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SECOND MESSENGER-ACTIVATED CALCIUM INFLUX IN RAT PERITONEAL MAST CELLS

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SUMMARY

1. To study the regulation of calcium influx in non-excitable cells, membrane currents of rat peritoneal mast cells were recorded using the whole-cell patch-clamp technique. At the same time, intracellular calcium concentration ($[Ca^{2+}]_i$) was monitored via the fluorescent calcium-indicator dye Fura-2, which was loaded into cells by diffusion from the patch pipette.

2. Stimulation of mast cells with secretagogues, such as compound 48/80 or substance P, caused release of Ca^{2+} from internal stores. In addition, external agonists also induced influx of external calcium in 26% of the cells investigated. The agonist-stimulated Ca^{2+} influx was increased during membrane hyperpolarization and was associated with small whole-cell currents.

3. Likewise, internal application of inositol 1,4,5-trisphosphate (Ins1,4,5P_3: 0·5–10 μM) elevated $[Ca^{2+}]_i$ due both to release of Ca^{2+} from internal stores and to influx of external calcium. The Ins1,4,5P_3-induced influx was greater at more negative membrane potentials, suggesting that Ins1,4,5P_3 opened a pathway through which calcium could enter at a rate governed by its electrochemical driving force.

4. Inositol 1,3,4,5-tetrakisphosphate (Ins1,3,4,5P_4) did not induce Ca^{2+} influx by itself nor did it facilitate or enhance Ins1,4,5P_3-induced Ca^{2+} entry. Calcium influx was also induced by inositol 2,4,5-trisphosphate. Since this inositol phosphate is a poor substrate for Ins1,4,5P_3 3-kinase it seems unlikely that Ins1,3,4,5P_4 plays a role in the regulation of the Ca^{2+}-influx pathway in mast cells.

5. The Ins1,4,5P_3-induced Ca^{2+} influx was associated with whole-cell currents of 1–2 pA or less, with no channel activity detectable in whole-cell recordings. The small size of the whole-cell current suggests either that the Ins1,4,5P_3-dependent influx occurs via small-conductance channels that are highly calcium specific or that the influx is not via ion channels.

6. Agonist stimulation also activated large-conductance (*ca* 50 pS) cation channels, through which divalent cations could permeate; thus, these channels represent a second pathway for Ca^{2+} influx. The slow speed of activation of the channels by agonists, their activation by internal guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), and the inhibition of agonist activation by internal guanosine 5′-*O*-(2-thiodi-

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phosphate) (GDP-β-S) all suggest that the 50 pS channels are regulated by a second messenger and/or a GTP-binding protein. The activity of the 50 pS channel in mast cells is not sensitive to either Ins1,4,5P_3 or Ins1,3,4,5P_4. Activity of the channel was inhibited by elevated $[Ca^{2+}]_i$.

7. Activation of 50 pS channels by agonists was prevented by incubation with the phorbol ester 4-β-phorbol 12-myristate, 13-acetate (an activator of protein kinase C) and by concentrations of internal neomycin that blocked agonist-induced Ca^{2+} transients. Internally applied phorbol ester had no effect on the activation of 50 pS channels by external agonists.

8. The two Ca^{2+} influx pathways gated by internal messengers in mast cells may carry out the function served by voltage-activated calcium channels in excitable cells. However, in contrast to the situation in excitable cells, where depolarization of the membrane is required to open calcium channels and thus stimulate Ca^{2+} influx, in mast cells membrane hyperpolarization is required to support calcium entry through voltage-independent pathways activated indirectly by external signals. Such hyperpolarization-driven Ca^{2+} influx has been observed in flux studies in many other non-excitable cells.

INTRODUCTION

The intracellular concentration of free calcium ($[Ca^{2+}]_i$) is important in the control of a variety of cellular functions, and an increase in $[Ca^{2+}]_i$ is a common mechanism by which external signals influence events inside the cell. An increase in cytoplasmic free calcium can arise either by influx of external calcium across the plasma membrane or by the release of calcium from intracellular sources. Influx of external calcium commonly occurs via voltage-activated calcium channels, which open upon membrane depolarization and allow calcium entry. Liberation of internal calcium is frequently stimulated by the intracellular second messenger inositol 1,4,5-trisphosphate (Berridge, 1987), a product of the hydrolysis of phosphoinositide membrane lipids. In excitable cells, calcium influx through voltage-activated channels has typically received emphasis, while in non-excitable cells, release of internal calcium and receptor-mediated Ca^{2+} influx, either by direct gating of receptor-operated channels or indirectly by second messenger-operated channels, have been considered the principal mechanisms of increasing $[Ca^{2+}]_i$ (for reviews see Hockberger & Swandulla, 1987; Meldolesi & Pozzan, 1987; Penner & Neher, 1988*a*; Hallam & Rink, 1989). Recently, inositol phosphates have been shown to trigger both release of calcium from intracellular stores and influx of external calcium in non-excitable cells (Kuno & Gardner, 1987; Llano, Marty & Tanguy, 1987; Morris, Gallacher, Irvine & Petersen, 1987; Penner, Matthews & Neher, 1988). However, the exact roles of the various inositol phosphates and the influx pathways they control in the different experimental systems remain unsolved.

To determine the membrane mechanisms regulating Ca^{2+} influx in the absence of voltage-gated calcium channels, we have made simultaneous patch-clamp recordings of membrane currents and Fura-2 measurements of $[Ca^{2+}]_i$ in rat peritoneal mast cells. These cells are non-excitable cells in which $[Ca^{2+}]_i$ modulates secretion (Gomperts, 1986; Neher, 1988; Penner & Neher, 1988*b*). We have found that

stimulation of mast cells with antigen or with the secretagogues substance P or compound 48/80 activated a non-specific cation channel through which divalent cations can enter the cell. Also, intracellular application of Ins1,4,5P_3 induced Ca^{2+} influx via a pathway that was not correlated with the non-specific channels and was not associated with detectable channel events or with large whole-cell currents. Although the total current was small (about 1–2 pA), this Ins1,4,5P_3-induced pathway was more effective than the non-specific cation channel in producing increases in $[Ca^{2+}]_i$, suggesting that it has high specificity for calcium. External agonists also activated a similar pathway in approximately one-quarter of the cells studied, producing a two-phase increase in $[Ca^{2+}]_i$. The first phase consisted of a transient increase that occurred rapidly after stimulation and was due to release of internal calcium, and the second phase consisted of a longer-lasting plateau of elevated $[Ca^{2+}]_i$ due to Ca^{2+} influx. Thus, these non-excitable cells have two pathways for Ca^{2+} influx, in addition to release of Ca^{2+} from internal stores. External agonists also activated a chloride current, which is described in the following paper (Matthews, Neher & Penner, 1989).

METHODS

Preparation of mast cells

Rat mast cells were obtained as described previously (Penner, Pusch & Neher, 1987). Briefly, a rat was anaesthetized with ether and decapitated. The peritoneum was opened and a suspension of cells of various types, including mast cells, was obtained by lavage. An aliquot of cells was plated directly onto glass cover-slips in an incubation Ringer solution containing (in mM): NaCl, 140; $NaHCO_3$, 45; KCl, 2·5; KH_2PO_4, 0·4; $CaCl_2$ 2; $MgCl_2$ 5; glucose, 11; streptomycin, 0·12 mg ml^{-1} and penicillin, 0·64 mg ml^{-1} and placed in an incubator at 37 °C and 10% CO_2. The remaining cells were centrifuged in a Percoll gradient at 4 °C for 20 min, and the band containing mast cells was collected and resuspended in Ringer solution of the above composition. The mast cell suspension was again briefly centrifuged to form a pellet of mast cells, the supernatant was removed, and the cells were resuspended in medium M199 supplemented with fetal calf serum (10%), $NaHCO_3$ (45 mM), glucose (2·5 mM), streptomycin (0·12 mg ml^{-1}), and penicillin (0·64 mg ml^{-1}). The purified mast cells were then plated onto glass cover-slips in modified medium M199 or in incubation Ringer solution and placed in the incubator.

Recordings were usually made within 0·5–7 h of plating from purified mast cells incubated in medium M199. In some experiments, recordings were also made from cells stored in incubation Ringer solution or from mast cells selected from among the mixed cells plated before purification. No differences in physiological characteristics were observed in mast cells taken from a given rat, regardless of the method used to isolate and incubate the cells.

External and internal solutions

The standard external solution was a modified Ringer solution (termed mast cell Ringer, or MCR) containing (in mM): NaCl, 140; KCl, 2·5; $CaCl_2$, 2; $MgCl_2$, 5; glucose, 5; HEPES–NaOH, 10; pH = 7·2. The Mg^{2+} concentration was elevated because it was found to help prevent the formation of ATP-induced membrane leak (Bennett, Cockcroft & Gomperts, 1981) caused by ATP diffusing from the tip of the patch pipette as the electrode approached the cell for seal formation. In some experiments, the divalent cation concentration of the external solution was altered as described in the text or figure captions. Solution changes were made by bath perfusion or by local superfusion of the recorded cell via an application pipette.

Patch pipettes for whole-cell recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were filled with a solution containing (in mM): potassium glutamate, 145; KCl, 1; NaCl, 8; $MgCl_2$, 1; HEPES–KOH, 10; Mg^{2+}-ATP, 0·5; Fura-2 penta-potassium salt (Molecular Probes, Inc.), 0·1;

pH = 7·2. In most experiments, the pipette solution also contained 0·3 mM-GTP. Ca^{2+}–EGTA and K_2–EGTA solutions for buffering intracellular calcium were prepared as described by Neher (1988).

The measured junction potential between the pipette solution and the external solution was −8 mV, and all voltages have been corrected to take this into account. Thus, to achieve a holding potential of +40 mV, the indicated voltage on the patch-clamp amplifier was set to +48 mV.

Data acquisition and analysis

The details of the measurement of Fura-2 fluorescence from single mast cells under whole-cell voltage clamp are given elsewhere (Almers & Neher, 1985; Neher, 1988, 1989). Cells were loaded with Fura-2 by diffusion from the patch pipette, and $[Ca^{2+}]_i$ was calculated from the ratio of emitted light at two excitation wavelengths, as described by Grynkiewicz, Poenie & Tsien (1985). A PDP 11 computer sampled the two fluorescence signals every 0·5 s and displayed the calculated $[Ca^{2+}]_i$ on-line during the experiment, together with membrane voltage and membrane current from the whole-cell voltage clamp (List, EPC-7). In addition, the variance of the membrane current was computed on-line at a wide bandwidth (2–500 Hz).

For analysis of single-channel events, membrane current and voltage were recorded on videotape via a digital pulse-code modulation encoder (Sony PCM-701 ES), replayed through an antialiasing filter (8-pole Bessel, 400–500 Hz), and digitized at 2000–2500 Hz. Channel amplitudes and durations were measured using the TAC program described by Colquhoun & Sigworth (1983). To determine single-channel conductances, straight lines were fitted to current–voltage curves using a least-squares criterion.

Drugs

Compound 48/80 (Sigma) was applied via local superfusion at a concentration of 5 μg ml^{-1} in external solution (usually MCR). Substance P (Sigma) was applied in the same way at 50 μg ml^{-1}. For antigenic stimulation, mast cells were first incubated for 1–3 h in modified medium M199 or in incubation Ringer solution containing 1 μg ml^{-1} IgE directed against bovine serum albumin conjugated with dinitrophenol (DNP–BSA); DNP–BSA was then applied locally to sensitized cells at 2–20 μg ml^{-1}. Both the IgE and the DNP–BSA were kind gifts of Dr H. Metzger (NIH). In some experiments, 10 μM-DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulphonate) was added to the external and internal solutions to block chloride current. Internal drugs were applied by diffusion from the recording-pipette solution. GTP-γ-S (kindly provided by Dr F. Eckstein, Göttingen) was applied at 40–100 μM, GDP-β-S (Boehringer) at 200 μM, and cyclic AMP and cyclic GMP (both Boehringer) typically at 50 μM. Inositol 1,4,5-trisphosphate (Ins1,4,5P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins1,3,4,5P_4) (both Amersham) were used at the concentrations indicated in the text or figure captions for each experiment. Inositol 2,4,5-trisphosphate (Ins2,4,5P_3) was a generous gift of Dr Robin Irvine. Neomycin (Sigma) was applied intracellularly at 0·1–1 mM. Both active (4-β-phorbol 12-myristate, 13-acetate) and inactive (4-α-phorbol 12,13-didecanoate; both from LC Services Corp., Woburn, MA, USA) phorbol esters were dissolved at 1 mM in DMSO and then diluted to 100 nM in MCR for external application to mast cells; cells were incubated with the phorbol ester in MCR for 25 min at 29–31 °C before recordings started. For internal application, the concentration was also 100 nM in the pipette solution.

RESULTS

In a previous publication (Penner *et al.* 1988), we demonstrated that stimulation of mast cells with external agonists can induce calcium influx via two distinct pathways: (1) a hyperpolarization-driven influx associated with total whole-cell currents less than 1–2 pA; and (2) a voltage-independent cation channel that is permeable to divalent cations and has a single-channel conductance of about 50 pS. The two pathways are differently expressed in different cells (see section on variability below), and thus they can be examined in relative isolation by selecting appropriate cells. The activation of the first pathway is demonstrated in Fig. 1, which shows the effect of the mast cell secretagogue compound 48/80 on intracellular

calcium concentration and on membrane current. Following the stimulus, there was a large, transient increase in $[Ca^{2+}]_i$, most likely due to release of Ca^{2+} from internal stores by inositol 1,4,5-trisphosphate ($Ins1,4,5,P_3$). After the agonist-induced Ca^{2+} transient, there was a plateau phase of elevated $[Ca^{2+}]_i$ during which increases in

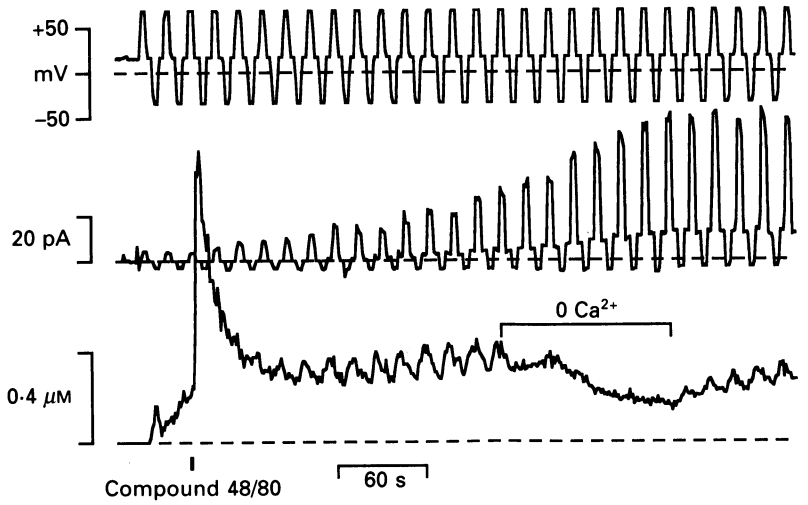

Fig. 1. Effect of compound 48/80 on membrane current and $[Ca^{2+}]_i$ in a mast cell. The bottom trace shows $[Ca^{2+}]_i$ as calculated from Fura-2 fluorescence, the middle trace shows the whole-cell membrane current, and the top trace shows the protocol of voltage-clamp command pulses. In each trace, the dashed line indicates the zero level. At the indicated time on the bottom trace, the mast cell secretagogue compound 48/80 (5 μM ml^{-1}) was applied, causing a large, transient rise in $[Ca^{2+}]_i$. Following the transient, each negative voltage pulse was associated with an increase in $[Ca^{2+}]_i$. For the indicated time, the external solution was switched from MCR to a similar solution without added Ca^{2+} or Mg^{2+}.

$[Ca^{2+}]_i$ occurred with each hyperpolarizing membrane voltage pulse. These hyperpolarization-driven increases in $[Ca^{2+}]_i$ were abolished when external calcium was removed (Fig. 1), indicating that they were due to influx of extracellular calcium. In unstimulated cells, $[Ca^{2+}]_i$ was unaffected by changes in membrane potential. Thus, agonist stimulation activated a pathway through which Ca^{2+} can enter the cell, provided membrane voltage is sufficiently negative to supply the needed electrical driving force. The properties of this influx pathway and its activation by $Ins1,4,5P_3$ will be described in the first major subsection of Results.

The membrane current trace in Fig. 1 also reveals a large, agonist-activated outward current during the positive membrane voltage pulses. This current, which develops slowly after stimulation, is carried by chloride ions and will be the subject of the following paper. It is blocked by the stilbene derivative DIDS, which was therefore used to suppress the chloride current in some experiments in this paper.

In addition to the Ca^{2+} entry pathway described above, agonist stimulation transiently activates non-specific cation channels that are permeable to divalent cations and should therefore contribute to total Ca^{2+} influx. The activation of these channels by compound 48/80 is illustrated in Fig. 2. In this experiment, membrane voltage was clamped to -50 mV, and activation of the channel resulted in an inward

current. Because of the relatively large single-channel conductance (about 50 pS), activation of the channels resulted in a large increase in the variance of the whole-cell membrane current (upper trace in Fig. 2*A*), which provided a useful signature for the activity of the 50 pS channel. As shown in Fig. 2*B*, the current through a single one of these channels can be sufficiently large to be resolved in whole-cell membrane current recordings, and their activity sums to produce a noisy inward current at negative membrane potentials. This channel will be the focus of later subsections of Results.

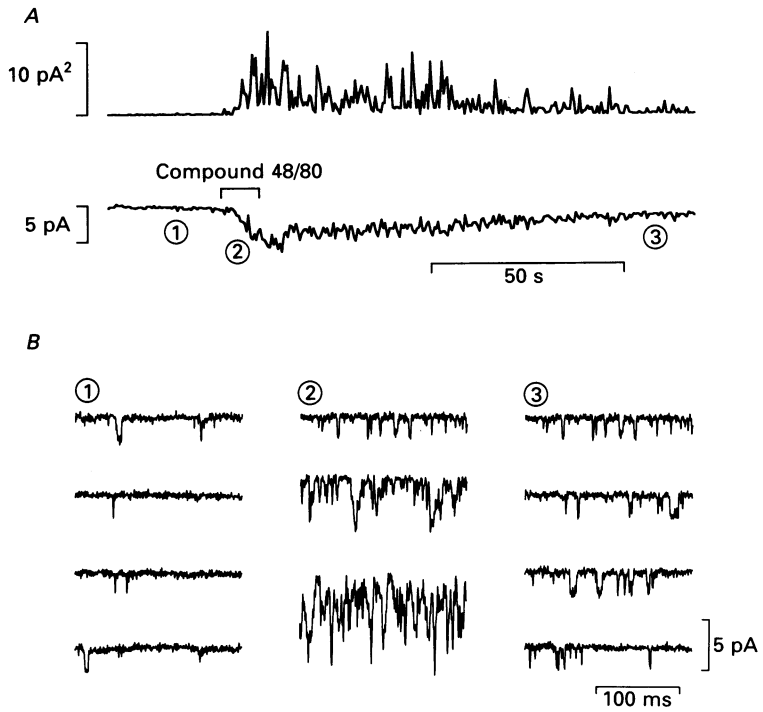

Fig. 2. Activation of large-conductance channels by compound 48/80. *A*, an overview of the experiment, showing the whole-cell current (bottom trace) at a steady holding potential of −50 mV. The current is smoothed by averaging over a 0·5 s sample period. The upper trace is the variance of the membrane current, calculated over the same 0·5 s periods at bandwidth 2–500 Hz. The timing of compound 48/80 application is shown above the bottom trace. *B*, higher resolution recordings of channel events observed in whole-cell currents. The numbers for each set of traces refer to the labelled periods in *A*. The traces in 1 are selected to show events; the actual rate of events was much lower during the pre-stimulation period. Bandwidth, 0–500 Hz.

Both pathways for calcium entry could be fully activated in cells in which exocytosis, or degranulation, was blocked. In most of the experiments reported here, secretion was inhibited by simply delaying the stimulus after breaking into the cell until wash-out of the secretory response had occurred (Penner *et al.* 1987). Thus, the effects of stimulation described here do not originate from vesicle membrane that becomes incorporated into the plasma membrane during secretion and instead represent agonist activation of existing plasma membrane mechanisms.

Ins1,4,5P$_3$ induces calcium influx

It is known that agonist stimulation of mast cells causes inositol phospholipid hydrolysis and release of Ins1,4,5P$_3$ (Kennerly, Sullivan & Parker, 1979; Beaven, Moore, Smith, Hesketh & Metcalfe, 1984; Nakamura & Ui, 1985). Intracellularly

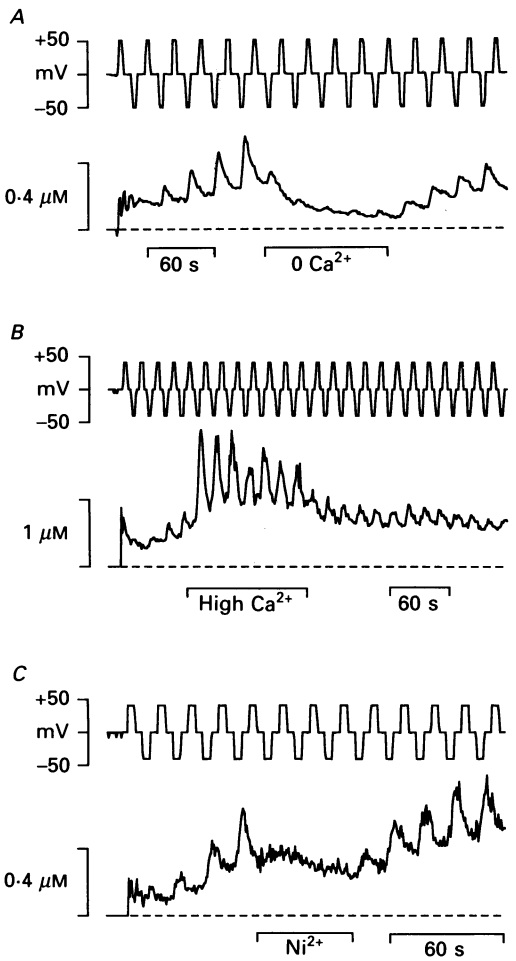

Fig. 3. Ca^{2+} influx stimulated by internal Ins1,4,5P$_3$. The bottom trace of each pair is $[Ca^{2+}]_i$ calculated from Fura-2 fluorescence, and the upper trace is the voltage protocol. The dashed baseline indicates the zero level. *A*, 0·5 μM-Ins1,4,5P$_3$. At the time indicated, the bath solution was changed from MCR to the same solution without added Ca^{2+} or Mg^{2+}. *B*, 10 μM-Ins1,4,5P$_3$. The external solution was switched from MCR to Ringer solution with 5 mM-Ca^{2+} and 0·5 mM-Mg^{2+} at the indicated time. *C*, 10 μM-Ins1,4,5P$_3$. The external solution was changed from MCR to MCR + 5 mM-Ni^{2+} at the indicated time.

supplied Ins1,4,5P$_3$ has been shown by Neher (1986, 1988) to mimic the agonist-stimulated release of calcium from internal stores in mast cells. To determine if the plateau phase of elevated calcium, with its concomitant hyperpolarization-driven calcium influx, might also be due to Ins1,4,5P$_3$, we included Ins1,4,5P$_3$ in the pipette solution dialysing the cells. Results from one experiment of this type are

shown in Fig. 3*A*. There were one or more transient increases in $[Ca^{2+}]_i$ immediately upon breaking into the cell with a pipette containing Ins1,4,5P_3. These Ins1,4,5P_3-induced transients were not correlated with changes in membrane voltage and presumably represent release from internal stores (Neher, 1988). After these early transients, hyperpolarization-driven increases appeared (Fig. 3) that were reminiscent of the hyperpolarization-driven calcium influx induced by agonist stimulation (Fig. 1). When external calcium was removed, the hyperpolarization-driven increases in internal calcium were eliminated (Fig. 3*A*), and when external calcium concentration was increased (Fig. 3*B*), the hyperpolarization-driven increases in $[Ca^{2+}]_i$ were larger, indicating that the increases in $[Ca^{2+}]_i$ were due to influx of external calcium. Consistent with this idea, the hyperpolarization-driven increase in $[Ca^{2+}]_i$ induced by Ins1,4,5P_3 was blocked by external application of nickel (Fig. 3*C*), cadmium or lanthanum.

The delay between the initial Ins1,4,5P_3-induced Ca^{2+} transients and the appearance of hyperpolarization-driven Ca^{2+} influx showed considerable variability across cells and could be quite long (> 100 s), although the rapid appearance of the transients demonstrated that Ins1,4,5P_3 had rapid access to the cell after whole-cell recording began (also confirmed by rapid rise of Fura-2 fluorescence as the dye diffused into the cell and by measurement of access resistance).

Because Ins1,3,4,5P_4 has been implicated in regulation of Ca^{2+} influx in other cells (Irvine & Moor, 1986; Morris *et al.* 1987), we considered the possibility that the delay might be due to metabolic accumulation of Ins1,3,4,5P_4. To test this, we made paired comparisons of the effects of 0·5 μM-Ins1,4,5P_3 and 0·5 μM-Ins1,4,5P_3 plus 10 μM-Ins1,3,4,5P_4 in ten pairs of cells from the same preparation of mast cells. There was no effect of Ins1,3,4,5P_4 on the prevalence, amplitude, or onset delay of hyperpolarization-driven Ca^{2+} influx, indicating that intracellular generation of Ins1,3,4,5P_4 from Ins1,4,5P_3 is unlikely to account for the delayed appearance of Ca^{2+} influx induced by Ins1,4,5P_3. Also, Ins1,3,4,5P_4 alone did not induce hyperpolarization-driven Ca^{2+} influx (nine cells). Moreover, to further clarify a possible role of Ins1,3,4,5P_4, we performed experiments with inositol 2,4,5-trisphosphate. Like Ins1,4,5P_3, this inositol phosphate is known to release Ca^{2+} from internal stores (Irvine, Brown & Berridge, 1984), although it is less potent. In contrast to Ins1,4,5P_3, inositol 2,4,5-trisphosphate is a poor substrate for the Ins1,4,5P_3 3-kinase and thus no production of Ins1,3,4,5P_4 is expected to occur upon its perfusion into the cell. Figure 4 clearly shows that inositol 2,4,5-trisphosphate can support hyperpolarization-driven Ca^{2+} influx. At low concentrations (5 μM) the Ca^{2+} influx phase is typically delayed by about 200–300 s ($n = 4$) while higher concentrations (50 μM) induce responses comparable to about 0·5–10 μM-Ins1,4,5P_3 (cf. Fig. 3; $n = 7$).

Ins1,4,5P_3-induced Ca^{2+} influx is driven by hyperpolarization

As with the Ca^{2+} influx activated by agonist stimulation, the Ins1,4,5P_3-induced influx occurred only when the membrane potential was sufficiently negative. This is illustrated in Fig. 5, which shows the membrane potential dependence of $[Ca^{2+}]_i$ in the presence of 10 μM-Ins1,4,5P_3. At such high concentrations of Ins1,4,5P_3, the initial transient release of Ca^{2+} from internal stores occurred so rapidly that only the

falling phase of the transient could be resolved. After this early transient, $[Ca^{2+}]_i$ declined to the low basal level typical of unstimulated cells and remained there for as long as the membrane potential was positive. Brief negative voltage steps, however, caused a rapid increase in $[Ca^{2+}]_i$ whose amplitude was larger at more negative potentials. In addition, the rate of change of $[Ca^{2+}]_i$ ($d[Ca^{2+}]_i/dt$; bottom trace of Fig. 5*A*) was greater at more negative potentials, consistent with influx at rates that increased with hyperpolarization. This dependence of the rate of Ca^{2+} influx on membrane potential is summarized for four experiments like that of Fig. 5*A* in Fig. 5*B*.

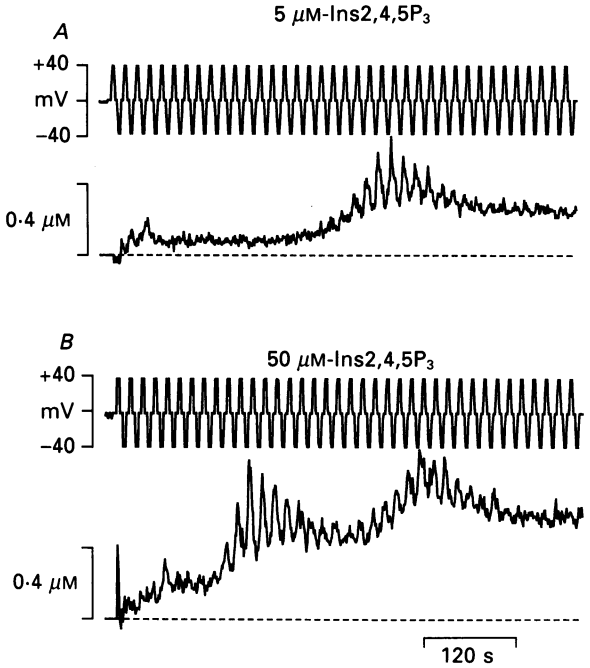

Fig. 4. Ca^{2+} influx stimulated by internal Ins2,4,5P_3. The bottom trace of each pair is $[Ca^{2+}]_i$ calculated from Fura-2 fluorescence, and the upper trace is the voltage protocol. The dashed baseline indicates the zero level. *A*, 5 μM-Ins2,4,5P_3. *B*, 50 μM-Ins2,4,5P_3

To compare results across experiments, the changes in $d[Ca^{2+}]_i/dt$ were normalized for each cell with respect to that observed with a pulse to −60 mV. At −40 mV, the mean change in $d[Ca^{2+}]_i/dt$ in these cells was 31 nM s^{-1}, with a mean inward current of −3·7 pA. In order to relate these changes to those induced by unspecific leak currents, we analysed $d[Ca^{2+}]_i/dt$ in seven cells where leak developed unrelated to any stimulus. In these cells, the mean change in $d[Ca^{2+}]_i/dt$ produced by the same voltage pulse protocol was 36 nM s^{-1}; however, the mean inward current required to induce these changes in $[Ca^{2+}]_i$ was 56 pA, more than 10 times the size of the current associated with Ins1,4,5P_3-induced changes in $[Ca^{2+}]_i$. These results suggest that Ins1,4,5P_3 opens a pathway through which external calcium can enter the cell. Negative membrane potentials apparently increase calcium entry through this pathway.

A simple explanation for the behaviour shown in Fig. 5 is that Ins1,4,5P_3

activates a voltage-independent, plasma membrane calcium conductance through which calcium enters the cell at a rate governed by its electrochemical gradient. We therefore examined whole-cell membrane current for evidence of this putative Ins1,4,5P_3- and agonist-activated calcium conductance. Because agonist stimulation

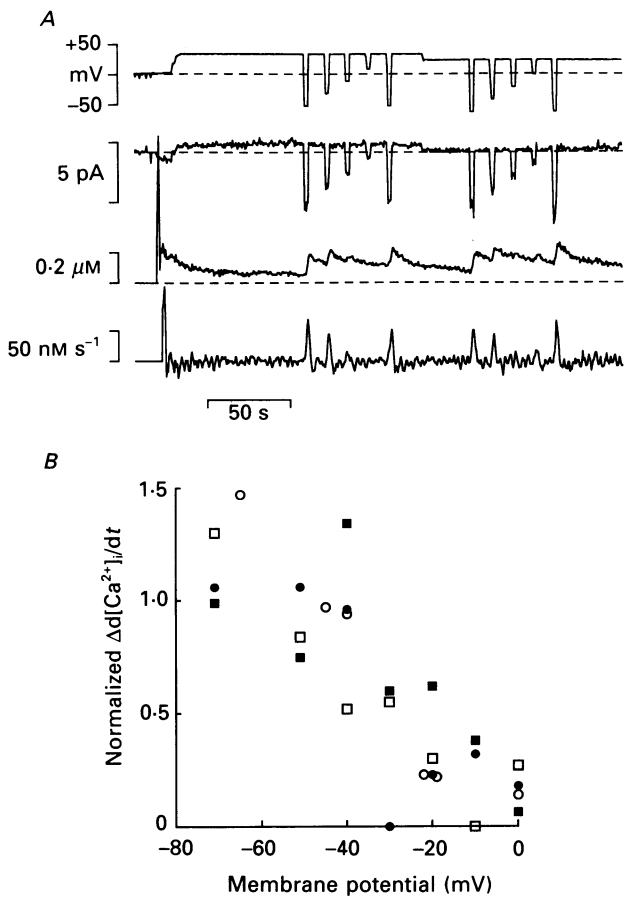

Fig. 5. Dependence of Ca^{2+} influx on membrane potential in the presence of internal Ins1,4,5P_3. *A*, an example of an individual experiment. From top to bottom, the traces show the voltage protocol, the whole-cell membrane current, the calculated $[Ca^{2+}]_i$, and the first time-derivative of the $[Ca^{2+}]_i$ trace ($d[Ca^{2+}]_i/dt$). Dashed lines indicate zero levels. *B*, summary of results of four experiments like that of *A* (each symbol represents a different cell). The abscissa gives the voltage during the test pulses, and the ordinate is the change in $d[Ca^{2+}]_i/dt$ produced by each pulse ($\Delta d[Ca^{2+}]_i/dt$, measured by subtracting the value of $d[Ca^{2+}]_i/dt$ during a 1·5 s period just before each pulse from the value of $d[Ca^{2+}]_i/dt$ in the 1·5 s period just before the end of each pulse. To compare results across experiments, the $d[Ca^{2+}]_i/dt$ values for each cell were divided by the measured value at −60 mV.

activates large-conductance channels (see Fig. 2) that are permeable to divalent cations (see below) and because Ins1,4,5P_3 has been reported to activate similar channels in lymphocytes (Kuno & Gardner, 1987), we investigated whether the Ins1,4,5P_3-induced calcium influx was mediated via the 50 pS channels described

above. Several lines of evidence, described in the next several paragraphs, indicate that this is not the case in mast cells.

Lack of correlation between activity of 50 pS channels and calcium influx

In experiments in which 50 pS channels were activated by compound 48/80 or by substance P, there was no relation between the amount of activity of 50 pS channels

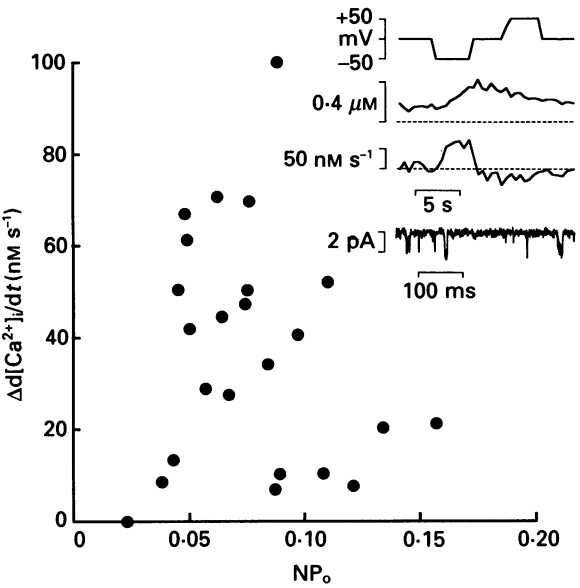

Fig. 6. Relation between Ca^{2+} influx and proportion of time that the 50 pS channel was open for an experiment with 4 μM internal Ins1,4,5P_3. The protocol of voltage pulses was as in Fig. 3, with 4 s alternating positive and negative pulses, separated by 4 s periods of 0 mV. Each data point shows the result for a single negative pulse in the series. The $d[Ca^{2+}]_i/dt$ was calculated as described in Fig. 5. NP_o is the proportion of the total period during a single pulse that one or more 50 pS channels were open (there were only a few overlapping openings of multiple channels at these low levels of single-channel activity). The inset shows one pulse cycle, with the change in $[Ca^{2+}]_i$, $d[Ca^{2+}]_i/dt$, and a sample of channel activity during the hyperpolarizing pulse.

and the presence of hyperpolarization-driven calcium increases following the initial calcium transient. Thus, we encountered cells in which their activity contributed 3–5 pA, but which showed no hyperpolarization-driven Ca^{2+} influx. Conversely, we found cells with large Ca^{2+} influx but little activity of 50 pS channels (current smaller than 2 pA). There was also no correlation between 50 pS channel activity and hyperpolarization-driven Ca^{2+} influx stimulated by intracellularly applied Ins1,4,5P_3. An example of this is shown in Fig. 6, in which Ca^{2+} influx elicited by a series of hyperpolarizing pulses to −50 mV is plotted against the activity of 50 pS channels during each pulse. As an index of Ca^{2+} influx, the difference between the rate of change of $[Ca^{2+}]_i$ (i.e. the first time-derivative of $[Ca^{2+}]_i$) just before and during each pulse was computed (see inset of Fig. 6). As a measure of 50 pS channel activity, the proportion of total time during which 50 pS channels were open was measured for

each voltage pulse (see inset). It is clear from Fig. 6 that there was no relation between the Ca^{2+} influx elicited by a pulse and the measured activity of the 50 pS channel during the pulse.

Total whole-cell current carried by 50 pS channels is too small to account for changes in $[Ca^{2+}]_i$

Activation of 50 pS channels by agonist stimulation typically produced a total membrane current of less than 5 pA in the whole cell at a holding potential of −50 mV, provided $[Ca^{2+}]_i$ was not buffered to low levels with EGTA. As will be demonstrated below, the reversal potential for the single-channel current is near 0 mV, and the channel is permeable to both monovalent and divalent cations, suggesting that this current is a non-specific cation current. Non-specific current through membrane 'leak,' either spontaneously occurring or induced by large voltage pulses or brief suction, had to be in the range of 10–20 pA in order to produce changes in $[Ca^{2+}]_i$ of the magnitude shown in Figs 1 and 3. Thus, unless the calcium current through the channel is considerably greater than that through leak under physiological conditions, the current through 50 pS channels activated by agonists is by itself too small to account for the large hyperpolarization-driven changes in $[Ca^{2+}]_i$ that can occur following agonist stimulation. As discussed later, however, activity of the 50 pS channel is reduced by elevated $[Ca^{2+}]_i$, and if $[Ca^{2+}]_i$ is buffered to essentially zero with EGTA, it is possible to observe larger whole-cell currents (e.g. Figs 7, 11 and 12). Thus, it is likely that 50 pS channels can contribute to Ca^{2+} influx at low $[Ca^{2+}]_i$. Unfortunately, this cannot be established without a specific blocker of the other, more prominent influx pathway.

Ins1,4,5P$_3$ does not activate 50 pS channels

In the presence of Ins1,4,5P$_3$ the total current carried by 50 pS channels is much smaller than after agonist stimulation. Indeed, even at high concentrations of Ins1,4,5P$_3$ that produce large hyperpolarization-driven increases in $[Ca^{2+}]_i$, only sporadic openings of individual channels are seen in whole-cell currents (e.g. Fig. 6), rather than the superimposed openings of many channels induced by agonists (e.g. Fig. 2). We found that within a given preparation of mast cells, the activity of 50 pS channels in cells dialysed with Ins1,4,5P$_3$ was the same as that in unstimulated control cells without Ins1,4,5P$_3$. In both cases, only occasional openings could be observed (e.g. Fig. 2*B* before stimulation and inset of Fig. 6), with the frequency of opening increasing slowly with time. Thus, Ins1,4,5P$_3$ did not activate the 50 pS channel. The same was true for Ins1,3,4,5P$_4$, whether applied alone or in combination with Ins1,4,5P$_3$. To determine if the failure to observe activation with Ins1,4,5P$_3$ or Ins1,3,4,5P$_4$ was due to rapid desensitization to inositol phosphates, we applied agonist to cells dialysed with high concentrations of both Ins1,4,5P$_3$ and Ins1,3,4,5P$_4$ (10 μM of each). As shown in Fig. 7, agonist activation of 50 pS channels was unaffected by this treatment; thus, Ins1,4,5P$_3$ and Ins1,3,4,5P$_4$ are not involved in the activation of the channel.

Ins1,4,5P$_3$-induced Ca^{2+} influx is associated with small whole-cell currents

For the reasons described in the previous section, the 50 pS channel cannot be the sole source of Ca^{2+} influx activated by either external agonist or internal Ins1,4,5P$_3$

in mast cells. However, there are no other recordable inward-current channels or large whole-cell currents that can account for the Ca^{2+} influx. Rather, the influx appears to be associated with whole-cell currents of not more than 1–2 pA, with no apparent channel fluctuations. An example of Ins1,4,5P_3-induced Ca^{2+} influx with

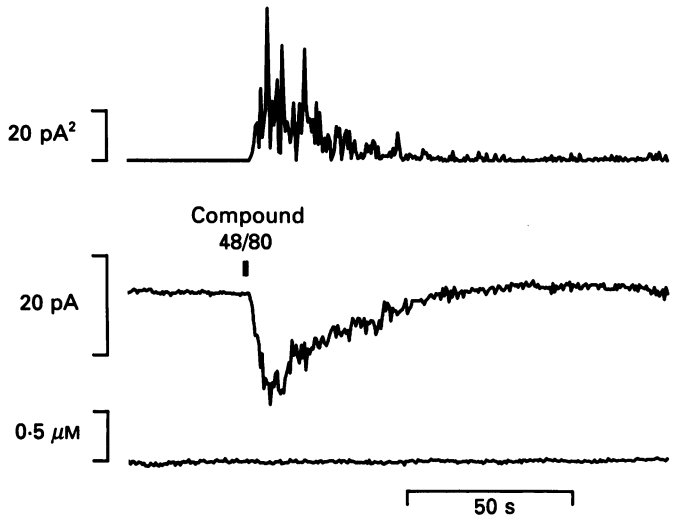

Fig. 7. Lack of effect of internal Ins1,4,5P_3, Ins1,3,4,5P_4 and EGTA on activation of the 50 pS channel by compound 48/80. The pipette solution contained 10 μM-Ins1,4,5P_3, 10 μM-Ins1,3,4,5P_4 and 2 mM-K_2–EGTA to clamp $[Ca^{2+}]_i$ near zero (bottom trace). Application of compound 48/80 at the indicated time activated a large whole-cell membrane current (middle trace), consisting of superimposed activity of many 50 pS channels. The upper trace is the variance of the membrane current, calculated as in Fig. 2. The steady holding potential was −50 mV.

minute inward current is shown in Fig. 8. In this cell, the first hyperpolarizing voltage pulse after breaking into the cell produced an inward current of 3·2 pA and a net increase of 0·26 μM in $[Ca^{2+}]_i$. With succeeding negative pulses, the amplitude of the inward current and the resulting increase in $[Ca^{2+}]_i$ increased slightly, until by the third pulse the current was 3·5 pA and the net change in $[Ca^{2+}]_i$ was 0·32 μM. At 0 mV, which was the holding potential between the alternating positive and negative voltage pulses, there was also a progressively increasing but smaller inward current. When the bath was perfused with solution containing 5 mM-Cd^{2+}, which eliminated the Ca^{2+} influx (also see Fig. 3*C*), the inward current at 0 mV was abolished and the current during the hyperpolarizing pulse was reduced to 0·8 pA. Thus, the total reduction in inward current caused by blocking Ins1,4,5P_3-induced Ca^{2+} influx in this cell was 2·7 pA, and we take this as a reasonable upper limit for the amount of inward current responsible for the Ca^{2+} influx.

In fact, the actual current underlying Ca^{2+} influx was likely to be smaller, because we found that Cd^{2+} also caused a small reduction in current in control cells without internal Ins1,4,5P_3, suggesting that at least part of its action was to reduce the resting cell conductance or the leak between the recording pipette and the membrane. Experiments like that of Fig. 8 were carried out on fifteen cells, which had an average Cd^{2+}-blockable current of 1·12 ± 0·20 pA (mean ± S.E.M.) at −40 mV.

Because the inward current underlying the Ins1,4,5P_3-induced Ca^{2+} influx is 1–2 pA or less, it must be highly specific for Ca^{2+} in order to produce the increases in $[Ca^{2+}]_i$ that we observe. We should point out, however, that calcium-specific currents need not be larger than about 1–2 pA to produce detectable increases in $[Ca^{2+}]_i$ in

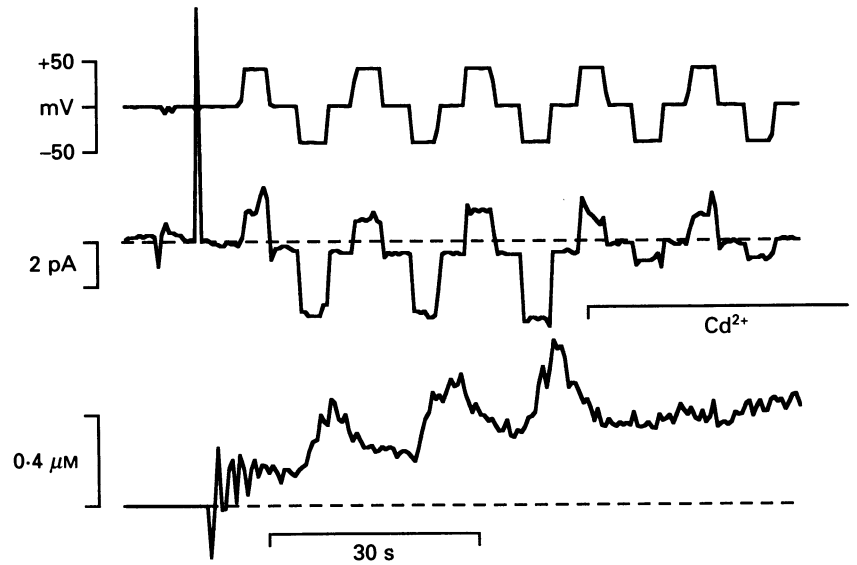

Fig. 8. Effect of cadmium on membrane current and Ca^{2+} influx induced by Ins1,4,5P_3. Top trace: voltage protocol; middle trace: whole-cell membrane current; bottom trace: $[Ca^{2+}]_i$. Dashed lines indicate zero levels. Internal solution contained 10 μM-Ins1,4,5P_3, and the external solution was MCR. Both internal and external solutions contained 10 μM-DIDS to block Cl^- current. The time of breaking into the cell is marked by the large artifact near the beginning of the current trace. At the indicated point, the bath was perfused with MCR containing 5 mM-Cd^{2+}.

mast cells (Neher, 1988); therefore, a small current on the boundary of detectability is feasible. The variance associated with the current was also small; for example, in the cell of Fig. 8, there was no detectable increase in variance during the first three negative voltage pulses, and no change in variance when the current was blocked with cadmium.

To obtain an estimate of the size of single-channel current we would be able to detect by looking at the variance of the membrane current, we analysed the variance increase associated with the agonist-activated chloride current, which can be seen in Fig. 1 and will be discussed in detail in the following paper (Matthews *et al.* 1989). We found that an increase in Cl^- current of 1·6 pA above baseline (which is similar in magnitude to the cadmium-blockable current underlying Ca^{2+} influx) was associated with a readily detectable increase in variance. The single-channel current for the Cl^- conductance is less than 0·1 pA (Matthews *et al.* 1989), and thus the single-channel current underlying Ca^{2+} influx is probably even smaller. This suggests a small single-channel conductance, if indeed the conductance pathway is via channels. The whole-cell currents associated with hyperpolarization-driven Ca^{2+} influx in mast cells are

sufficiently small that other mechanisms are possible, although the simplest way to explain the dependence of influx on hyperpolarization is with calcium channels that allow calcium influx down its electrochemical gradient.

Properties of the 50 pS cation channel

Although the 50 pS channel cannot account for the bulk of hyperpolarization-driven Ca^{2+} influx in mast cells, its properties are such that it should contribute a

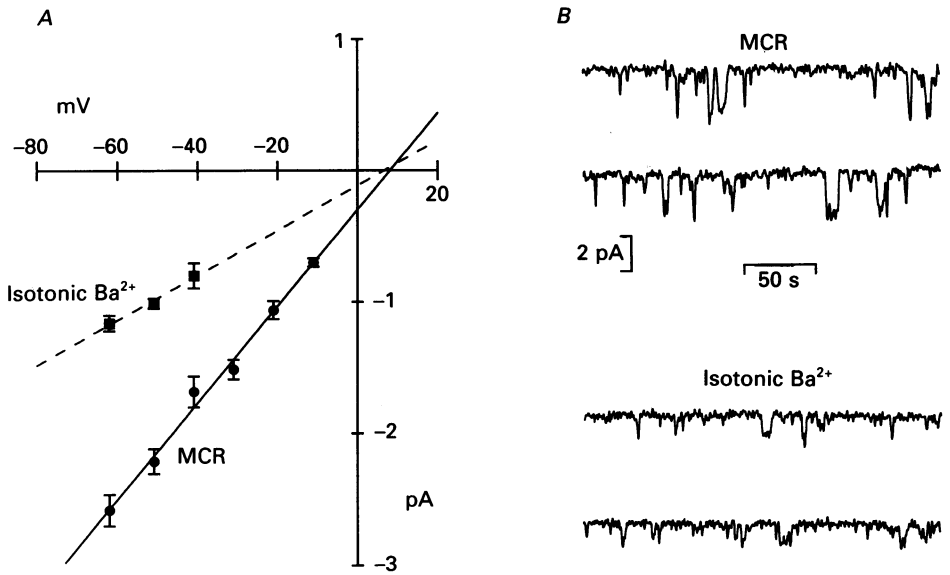

Fig. 9. *A*, relation between single-channel current and membrane voltage for large agonist-activated channels observed in whole-cell recordings of membrane current. Each data point is the mean of five to eleven experiments in MCR (●) or three to five experiments in isotonic Ba^{2+} (■). The vertical lines show ±1 standard deviation. The straight lines were fitted to the data using a least-squares criterion. Isotonic Ba^{2+} contained (in mM): $BaCl_2$, 95; HEPES–NaOH, 10; glucose, 11; pH 7·2. *B*, examples of channel activity in MCR (upper pair of traces) and after switching the bathing solution to isotonic Ba^{2+} (lower pair of traces). Bandwidth, 0–400 Hz; membrane potential, −60 mV.

component of calcium current, particularly after agonist stimulation in cells with very low resting $[Ca^{2+}]_i$. In this section, we will discuss the characteristics of the channel that suggest its contribution to Ca^{2+} influx.

The current–voltage relation for the 50 pS channel in our usual external solution (mast cell Ringer solution) is shown in Fig. 9. The extrapolated single-channel current reversed near 0 mV, which suggests that the channel is non-specific. The slope of the current–voltage relation corresponded to an average single-channel conductance of 37 pS in mast cell Ringer solution, which had an elevated concentration of Mg^{2+} (see Methods). When external divalent ions were removed, the single-channel conductance increased to 60 pS (seven cells). This is similar to the action of external divalent ions on the conductance of other non-specific channels (Haynes, Kay & Yau, 1986; Matthews, 1986, 1987; Mayer & Westbrook, 1987),

where the conductance is typically somewhat lower in the presence of divalent cations. In a saline with more conventional concentrations of Mg^{2+} (2 mM) and Ca^{2+} (1 mM), the single-channel conductance averaged 44 pS (three cells). We will refer to this channel as the 50 pS channel, a conductance that is a round number intermediate between the measured conductances in elevated-Mg^{2+} and in low-divalent Ringer solutions.

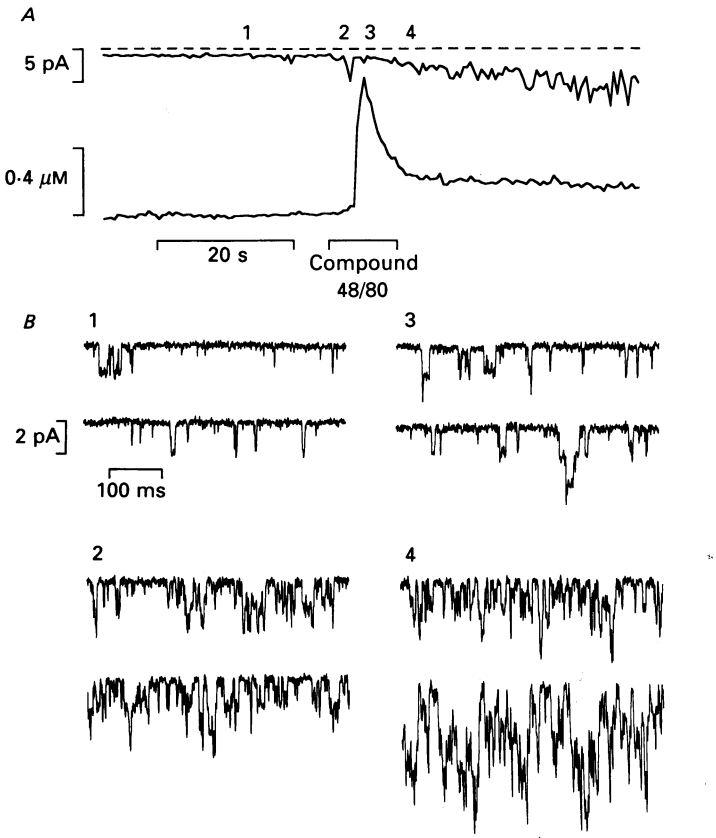

Fig. 10. Inhibition of 50 pS channel activity during Ca^{2+} transient. *A*, an overview of the experiment. The upper trace shows whole-cell membrane current and the lower trace $[Ca^{2+}]_i$ in response to external application of compound 48/80 at the indicated time. *B*, samples of single-channel activity from the times indicated by the corresponding numbers in *A*. Pair 1, activity in the resting state before stimulation. Pair 2, increased activity of 50 pS channels after applying compound 48/80 but before the Ca^{2+} transient. Pair 3, inhibition of activity during the Ca^{2+} transient. Pair 4, after the Ca^{2+} transient subsided, large activation of 50 pS channels was apparent. Holding potential, −40 mV. Bandwith, 0–400 Hz.

To determine the conductivity of the channel to divalent cations, we also measured single-channel conductance in isotonic Ba^{2+} solution, as illustrated in Fig. 9. The conductance was reduced from an average of 37 pS in mast cell Ringer solution to an average of 17 pS when the solution was switched to isotonic Ba^{2+}. Thus, like other non-specific cation channels, the 50 pS channel is capable of carrying appreciable divalent cation current.

Activation of the 50 pS channel

We found that 50 pS channels could be activated by external application of compound 48/80, substance P, or DNP–BSA to which cells were sensitized by incubation with IgE directed against DNP–BSA. Because the channel is activated in

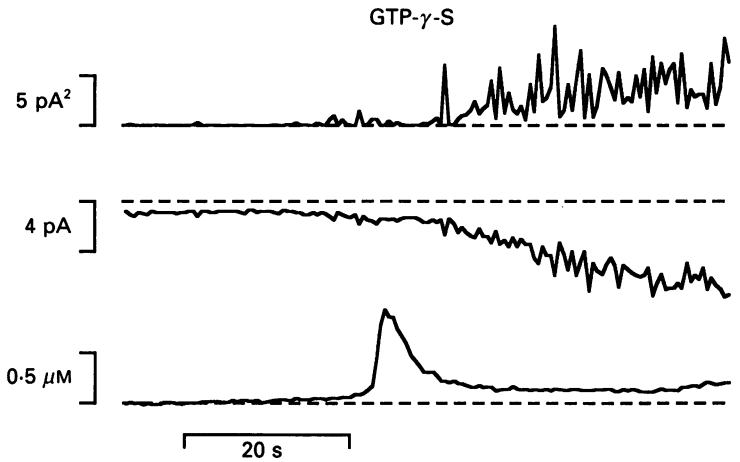

Fig. 11. Internally applied GTP-γ-S (40 μM) activated the 50 pS channel and induced a Ca^{2+} transient. Top trace, variance of the membrane current. Middle trace, whole-cell membrane current. Bottom trace, $[Ca^{2+}]_i$.

a similar fashion by a variety of agonists and because the activation is slow, often reaching a peak several seconds after application of agonist has ceased, we consider it likely that the channel is gated by an internal messenger rather than directly by agonist. However, we have not yet been able to identify that messenger. We showed above (Fig. 7) that Ins1,4,5P_3 and Ins1,3,4,5P_4 are not involved in channel gating. Similarly, cyclic AMP and cyclic GMP do not activate the channel (cyclic AMP, fifty-seven cells; cyclic GMP, sixteen cells) or prevent its activation by agonists (cyclic AMP, six cells; cyclic GMP, eight cells). The channel is not activated by the elevated $[Ca^{2+}]_i$ that accompanies agonist stimulation, because channel activation is not inhibited when $[Ca^{2+}]_i$ is clamped to essentially zero with internal EGTA (Figs 2 and 7).

In fact, elevated $[Ca^{2+}]_i$ seems to reduce a channel activity, as illustrated in Fig. 10. Activity of the 50 pS channel typically began to increase just before the Ca^{2+} transient elicited by application of agonist (trace 1, Fig. 10*B*); however, during the transient, activity ceased, resuming again during the falling phase of the transient (traces 2 and 3, Fig. 10*B*; see also Fig. 11). Activity of the 50 pS channel could also be reduced when $[Ca^{2+}]_i$ was increased with ionomycin, indicating that it is elevated $[Ca^{2+}]_i$ *per se* that inhibits activity, rather than some other event associated with agonist stimulation of the cell.

Although we have not identified a second messenger that can activate the 50 pS channel, we have found that the non-hydrolysable GTP-analogue GTP-γ-S can, in some cells, induce activity of the 50 pS channel that mimics activation by external agonist. An example is shown in Fig. 11. This suggests that a GTP-binding protein

is involved in the linkage between agonist and activation of the channel. Results like that in Fig. 11 were obtained in 21% of the cells that were dialysed with internal solutions containing 40 μM-GTP-γ-S. In the other cells, GTP-γ-S prevented activation of 50 pS channels by compound 48/80, demonstrating that GTP-γ-S had

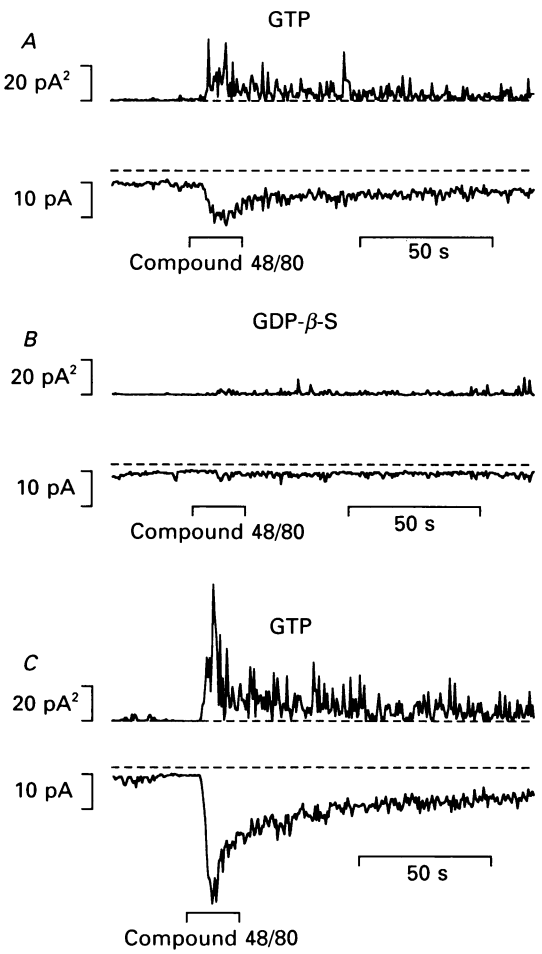

Fig. 12. Inhibition by GDP-β-S of the activation of 50 pS channels by compound 48/80. The pairs of traces show recordings from three consecutive cells from the same group of mast cells with 0·3 mM-GTP (*A*), no GTP and 0·2 mM-GDP-β-S (*B*), and 0·3 mM-GTP again (*C*). The bottom trace of each pair is the membrane current, and the top trace is the variance of the current, as in Fig. 2. Compound 48/80 was applied as indicated. Holding potential, −50 mV. The pipette solution contained 2 mM-K_2–EGTA to maintain $[Ca^{2+}]_i$ at a low level.

interfered with the link between agonist binding and channel activation, without itself inducing channel activity. One possible explanation is that there might also be a GTP-γ-S-sensitive inhibitory step, in analogy to adenylate cyclase. GTP-γ-S might activate both excitatory and inhibitory linkages simultaneously, with the net result depending on the balance achieved in a particular cell.

We further examined the role of GTP in the linkage between external agonists and activation of the 50 pS channel by including the non-hydrolysable GDP-analogue GDP-β-S in the pipette solution. As illustrated in Fig. 12, GDP-β-S reduced the activation of the channel by compound 48/80. Results like that shown were obtained

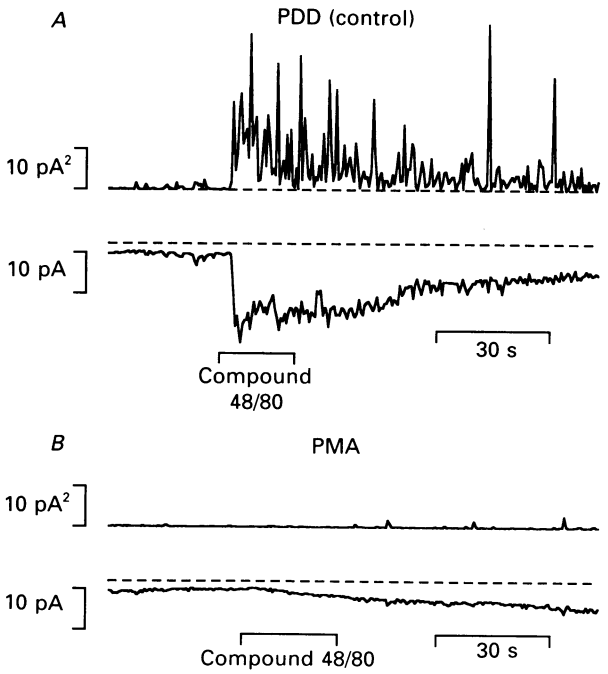

Fig. 13. Effect of phorbol esters on activation of 50 pS channels by compound 48/80. Recordings were from two mast cells in the same preparation. *A*, incubation for 31 min in MCR containing 0·1 μM-PDD (an inactive phorbol ester). Upper trace shows the variance of the membrane current, as in Fig. 2, and the lower trace is the membrane current. Timing of compound 48/80 application was as indicated. Holding potential, -50 mV. Internal solution contained 2 mM-K_2–EGTA. *B*, as in *A*, except that the cell was incubated for 32 min in MCR containing 0·1 μM-PMA.

in three out of four preparations (across preparations, compound 48/80 activated 50 pS channels in twenty out of twenty-two control cells and two out of eight cells with GDP-β-S). This is further indication of the involvement of a G protein in the activation of 50 pS channels by agonists.

Generation of Ca^{2+} transients by agonist stimulation can be prevented in mast cells by activation of protein kinase C with phorbol ester (Penner, 1988). This treatment also prevents the activation of 50 pS channels by compound 48/80, as shown in Fig. 13. Results from two cells in the same preparation, incubated for equal amounts of time in either active phorbol ester (4-β-phorbol 12-myristate, 13-acetate; PMA) or the inactive control phorbol ester (4-α-phorbol 12,13-dideconoate; PDD), are shown in Fig. 13. Activation of 50 pS channels was eliminated by PMA incubation but not by PDD, a result observed in seven out of seven preparations. The effect of PMA

required incubation for 25–30 minutes and may reflect feedback inhibition of polyphosphoinositide break-down.

Agonist-induced Ca^{2+} transients can also be prevented in mast cells by internally applied neomycin (Penner, 1988), which blocks inositol phospholipid turnover

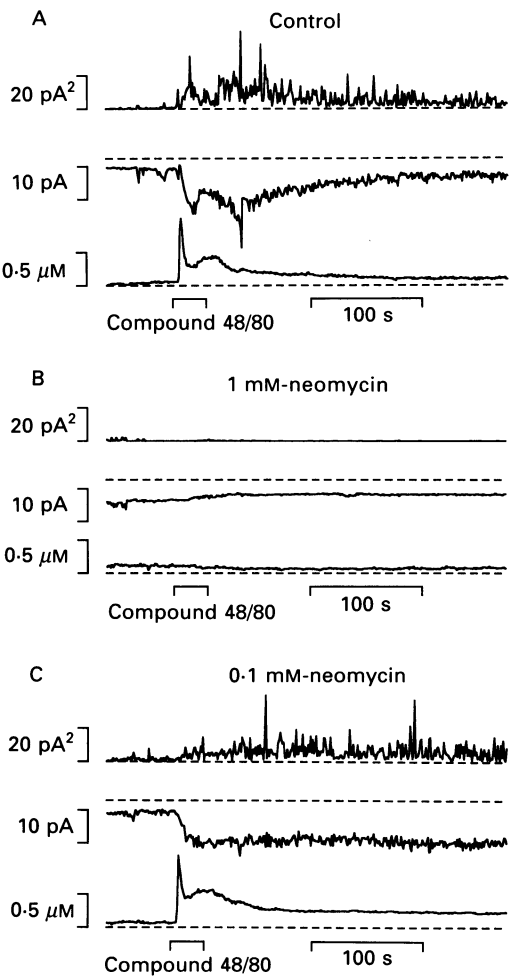

Fig. 14. Effect of internal neomycin on activation of 50 pS channels by compound 48/80. Recordings were from three mast cells in the same dish. In each group of three traces, the top is the variance of the membrane current, the middle is the membrane current, and the bottom is $[Ca^{2+}]_i$. *A*, standard intracellular solution. *B*, standard internal solution with 1 mM-neomycin added. *C*, standard internal solution with 0·1 mM-neomycin. Compound 48/80 was applied as indicated. Holding potential, −50 mV. Dashed lines indicate zero levels.

(Cockcroft, Howell & Gomperts, 1987). Internal neomycin also blocked activation of 50 pS channels by compound 48/80, as shown in Fig. 14, which shows recordings obtained a few minutes apart from three cells in the same mast cell preparation. A high concentration of neomycin (1 mM), which blocked generation of the agonist-induced Ca^{2+} transient, also prevented activation of 50 pS channels, but when the

concentration of neomycin was reduced to the point where the Ca^{2+} transient reappeared, activation of the 50 pS channel was also restored. The results in Figs 13 and 14 suggest some connection between generation of Ca^{2+} transients, and thus inositol phospholipid hydrolysis, and activation of 50 pS channels; however, a role for Ins1,4,5P_3 and Ins1,3,4,5P_4 has already been eliminated. To test for a possible direct action of phorbol esters on the channel, we included PMA in the pipette solution but found no activation of 50 pS channels and no effect on the activation of the channels by compound 48/80.

Variability among mast cells in agonist-induced Ca^{2+} influx and activation of 50 pS channels

The effects of external agonists on membrane currents and Ca^{2+} influx varied considerably among mast cells, and not all cells showed all aspects of the agonist-induced changes in membrane current and $[Ca^{2+}]_i$ described in this paper. Thus, hyperpolarization-driven increases in $[Ca^{2+}]_i$ upon agonist-stimulation were found in 26% of ninety-eight cells. The other 74% of the cells showed only the initial Ca^{2+} transient in response to agonist, without the plateau phase of elevated $[Ca^{2+}]_i$ and its associated hyperpolarization-driven Ca^{2+} influx. We do not know whether the variability in the appearance of agonist-induced Ca^{2+} influx is due to some uncontrolled aspect of our experimental procedures, such as wash-out of an important intracellular factor, or to real physiological variation among mast cells.

Activation of 50 pS channels by agonists was more regularly observed (82% of 168 cells), but the total whole-cell current due to activation of these channels varied considerably, ranging from 0·5 to 40 pA, even when $[Ca^{2+}]_i$ was buffered to essentially zero with EGTA to eliminate possible variation stemming from inhibition of the channel by elevated $[Ca^{2+}]_i$. There was typically less variability in both agonist-induced Ca^{2+} influx and in activation of 50 pS channels among cells of a given preparation than across preparations. Because of this greater uniformity, we took care to make experimental comparisons only within a single preparation of mast cells whenever possible. The effect of internally applied Ins1,4,5P_3 on Ca^{2+} influx was also more reproducible: 10 μM-Ins1,4,5P_3 caused hyperpolarization-driven Ca^{2+} influx in 80% of 103 cells. At a lower concentration of Ins1,4,5P_3 (0·5 μM), hyperpolarization-driven Ca^{2+} influx was observed less frequently (55% of eighty cells), and the delay between breaking into the cell and the first appearance of hyperpolarization-driven increases in $[Ca^{2+}]_i$ was more variable.

DISCUSSION

In agreement with previous electrophysiological studies (Lindau & Fernandez, 1986), we found no indication of voltage-activated ion channels that control Ca^{2+} influx in mast cells. Instead, influx of external calcium occurred via two pathways that are activated by agonist stimulation and regulated via internal messengers. Of the two Ca^{2+} influx pathways, the more potent can be activated by internally applied Ins1,4,5P_3, as well as by external secretagogues, and does not involve large channels or large whole-cell currents. The other pathway, which can be activated by internal GTP-γ-S as well as by external agonists, is via large-conductance cation channels;

however, this pathway by itself does not typically produce sufficient Ca^{2+} influx to cause changes in intracellular calcium concentration, although it may contribute to Ca^{2+} influx, particularly at low levels of $[Ca^{2+}]_i$.

Ins1,4,5P_3-induced pathway

In the presence of internal Ins1,4,5P_3 (0·5–10 μM), membrane hyperpolarization caused increases in $[Ca^{2+}]_i$ due to influx of external Ca^{2+}, and the rate of influx increased with increasing hyperpolarization. A simple explanation of this observation is that Ins1,4,5P_3 opens a calcium conductance in the plasma membrane, through which Ca^{2+} can enter the cell down its electrochemical gradient. However, no channel activity associated with the Ca^{2+} influx could be detected, and large hyperpolarization-driven increases in $[Ca^{2+}]_i$ sometimes occurred in the presence of total inward membrane currents of less than 1–2 pA in the whole cell. The small size of the measured current requires that the conductance be specific for Ca^{2+}, and the absence of fluctuations in the current suggests a small single-channel conductance. If the influx pathway were through channels, these channels could in principle be located in the plasma membrane or possibly constitute a direct connection between extracellular space and intracellular Ca^{2+} storage organelles as suggested by the capacitative model for Ca^{2+} release and uptake (Putney, 1986). In this case the observed Ca^{2+} influx would first enter the Ca^{2+} stores, which could not retain the Ca^{2+} due to the continued presence of Ins1,4,5P_3. In order to explain the voltage dependence of Ca^{2+} influx in this latter mechanism one would have to postulate electrical continuity between plasma membrane and the membrane of the sequestration organelles.

Another alternative, however, is that the Ins1,4,5P_3-gated Ca^{2+} influx occurs via a pump or carrier rather than a Ca^{2+} channel. Again, to account for the voltage sensitivity of the influx, it is necessary to postulate either that Ins1,4,5P_3 activates an inwardly directed Ca^{2+} transporter whose transport rate increases with hyperpolarization or that Ins1,4,5P_3 inhibits an outward Ca^{2+} transporter whose rate similarly depends on hyperpolarization. The latter scheme could arise, for example, if Ins1,4,5P_3 inhibited Na^+–Ca^{2+} exchange. In the cell without Ins1,4,5P_3 increased Ca^{2+} influx through 'leak' during hyperpolarization might be balanced by increased efflux via Na^+–Ca^{2+} exchange so that no increase in $[Ca^{2+}]_i$ is observed at negative membrane potentials. If, in the presence of Ins1,4,5P_3, the efflux is inhibited and the influx unchanged, hyperpolarization-driven increases in $[Ca^{2+}]_i$ like those observed might result. At present, we have no good basis for selecting among the alternative mechanisms of Ins1,4,5P_3-induced Ca^{2+} influx.

The onset of hyperpolarization-driven Ca^{2+} influx induced by Ins1,4,5P_3 was usually delayed following break-in, in some cells by more than 100 s, even with a high concentration of Ins1,4,5P_3 in the pipette and even though Ca^{2+} transients stimulated by Ins1,4,5P_3 occurred rapidly. This slow onset might reflect slow gating of the influx mechanism by Ins1,4,5P_3. Another possibility for the slow activation of Ca^{2+} influx is that some inhibiting factor must wash out of the cell before the activation of the influx mechanism by Ins1,4,5P_3 can occur; in such a scheme, variation among cells in the amount of the putative inhibitor might account for the fact that some cells show rapid appearance of Ins1,4,5P_3-induced Ca^{2+} influx, while

others show pronounced delay. If the Ca^{2+} influx were through the calcium stores (see above), one could explain the slow activation of Ca^{2+} influx by the different rates involved in dumping the intracellular Ca^{2+} pools (determined by the properties of the Ins1,4,5P_3-gated Ca^{2+} release channels) and the slower rate of refilling the stores (determined by the properties of pathways that connect extracellular space with the Ca^{2+} stores).

Alternatively, the delay might be due to the slow build-up of some factor that either itself activates the influx or is a necessary co-factor for the activation by Ins1,4,5P_3. Ins1,3,4,5P_4 is probably not this factor in mast cells, because the addition of Ins1,3,4,5P_4 to pipette solutions containing Ins1,4,5P_3 had no apparent effect. The contention that Ins1,3,4,5P_4 does not play a significant role for Ca^{2+} influx, at least in mast cells, is corroborated by the finding that Ins2,4,5P_3 could activate Ca^{2+} influx. When using this inositol phosphate, which is a poor substrate for the Ins1,4,5P_3-kinase (Irvine & Moor, 1986), there will be no generation of Ins1,3,4,5P_4, suggesting that Ins1,3,4,5P_4 is not required for Ca^{2+} influx. Similar results have been obtained in *Xenopus* oocytes in which Ins1,4,5P_3 and Ins2,4,5P_3, but not Ins1,3,4,5P_4, supported Ca^{2+} influx (Snyder, Krause & Welsh, 1988, but see Irvine & Moor, 1986). In other cell types, such as lacrimal gland cells, Ins1,3,4,5P_4 appears to be of more importance for Ca^{2+} influx (Morris *et al.* 1987).

Although the stimulation of Ca^{2+} influx by Ins1,4,5P_3 was consistently observed across preparations and although virtually all cells stimulated with agonists show Ca^{2+} transients that we take to be diagnostic of intracellular formation of Ins1,4,5P_3, activation of the influx by agonists was found in relatively few cells (26% of all cells). This might arise because of some technical difficulty, such as washing out of a required factor or of endogenously generated Ins1,4,5P_3 itself, or it might represent real physiological diversity among mast cells. Diversity might easily arise, for example, from variation in the amount of the Ins1,4,5P_3-gated influx mechanism present in the cells, from variation in the Ca^{2+} buffering or pumping capacity of the cell, and/or from differences in the amount of Ins1,4,5P_3 released by a stimulus. The fact that with direct administration of Ins1,4,5P_3 a much higher percentage of cells (80% with 10 μM-Ins1,4,5P_3) showed Ca^{2+} influx may be considered as favouring the last of these alternatives. Even with a high concentration of Ins1,4,5P_3, the amount of current underlying Ins1,4,5P_3-induced Ca^{2+} influx (1–2 pA; see above) is close to the threshold amount required to produce an increase in $[Ca^{2+}]_i$ within a few seconds, given the approximate endogenous Ca^{2+}-buffering capacity (Neher, 1988). Thus, a relatively small change in any of the relevant parameters might reduce Ca^{2+} influx sufficiently to prevent an increase in $[Ca^{2+}]_i$.

Variation in the amount of Ca^{2+} influx may have meaning for the phsyiological functioning of the mast cell. The sensitivity of secretion to $[Ca^{2+}]_i$ becomes greater 10–30 s after the cell is stimulated (Neher, 1988). This period of heightened sensitivity comes after the initial Ca^{2+} transient elicited by stimulation has already subsided, and thus the transient is unlikely to be able to accelerate secretion. However, the period of hyperpolarization-driven Ca^{2+} influx coincides with the post-stimulus increase in sensitivity. Therefore, regulation of the amount of influx might be a particularly effective way to modulate the speed of secretion after receipt of a stimulus.

50 pS channel

It seems likely that the 50 pS cation channel is controlled by an internal messenger rather than directly gated by external agonists. The channel activates slowly upon application of agonist and remains active long after agonist is removed, so its activation is not tightly coupled to the presence of agonist in the bath. Also, the channel can be activated by GTP-γ-S (though not in all cells), and the activation by agonists can be blocked by GDP-β-S, signs for the involvement of a GTP-binding protein in the linkage between agonist and the channel. Ins1,4,5P_3, Ins1,3,4,5P_4, cyclic AMP, and cyclic GMP all fail to activate the channel or to prevent its activation by agonists, whereas an increase in $[Ca^{2+}]_i$ inhibits the channel; so none of these potential messengers is evidently involved in the activation of the channel. The inhibition of the channel by incubation with phorbol ester or by intracellular application of neomycin, both of which also block agonist-induced Ca^{2+} transients, suggests that some factor related to inositol phospholipid turnover may be involved in control of the 50 pS channel. However, we do not yet know what messenger gates this channel.

Activation of 50 pS channels could produce whole-cell currents of 10–40 pA when $[Ca^{2+}]_i$ was buffered to low levels with EGTA (e.g. Figs 10, 12, and 13). Because the channel is permeant to divalent cations (Fig. 8), its activation might therefore cause changes in $[Ca^{2+}]_i$ in cells in which the increase in $[Ca^{2+}]_i$ from other sources is small or absent. Without EGTA in the pipette solution, however, the whole-cell current contribution from 50 pS channels rarely exceeded 5 pA at -50 mV, which is not by itself sufficient to produce increases in $[Ca^{2+}]_i$ (see discussion of Fig. 5 in Results).

Dependence of Ca^{2+} influx on membrane hyperpolarization

Influx of external calcium stimulated either by agonists or by internal Ins1,4,5P_3 occurred only at negative membrane potentials (Fig. 5). However, both the 50 pS channel, which has an extrapolated reversal potential near 0 mV (see Fig. 8), and the small current associated with Ins1,4,5P_3-induced Ca^{2+} influx, which was inward at 0 mV (see Fig. 7), would tend to move the membrane potential out of the range where detectable increases in $[Ca^{2+}]_i$ occur. At least under the conditions of our whole-cell recordings, the zero-current potential of an unstimulated mast cell is near 0 mV, so there seem to be no resting conductances that would provide the necessary negative potential to support Ca^{2+} influx. But following stimulation, a large current was usually activated that was outward at 0 mV (e.g. Fig. 1) and had a negative reversal potential. The role of this current may be to establish the negative membrane potential necessary for Ca^{2+} influx to occur in the stimulated mast cell. The properties of this current will be the subject of the following paper.

After submission of this manuscript, a patch-clamp study reported secretagogue-induced currents in rat peritoneal mast cells (Kuno, Okado & Shibata, 1989). The paper demonstrates compound 48/80-induced whole cell currents (cf. our Figs 2, 7 and 13) and the authors suggest these currents to be carried by non-selective cation channels of 28–45 pS conductance and Ca^{2+}-specific channels (measured as barium currents through single channels in the cell-attached recording configuration) of 10 pS conductance. These conductance values are similar to those of the non-specific cation channel reported by us here and in a previous publication (Penner *et al.* 1988). In contrast to our interpretation, Kuno *et al.* discuss these conductance properties as representative of two different channel types, one of which accounts for Ca^{2+} entry. From our data there is no

evidence that the non-specific cation currents and the Ba^{2+} currents are carried by different channels. We infer that they contribute to Ca^{2+} influx but that they are not the main source for the bulk of Ca^{2+} influx in mast cells. Instead there is evidence for a distinct, Ins1,4,5P_3-mediated Ca^{2+} influx pathway which is not associated with single channels of large conductance.

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