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Regulation of calcium influx by second messengers in rat mast cells

Reinhold Penner, Gary Matthews & Erwin Neher

Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen, FRG

Biphasic increases in the free intracellular calcium concentration, consisting of a large initial transient followed by a sustained elevation, are frequently observed in non-excitable cells following stimulation. In rat peritoneal mast cells a cAMP- and Ca-activated chloride current can interact with IP_3-dependent calcium influx to provide the sustained elevation of intracellular Ca concentration following transient IP_3-induced release of calcium from intracellular stores. This novel combination of second messenger systems provides a flexible means to modulate calcium-dependent processes such as exocytosis.

Changes in intracellular calcium concentration are important in the regulation of a variety of cellular functions, including secretion, contraction and hormone actions. Increases in $[Ca]_i$ in excitable cells arise mainly via voltage-activated calcium channels, whereas in non-excitable cells release of intracellular calcium by the second messenger inositol trisphosphate (IP_3) is prominent[1]. In addition to this typically transient increase in $[Ca]_i$, which is independent of extracellular calcium, many non-excitable cells show a sustained phase of elevated $[Ca]_i$ due to influx of extracellular calcium[2,3]. Electrophysiological and pharmacological evidence suggests that this influx is mediated by second messengers rather than by classic voltage-dependent calcium channels. A potential role in mediating calcium influx has been attributed to $[Ca]_i$ (ref. 4) and IP_3 (refs 5-7). Also IP_4 may be involved in calcium influx[8,9], and cAMP can influence calcium influx in non-excitable cells by unknown mechanisms[10-12].

We have performed simultaneous patch-clamp and calcium-indicator dye (fura-2) measurements in rat peritoneal mast cells in order to investigate molecular mechanisms underlying second messenger mediated calcium signals. In these non-excitable cells, previously thought to be devoid of physiologically relevant ion channels[13], we have discovered three ionic mechanisms that are activated following receptor stimulation and that may enhance secretion by maintaining elevated $[Ca]_i$: (1), a voltage-independent cation channel of ~50 pS conductance through which divalents can permeate according to their thermodynamic driving force; (2), a hyperpolarization-driven calcium influx activated both by external stimuli and by intracellularly applied IP_3—if this influx is mediated by ion channels, they must be very calcium-specific because large changes in $[Ca]_i$ occur with whole cell currents of 1-2 pA or less; (3) a chloride current of 0.5-1 pS single-channel conductance, activated following external stimulation and also induced by internally applied cAMP and by high $[Ca]_i$. This current will clamp the membrane potential of an intact cell to negative values, thus providing driving force for calcium influx through mechanisms (1) and (2).

Conductance and $[Ca]_i$ following agonist

An overview of the effects of secretory stimulation on $[Ca]_i$ and on membrane conductance is shown in Fig. 1*a*. Following stimulation of mast cells by secretagogues such as compound 48/80 or substance P, we observed a large, transient increase in $[Ca]_i$ that was independent of external calcium. It was often followed by a plateau of elevated $[Ca]_i$ (0.3-0.8 μM) lasting tens of seconds. This latter sustained phase of elevated $[Ca]_i$ was abolished as external calcium was removed from the bath solution (not illustrated, but see Fig. 4*b*), suggesting that it results from influx of extracellular calcium. Changes in voltage had no influence on $[Ca]_i$ before stimulation, but during the plateau, $[Ca]_i$ increased with hyperpolarization and decreased with depolarization, further indicating that membrane calcium conductance was increased. As will be pointed out below, the pathway for calcium influx is opened by second messengers rather than being voltage-gated. However, the amount of calcium flowing through this pathway may be termed 'potential-dependent' in that the influx depends on the electrical driving force as long as the pathway remains activated. It is therefore not surprising that membrane hyperpolarization promotes calcium influx, whereas less calcium enters upon depolarization, contrary to the case of excitable cells, where depolarization opens voltage-activated calcium channels. The phenomenon that calcium influx following agonist stimulation is reduced by depolarization and increased by hyperpolarization has been recognized in various non-excitable cells[3,14-17].

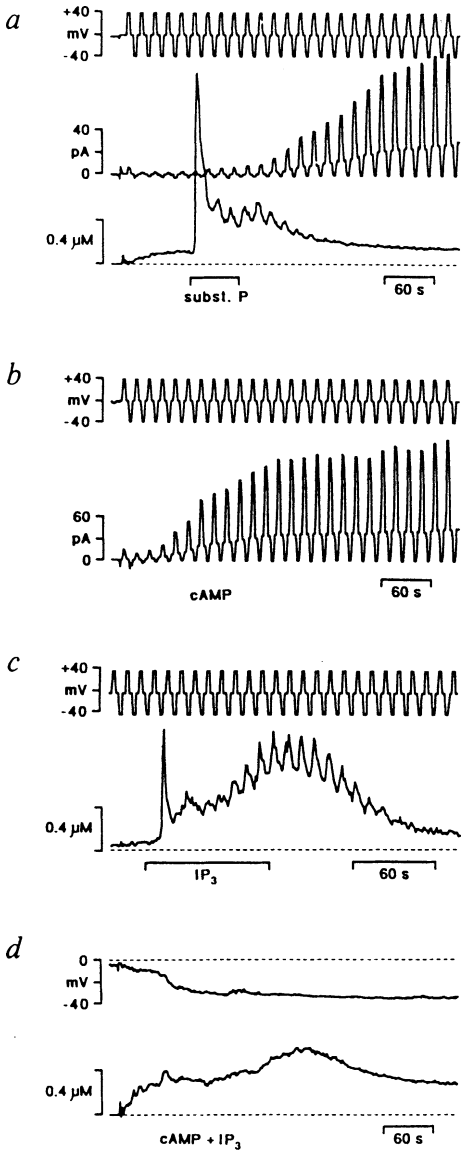
The most prominent conductance change following stimulation was a slowly developing, outwardly rectifying current that was responsible for the large increase in outward current during positive voltage pulses in Fig. l*a*. As will be shown below, this current was carried mainly by chloride ions and will be referred to as the delayed chloride current. Inward currents that might account for the plateau phase of elevated $[Ca]_i$ were much smaller in amplitude and barely visible in Fig. l*a*. However, we did find that secretagogue stimulation increased the activity of 50-pS cation channels through which calcium may permeate (see below), but as discussed later, these channels are likely not the primary source of hyperpolarization-driven calcium influx during the plateau phase. In a few favourable experiments, close inspection of current records revealed small, smooth changes in the DC level on which the above-mentioned channels superimposed. This small inward current may be more closely related to the calcium influx than the large channels. A more detailed description of these currents will follow below.

cAMP and IP_3 mimic agonist-induced responses

We attempted to 'reconstitute' the physiological responses in the absence of agonist-mediated stimulation by introducing second messengers known to play a role in signal transduction in mast cells, notably IP_3 and cAMP. The effects of dialysing a cell with a pipette solution containing 50 μM cAMP are illustrated in Fig. l*b*. Cyclic AMP induced a slowly developing current indistinguishable from the delayed chloride current seen after secretagogue stimulation, suggesting that the delayed chloride current in stimulated cells is induced by increased levels of cAMP. This current is, however, likely to be subject to complex regulation, including modulation by $[Ca]_i$. Cyclic AMP did not cause changes in $[Ca]_i$.

* Permanent address: Department of Neurobiology, SUNY, Stony Brook, New York 11794, USA.

Fig. 1 Changes in current and $[Ca]_i$ in response to agonist stimulation are mimicked by cAMP and IP_3. *a*, Substance P (50 μg ml^{-1}) was applied for the time indicated. The cell was voltage-clamped to different potentials (top trace) and the resulting currents (middle trace) and changes in $[Ca]_i$ (bottom trace) are shown. *b*, The cell was perfused with standard internal solution supplemented with 50 μM cAMP and subjected to the same voltage protocol. *c*, The cell was initially perfused with standard internal solution. For the time indicated, the cell was dialysed with a solution that contained IP_3 (10 μM) using micro-pipette perfusion. *d*, The cell membrane potential was monitored in current clamp while cAMP (50 μM) and IP_3 (0.5 μM) were included in the pipette-filling solution.



Methods. Rat peritoneal mast cells were purified on a percoll gradient as described[38] and suspended in culture medium M199 supplemented with fetal calf serum (10%), $NaHCO_3$ (45 mM), glucose (2.5 mM), streptomycin (0.12 mg ml^{-1}), penicillin (0.64 mg ml^{-1}), pH 7.2. Cells were plated onto cover slips placed inside 35-mm culture dishes and stored in an incubator at 37 °C and 10% CO_2 for 1-6 h until use. Experiments were performed at 23-26 °C in a Mg-rich saline of the following composition (in mM): NaCl 140, KCI 2.5, $CaCl_2$ 2, $MgCl_2$ 5, glucose 11, HEPES·NaOH 10, pH7.2. In some experiments 10 μM DIDS (4,4'-diisothiocyano-2,2'-stilbene-disulphonate) was added to external solutions. Patch-clamp measurements were performed with Sylgard-coated pipettes in the tight-seal whole-cell configuration[39]. Pipette resistance, after filling with the solution given below, ranged between 3 MΩ and 5 MΩ. The solution for filling pipettes (intracellular solution) contained (in mM): K-glutamate 135, NaCl 20, $MgCl_2$ 1, HEPES·NaOH 10, Na_2-ATP 0.2, GTP 0.3, fura-2 pentapotassium salt 0.1 (Molecular Probes). Occasionally DIDS (10 μM) was also included. Secretagogues 48/80 (5 μg ml^{-1}) and substance P (50 μg ml^{-1}) were dissolved in extracellular solution and applied directly onto the cell under investigation via pressure ejection from a second pipette. GTP-γ-S (kindly provided by Dr F. Eckstein, Göttingen) was used at 40-100 μM. Inositol 1,4,5-trisphosphate (IP_3), inositol 1,3,4,5-tetrakisphosphate (IP_4) (both Amersham, UK) and cAMP (Sigma) were used at the concentrations indicated. The concentration of intracellular calcium $[Ca]_i$ was measured by use of the fluorescent indicator dye fura-2. Cells were loaded with fura-2 pentapotassium salt by diffusion from the recording pipette. Fluorescence of fura-2 was excited alternately by light at 360 and 390 nm by means of a rotating filter wheel fitted to a slot in the excitation pathway. $[Ca]_i$ was calculated from the fluorescence ratio[40]. The calibration procedure and details of the measurement are described else-where[18,41]. A total of four signals (two fluorescence intensities; DC current and DC voltage) were fed to the computer and sampled every 0.5 s. In addition, signals were recorded on magnetic tape for later noise analysis and single-channel measurements. Single-channel conductance was calculated from variance and mean current at 2-500 Hz bandwidth assuming low opening probability of channels.

When IP_3 was perfused into the cell (Fig. l*c*), there were one or more pulsatile increases in $[Ca]_i$ that were not synchronized with membrane hyperpolarizations. These likely represent IP_3-induced release of calcium from internal stores[18]. After a delay, transient increases in $[Ca]_i$ occurred during hyperpolarizing voltage pulses, in a manner similar to the plateau phase of $[Ca]_i$ following secretagogue stimulation (Fig. l*a*). These potential-dependent changes in $[Ca]_i$ induced by IP_3 required extracellular calcium (see Fig. 4*b*).

Thus, cAMP mimicked the stimulation-induced delayed chloride current, while IP_3 reproduced the changes in $[Ca]_i$ that follow stimulation. If cAMP and IP_3 indeed cause the changes in membrane properties that occur with secretagogue stimulation, a combination of cAMP and IP_3 in the pipette solution should mimic the physiological events. The experiment shown in Fig. 1*d* was carried out to test this under current clamp, with membrane potential allowed to vary according to the currents flowing. The membrane potential following patch rupture was close to 0 mV and slowly hyperpolarized to -40 mV as cAMP diffused into the cell and activated the delayed chloride current, which reverses at about –40 mV (see next section). At this voltage, the driving force for extracellular calcium was substantial, and after a delay, $[Ca]_i$ increased to produce a plateau reminiscent of the sustained phase of $[Ca]_i$ following agonist stimulation. It therefore appears likely that the two second messengers act in concert to bring about the changes in $[Ca]_i$: IP_3 opens a pathway through which calcium enters the cell and cAMP activates a chloride current, which provides the driving force for calcium entry.

In experiments where second messengers were perfused into the cell we could regularly obtain consistent and reproducible activation of the various mechanisms. Thus, we could induce calcium influx by 5-10 μM IP_3 in 79% of the cells (n = 68) and the delayed chloride current was activated by cAMP in 77% of the cells investigated (n = 96). Responses following agonist stimulation showed some degree of variability across preparations, although being quite uniform among cells of a given preparation. Activation of the cation channels to variable extents was evident in 83% of the cells (n = 111). The hyperpolarization-driven plateau phase of elevated $[Ca]_i$ was seen in 26% of the cells (n = 93), the others showing only the initial calcium transient. This variable occurrence of the plateau phase may reflect true variability in the physiological state of mast cells that is meaningful in determining the responsiveness towards secretagogue stimulation. Alternatively, there may be inadvertent variations in our experimental conditions (for example, washout) that prevent the manifestation of calcium influx in some cells.

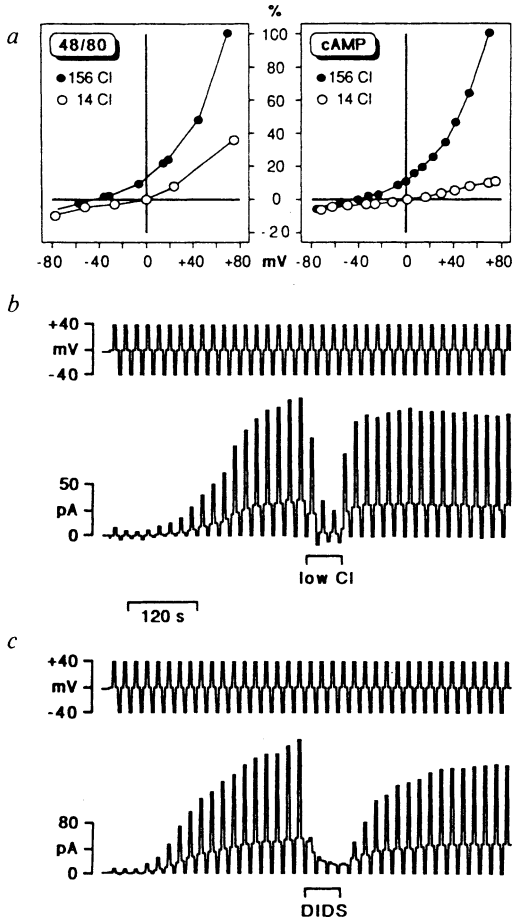


Fig. 2 The properties of the delayed chloride current activated by agonist stimulation and cAMP. *a*, Current-voltage relationships of the delayed chloride current activated by compound 48/80 (5 μg ml^{-1}) and cAMP (50 μM) with standard external saline (•) and low-chlonde saline (◦), where NaCl was replaced by Na-glutamate. Current amplitudes were normalized by setting the current amplitude at +70 mV in normal chloride to 100% in each graph (70 pA, 48/80; 110 pA, cAMP). *b*, The delayed chloride current was maximally activated by internal cAMP (100 μM). At the time indicated, the cell was flushed with low-chloride external saline as in (*a*). *c*, Same as *(b)*, except that the cell was flushed with standard external saline containing 10 μM DIDS. Following removal of DIDS, the current recovered, which contrasts with the

[19]

Delayed chloride current activated by cAMP

A typical current-voltage relationship for the delayed chloride current induced by compound 48/80 is illustrated in Fig. 2*a* (left panel); it shows outward rectification and reversal near -40 mV. As shown in Figs 2*b* and *c*, this current could also be maximally activated by 50- l00 μM internal cAMP in the absence of secretagogue. The current-voltage relation for the cAMP current is shown in Fig. 2*a* (right panel). At +50 mV, the chord conductance of the single channels underlying the delayed chloride current was 0.5-1 pS as determined by noise analysis. When external chloride concentration was reduced from 156 to 14 mM (internal concentration was 11 mM), there was a strong reduction in outward current, and the reversal potential shifted to near 0 mV for both agonist-induced and cAMP-induced currents (Fig. 2*a*). The effects of reducing external chloride on the cAMP-induced current are illustrated in Fig. 2*b*. Voltage pulses to three different potentials elicited either no current (at the usual reversal potential of -40 mV) or large outward currents under conditions of high external chloride concentration. For the time indicated by the bar, low chloride solution was applied to the cell via local perfusion, shifting the reversal potential to about 0 mV and reducing outward current.

Adding 4,4'-diisothiocyano-2,2'-stilbenedisulphonate (DIDS), which has been shown to block chloride ehannels[19], strongly reduced the delayed chloride conductance, as demonstrated in Fig. 2*c*. This experiment was carried out under identical conditions as the one in Fig. 2b, except that the cell was superfused with an external solution containing 10 μM DIDS, instead of low-chloride solution.

While cAMP was the most potent and reliable activator, the delayed chloride current also frequently developed in nonstimulated cells under a variety of experimental conditions, although it was small and greatly delayed. For example, high $[Ca]_i$, in the range 0.6-2 μM, was capable of inducing the current (not illustrated), but the onset lagged markedly behind the rise in $[Ca]_i$ and maximal activation was obtained only after hundreds of seconds. Elevated $[Ca]_i$ may account for the finding that the delayed current also frequently developed in the presence of IP_3, as the IP_3-induced delayed current was almost completely abolished when internal ealeium was buffered to low levels with 2 mM EGTA. Cyclic AMP could fully activate the delayed chloride current even in the presence of 2 mM EGTA (not illustrated), showing that increased $[Ca]_i$ is not necessary for the linkage between cAMP and the chloride conductance.

Antigenic stimulation, which is known to cause a transient increase in cAMP levels[20], induces Ca influx[12]. In the case of 48/80 stimulation, however, cAMP levels either decrease or remain unchanged[21]. Hence, cAMP may play an important role in supporting Ca influx following antigenic stimulation, whereas it may not do so following stimulation with 48/80. It thus appears that another mechanism is responsible for the activation of the delayed chloride current in 48/80-stimulated cells. One may argue that the initial calcium transient induced by IP_3 activates this current. However, the comparatively rapid onset of the chloride current following stimulation is in contrast to the delayed onset of the current when activated by high $[Ca]_i$ alone. This delayed activation by high $[Ca]_i$ indicates that the current may not be directly gated by calcium, but possibly by some, at present unknown, calcium-dependent process. In addition, it seems unlikely that cAMP directly activates the chloride current, since cAMP levels in the cytosol (imposed by the intra-pipette solution) are high long before full activation of the delayed current. This delayed gating may reflect the activation of a process secondary to the increase in cAMP, possibly involving phosphorylation processes mediated by cAMP-dependent protein kinase. It is also conceivable that activation of protein kinase C may enhance the delayed chloride current following agonist stimulation. Chloride channels activated by calcium and cAMP have been described in epithelial cells[22], but their single-channel conductance is larger[23] than that of the channels underlying the delayed chloride current.

The delayed chloride current, which was present in the vast majority of cells, was preceded in about 10% of the cells by another, fast chloride current that was activated directly by $[Ca]_i$ and was particularly prominent during the initial calcium transient following secretagogue stimulation. This chloride current was clearly distinct from the delayed current, since it activated and deactivated extremely fast, closely following the changes in $[Ca]_i$, thus resembling calcium-activated chloride channels in lacrimal gland cells[24] and oocytes[25].

Agonist-activated nonspecific cation channels

We typically observed single channels that opened with increased frequency immediately after application of compound 48/80 or substance P (Fig. 3*a*). Also GTP-γ-S (40-100 μM) was able to induce these channels, indicating them to be G protein- or second-messenger-gated rather than receptor-activated. Perfusing cells with cAMP did not induce the opening of the cation channels. They had a reversal potential near 0 mV, suggesting cations as current carriers, and a linear

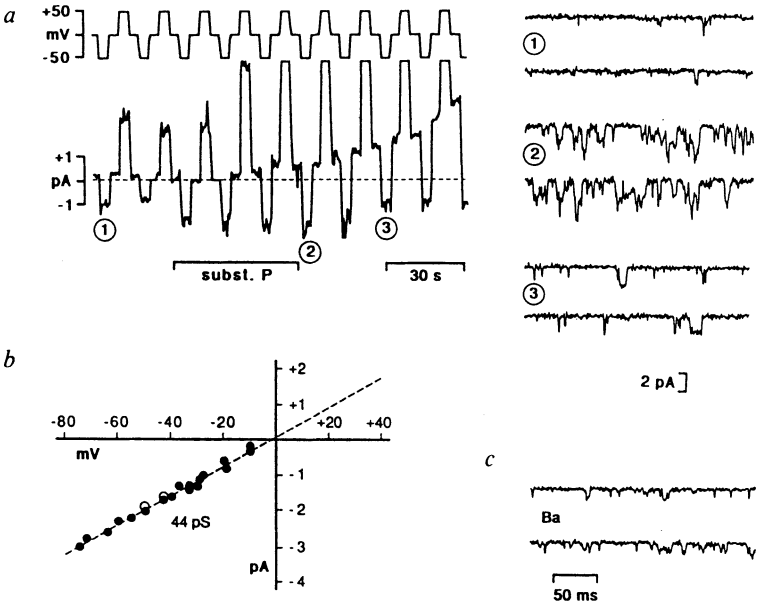


Fig. 3 The characteristics of single-channel events following agonist stimulation. *a*, The cell was stimulated with substance P (50 μg ml^{-1}) for the time indicated and subjected to voltage steps. Note the transient increase in inward current following agonist stimulation. The delayed chloride current, which also activated in this experiment, is truncated at depolarized voltage pulses as it exceeded 5 pA. Representative traces were taken before (1), during (2) and after (3) the inward current change, showing single-channel events detected in the whole-cell mode during hvperpolarizing episodes at -50 mV. *b*, Current voltage relationship for agonist-induced single channels from different cells (48/80-induced (•) and substance P-induced (◦)). Single-channel amplitudes and the chord conductance of 44 pS pertain to standard internal and external solutions. Note that our standard external saline contains 5 mM $MgCI_2$. In a more 'conventional' saline (2 mM $MgC1_2$) we measured 53 pS slope conductance and in a low-divalent saline (0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$) we measured 67 pS. *c*, Single channels as in (*a*), but activated by compound 48/80 and recorded at -60 mV in external saline of the following composition (in mM) $BaCI_2$ 95, HEPES·NaOH 10, glucose 11, pH 7.2.

current-voltage relation between -20 and -60 mV, with a conductance of ~50 pS (Fig. 3*b*). When the standard bath saline was exchanged for isotonic barium solution, channel amplitude was reduced to 16 pS (Fig. 3*c*), suggesting that the channels are permeable to divalent cations. The single-channel current increased when external divalent cations were removed, as with other nonspecific calcium-permeable channels[26,27]. These channels were observed following stimulation when $[Ca]_i$ was chelated to virtually zero (1-2 mM EGTA), so they are not activated by the secretagogue-induced increase in $[Ca]_i$. In fact, the opening frequency of these channels was drastically reduced as $[Ca]_i$ increased above 0.5 μM, similar to IP_3-gated channels in lymphocytes[7].

In view of the apparent similarity to IP_3-activated cation channels in lymphocytes, we tested if the 50-pS channels are activated by IP_3. With a wide range of IP_3 concentrations (0.5-10 μM), however, we found no significant increase in channel activity over unstimulated control cells. Without external stimulation, the frequency of single-channel openings was low and tended to increase slightly with time, both in the presence and absence of IP_3.

Given the properties of the 50-pS cation channels, they should contribute to calcium influx at hyperpolarized voltages, but as their whole-cell currents rarely exceeded 10 pA, they cannot entirely account for the observed changes in $[Ca]_i$. This is because currents through nonspecific channels should behave like leak currents (either spontaneous or experimentally induced), which we find must be in the range of 10-20 pA in order to produce increases in $[Ca]_i$. Also, there was no tight correlation between the activity of the 50-pS channel and changes in $[Ca]_i$. Increases in $[Ca]_i$ were not present in all cells that showed substantial channel activity (currents up to 4 pA). Conversely, we encountered cells in which there was little activity of the 50-pS cation channels, but normal development of hyperpolarization-driven increases in $[Ca]_i$. It is inferred that changes in $[Ca]_i$ may be more closely associated with a small, smooth inward current that can be discerned under favourable circumstances (see next section).

Calcium influx induced by IP_3

The agonist-induced changes in $[Ca]_i$ could be mimicked by IP_3 applied in the pipette solution (Figs 1*c* and 4*a*). Whereas the mobilization of calcium from internal stores was extremely fast (at concentrations larger than 1 μM), such that quite often only the trailing edge of the calcium transient was seen, the voltage-dependent changes in $[Ca]_i$ developed rather slowly (Fig. 4*a*) and were abolished upon removal of extracellular calcium (Fig. 4*b*). The slow onset of voltage-dependent changes in $[Ca]_i$ may be due to slow gating kinetics of the calcium influx mechanism or to build-up of a required co-factor. IP_4 has been suggested to induce calcium influx by itself or synergistically with IP_3[8,9]. We cannot rule out a possible role for IP_4 in supporting IP_3-induced calcium influx, since it may be generated from IP_3 by the IP_3-kinase. However, perfusing cells with 10 μM IP_4 did not cause IP_3-like changes in $[Ca]_i$ (n = 9) and application of IP_4 together with IP_3 was not noticeably different from application of IP_3 alone. That is, the response pattern of a low concentration of IP_3 (0.5 μM), which caused hyperpolarization-driven increases of $[Ca]_i$ with variable delays in about 50% of the cells, was not enhanced in any way by the additional presence of a high concentration of IP_4 (10 μM) in 10 paired experiments.

We rarely observed clear and unequivocal inward currents following application of IP_3. This may not speak against IP_3-activated currents, because the amplitude of a calcium-specific current necessary for an effect on $[Ca]_i$ is in the range of only 1-2 pA (ref. 18), as compared to 10-20 pA for a non-specific current. Often, the delayed chloride current, activity of the large cation channel, or an initial or developing leak between the pipette and the plasma membrane prevented detection of such small IP_3-induced currents. On favourable occasions, however, where chloride currents were blocked by DIDS and the leak current was small and unaltered after the effects of IP_3 had subsided, we observed a small increase in inward current correlated with the phase of voltage-dependent calcium entry (see bar graph in Fig. 4*a*). In the cell of Fig. 4*a*, there was no correlation between $[Ca]_i$ and the frequency of opening of the 50-pS cation channels (previous section) that were riding on top of the IP_3-induced current. Representative traces that show single-channel activity before, during and after the phase of voltage-dependent phase of calcium entry are provided in Fig. 4*a*. Thus, the calcium influx seemed related to a 1-2 pA, smooth inward current not associated with visible channel events, rather than to the activity of 50-pS channels.

Functional significance for secretion

Stimulation of rat peritoneal mast cells by secretagogues initiate complex mechanisms for the regulation of $[Ca]_i$. Our results suggest that substance P and compound 48/80 stimulate Pl breakdown, liberating IP_3, which releases internal calcium and produces an initial transient increase in $[Ca]_i$. Possibly in conjunction with another co-factor, IP_3 also activates a pathway for external calcium entry that does not produce large inward currents. In addition, calcium may permeate through

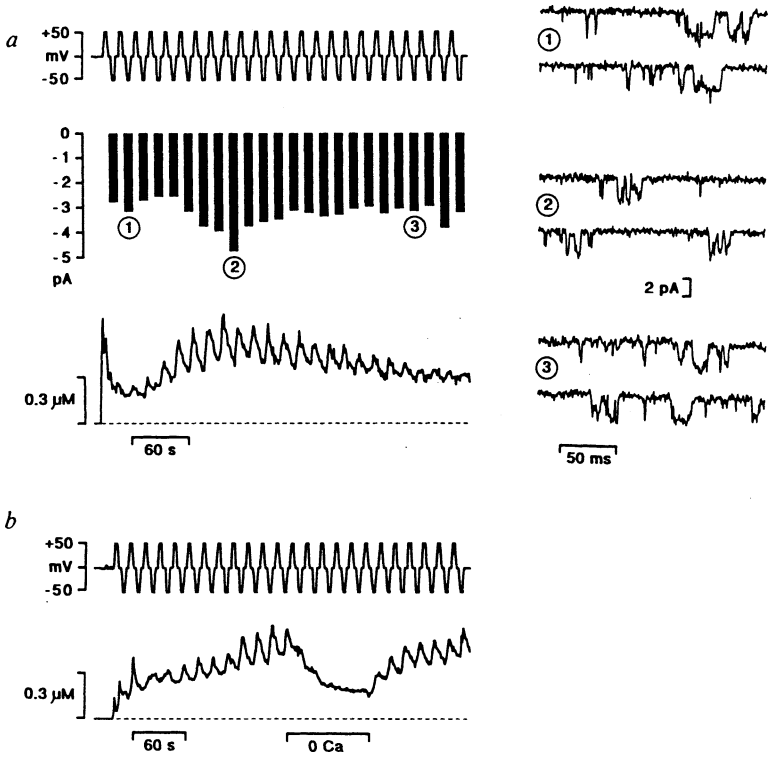


Fig. 4 Effects of IP_3 on inward current and single-channel activity. *a*, The cell was perfused with standard internal solution containing 4 μM IP_3 and voltage-clamped to three different potentials. Inward current for hyperpolarizing voltage pulses are illustrated as a bar graph. Note the temporal correlation between inward current amplitude and the resulting voltage-dependent changes in $[Ca]_i$. In addition, representative traces showing single-channel events before (1), during (2) and after (3) the voltage-dependent plateau phase of $[Ca]_i$ were taken during hyperpolarizing episodes. In this experiment, 10 μM DIDS was present in internal and external solutions to reduce the contribution of the delayed chloride current. *b*, Experimental protocol as in (*a*), but with 0.5 μM IP_3 and no external or internal DIDS. At the time indicated, the standard bath solution was replaced by nominally calcium-free saline to illustrate the dependence of IP -induced changes in [Ca] on external calcium.

nonspecific cation channels that are activated by an as yet unidentified mechanism. These influx pathways represent the mechanism for the sustained phase of $[Ca]_i$.

In current-clamp experiments, we observed membrane hyperpolarization to about -40 mV following activation of the chloride current, with a concomitant rise in $[Ca]_i$. This suggests that activation of the chloride current in intact cells will increase calcium influx by providing electrical driving force. In the absence of chloride conductance, the membrane potential after secretagogue stimulation would approach the reversal potential for the agonist-activated conductances, that is positive values for a calcium-specific mechanism or values close to 0 mV for a non-selective cation conductance. But the large delayed chloride current, the dominant conductance in stimulated cells, will bring the membrane potential near the chloride equilibrium potential, E_{Cl}. We do not know the exact value of E_{Cl} in the intact cell. In other non-excitable cells, however, where E_{Cl} can be measured more accurately, it is -30 to -40 mV (refs 28, 29), which is the range of potentials in which we observed hyperpolarization-driven calcium increases. Thus, activation of the chloride current would likely clamp the potential sufficiently negative to support calcium influx.

The role of cAMP in mast-cell secretion has been the subject of much research but remains a matter of controversy and confusion. We suggest a stimulatory role for cAMP by virtue of providing electrical driving force for calcium influx. Most pharmacological evidence, however, points towards an inhibitory action, since drugs that are known to elevate cAMP suppress secretion[30]. Patch-clamp experiments using capacitance measurement in fact confirm such an inhibitory mechanism of cAMP on secretion at fixed membrane voltage and $[Ca]_i$ (unpublished observations). The discrepancies may be resolved by differential effects of cAMP on the mechanisms that govern secretion and on calcium influx. The balance between inhibitory actions on secretion and facilitatory effects on calcium influx may determine the resulting secretory response and may explain some of the controversial reports on the role of cAMP in mast cell secretion. In one study, however, the inhibitory actions of cAMP on secretion have been attributed to its ability to suppress antigen-induced calcium influx, based on tracer-flux studies in the presence of dibutyryl-cAMP and forskolint[12]. At present we have no convincing explanation for this discrepancy.

We have identified two conductance pathways for calcium entry, one of which can be mimicked by IP_3. Single channels induced by IP_3 have long been searched for, but so far only one study has reported the presence of calcium-specific single channels activated by micromolar concentrations of IP_3 in excised membrane patches[7]. We have been unable to find single-channel events induced by IP_3. Instead, we have found a very small current associated with IP_3-induced calcium influx. This current could not be resolved into single-channel events, indicating a small single-channel conductance or a different mechanism of Ca entry[31]. Small calcium-specific channels have been observed in reconstituted membranes from mast cells and lymphocytes[32,33].

In addition, we found increased activity of 50-pS cation channels following stimulation with secretagogues, but these channels were not activated by IP_3. It is likely that a second messenger is involved in the gating of these channels, because they are also activated by GTP-γ-S and do not show the brisk onset and decay of activity with stimulation that is typical for directly ligand-activated channels. Alternatively, these channels may be directly gated by G proteins. Their activation even at low intracellular calcium (1-2 mM EGTA) and the inability of IP_3 to activate them distinguishes these channels from previously described second-messenger-gated cation conductances in neutrophils[4], where $[Ca]_i$ activates channels, and in lymphocytes[7], where IP_3 has been suggested to open plasma membrane channels.

Taken together, it is clear that in addition to the mobilization of calcium from internal stores, there is a prominent calcium influx in mast cells following stimulation. Optimal conditions for influx are provided by interactive and synergistic actions of the second messengers IP_3 and cAMP. Although a rise of $[Ca]_i$ into the range of that observed under physiological conditions is not sufficient by itself to induce secretion in mast cells[34,35],

there is general agreement that increased $[Ca]_i$ can dramatically enhance secretion in the presence of an additional signal[36,37]. In particular, calcium sensitivity of the secretory mechanism increases some 20-40 s after a stimulust[18]. Unlike the fast calcium transient, which occurs too early to contribute significantly to secretion, the plateau phase caused by calcium influx is ideally timed to influence the secretory response during this period of heightened sensitivity.

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