. & CASTEELS, R. (1990). Agonist-dependent Ca^{2+} and Mn^{2+} entry dependent on state of filling of Ca^{2+} stores in aortic smooth muscle cells of the rat. *Journal of Physiology* **427**, 171–186.

MITSUHASHI, M. & PAYAN, D. G. (1989). Solubilization and characterization of the pyrilamine-binding protein from cultured smooth muscle cells. *Molecular Pharmacology* **35**, 751–759.

MOLLEMAN, A., HOITING, B., DUIN, M., VAN DEN AKKER, J., NELEMANS, A. & DEN HERTOG, A. (1991*a*). Potassium channels regulated by Ins(1,3,4,5)-tetrakisphosphate and internal calcium in DDT_1 MF-2 smooth muscle cells. *Journal of Biological Chemistry* **266**, 5658–5663.

MOLLEMAN, A., NELEMANS, A. & DEN HERTOG, A. (1989). P_2-purinoceptor operated conductances in DDT_1 MF-2 smooth muscle cells. *European Journal of Pharmacology* **169**, 167–174.

MOLLEMAN, A., NELEMANS, A., VAN DEN AKKER, J., DUIN, M. & DEN HERTOG, A. (1991*b*). Voltage-dependent sodium and potassium, but not calcium conductances in DDT_1 MF-2 smooth muscle cells. *Pflügers Archiv* **417**, 479–484.

MORRIS, A. P., GALLACHER, D. V., IRVINE, R. F. & PETERSEN, O. H. (1987). Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. *Nature* **330**, 653–655.

MUALLEM, S., PANDOL, S. J. & BEEKER, T. G. (1989). Hormone-evoked calcium release from intracellular stores is a quantal process. *Journal of Biological Chemistry* **264**, 205–212.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990). Reversible and selective antagonism by suramin of ATP-activated inward current in PC12 phaeochromocytoma cells. *British Journal of Pharmacology* **101**, 224–227.

NELEMANS, A., HOITING, B., MOLLEMAN, A., DUIN, M. & DEN HERTOG, A. (1990). α-Adrenoceptor regulation of inositol phosphates, internal calcium and membrane current in DDT_1 MF-2 smooth muscle cells. *European Journal of Pharmacology* **189**, 41–49.

PETERSEN, O. H. (1989). Does inositol tetrakisphosphate play a role in the receptor-mediated control of calcium mobilization? *Cell Calcium* **10**, 375–383.

PETERSEN, O. H. & WAKUI, M. (1990). Oscillating intracellular Ca^{2+} signals evoked by activation of receptors linked to inositol lipid hydrolysis: mechanism of generation. *Journal of Membrane Biology* **118**, 93–105.

PUTNEY, J. W. JR (1986). A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1–12.

REYNOLDS, E. W. & DUBYAK, G. R. (1986). Agonist-induced transients in cultured smooth muscle cells: measurements with Fura-2 loaded monolayers. *Biochemical and Biophysical Research Communications* **136**, 927–934.

SINGER, J. J. & WALSH, J. V. JR. (1987). Characterization of calcium-activated potassium channels in smooth muscle cells using the patch-clamp technique. *Pflügers Archiv* **408**, 98–111.

VOLPE, P., KRAUSE, K.-H., HASHIMOTO, S., ZORZATO, F., POZZAN, T., MELDOLESI, J. & LEW, D. P. (1988). 'Calciosome', a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of non-muscle cells? *Proceedings of the National Academy of Sciences of the USA* **85**, 1091–1095.