# Sequential activation of different $Ca^{2+}$ entry pathways upon cholinergic stimulation in mouse pancreatic acinar cells

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1. We have studied capacitative calcium entry (CCE) under different experimental conditions in fura-2-loaded mouse pancreatic acinar cells by digital microscopic fluorimetry. CCE was investigated during $[Ca^{2+}]_i$ decay after cell stimulation with a supramaximal concentration of ACh (10 $\mu$M) or during $Ca^{2+}$ readmission in $Ca^{2+}$-depleted cells (pretreated with thapsigargin or ACh).

2. $La^{3+}$ and $Zn^{2+}$ (100 $\mu$M) inhibited CCE during $Ca^{2+}$ readmission but had negligible effects during ACh decay. In contrast flufenamic acid (100 $\mu$M), an inhibitor of non-selective cation channels, genistein (10 $\mu$M), a broad-range tyrosine kinase inhibitor, and piceatannol (10 $\mu$M), an inhibitor specific for non-receptor Syk tyrosine kinase, inhibited CCE during ACh decay but not during $Ca^{2+}$ reintroduction.

3. Simultaneous detection of $Mn^{2+}$ entry and $[Ca^{2+}]_i$ measurement showed that, in the presence of extracellular calcium, application of 100 $\mu$M $Mn^{2+}$ during ACh decay resulted in manganese influx without alteration of calcium influx, whilst when applied during $Ca^{2+}$ readmission, $Mn^{2+}$ entry was significantly smaller and induced a clear inhibition of CCE.

4. Application of the specific protein kinase C inhibitor GF109293X (3 $\mu$M) reduced CCE in $Ca^{2+}$-depleted cells, whereas the activator phorbol 12-myristate, 13-acetate (3 $\mu$M) increased $Ca^{2+}$ entry.

5. Based on these results we propose that cholinergic stimulation of mouse pancreatic acinar cells induces $Ca^{2+}$ influx with an initial phase operated by a non-specific cation channel, sensitive to flufenamic acid and tyrosine kinase inhibitors but insensitive to lanthanum and divalent cations, followed by a moderately $Ca^{2+}$-selective conductance inhibited by lanthanum and divalent cations.

Cytosolic calcium concentration ($[Ca^{2+}]_i$) is a key regulatory factor for a large number of cellular proccesses such as secretion, contraction, metabolism or even gene expression and apoptosis. Many hormones and neurotransmitters increase $[Ca^{2+}]_i$ via activation of phospholipase C, which results in generation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ releases calcium from intracellular pools by binding to specific receptors, and diacylglycerol activates several isoforms of protein kinase C (PKC), an enzyme involved in the control of numerous cellular functions.

However, to maintain $Ca^{2+}$ signals and refill intracellular pools, agonists also activate entry of extracellular calcium following its electrochemical gradient through plasma membrane channels. In non-excitable cells, and also in excitable tissues, the main mechanism for calcium influx is depletion of intracellular $Ca^{2+}$ stores, which opens plasma membrane $Ca^{2+}$ channels using an unknown signal. This mechanism has been termed capacitative calcium entry (CCE) by Putney, and although a number of theories have been proposed, there is no convincing explanation (Clapham, 1995), in spite of its evident physiological and clinical relevance.

The identity of the plasma membrane $Ca^{2+}$ channel involved in CCE is also controversial. Electrophysiological studies have described in mast cells an inward $Ca^{2+}$ release-activated $Ca^{2+}$ current ($I_{CRAC}$) (Hoth & Penner, 1992), highly $Ca^{2+}$ selective and sensitive to inhibition by $La^{3+}$ and divalent cations (Hoth & Penner, 1993). $I_{CRAC}$ shows both fast and slow negative feedback due to inhibition by $Ca^{2+}$ and PKC (Parekh & Penner, 1995; Zweifach & Lewis, 1995). However, different $Ca^{2+}$ entry currents have been subsequently described, with the selectivity for calcium varying from moderate to high, depending on cell type (Clapham, 1995).

In pancreatic acinar cells there are conflicting reports regarding the possible existence of $I_{CRAC}$ channels. Although a moderately $Ca^{2+}$-selective $I_{CRAC}$ has been described in rat

pancreas (Bahnson *et al.* 1993), a recent paper describes in mouse acinar cells a $La^{3+}$-insensitive non-selective cation channel as the main route for CCE (Krause *et al.* 1996). This current, termed $I_{NCRAC}$ (for non-selective calcium release-activated current), can be inhibited by the tyrosine kinase inhibitor genistein, and is different from the non-selective cation channel activated by cytosolic calcium previously described in rodent exocrine pancreas (Pfeiffer *et al.* 1995).

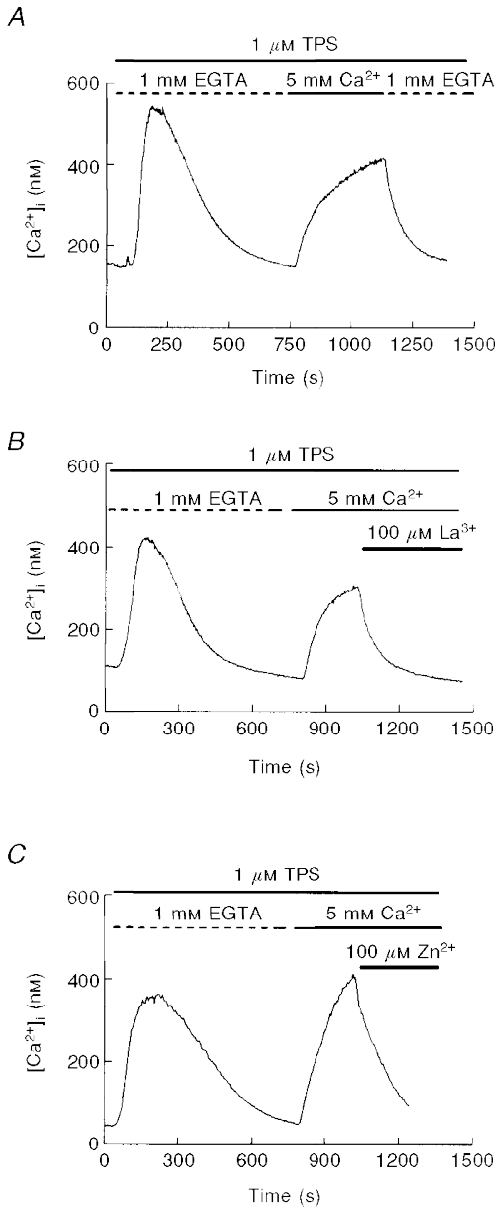


**Figure 1. Capacitative calcium entry in pancreatic acinar cells is inhibited by lanthanum and zinc**

*A*, capacitative calcium entry in mouse pancreatic acinar cells. After depletion of intracellular $Ca^{2+}$ pools with thapsigargin (TPS; 1 $\mu$M) in a $Ca^{2+}$-free solution, application of 5 mM extracellular calcium results in a $[Ca^{2+}]_i$ plateau due to $Ca^{2+}$ influx. *B*, effect of 100 $\mu$M $LaCl_3$ on $Ca^{2+}$ influx. Lanthanum was added to the $Ca^{2+}$-containing solution. *C*, effect of 100 $\mu$M $ZnCl_2$ during thapsigargin-induced $Ca^{2+}$ entry. All the traces are representative of at least 4 (*C*) or 6 (*A*, *B*) independent experiments.

The aim of our study was to characterize the route involved in $Ca^{2+}$ influx in mouse pancreatic acinar cells. Our data indicate that, upon $Ca^{2+}$ mobilization, there is a sequential activation of at least two different $Ca^{2+}$ entry pathways or alternatively a single channel with two different states: an initial non-specific conductance, sensitive to flufenamic acid and genistein and scarcely sensitive to divalent cations and $La^{3+}$, and a late conductance moderately specific for $Ca^{2+}$ and inhibited by lanthanum and manganese, similar to the previously described $I_{CRAC}$. In addition, we present evidence that PKC is a putative modulator of this $Ca^{2+}$ influx pathway.

Some of these data were presented to the Physiological Society Meeting in Liverpool (Camello *et al.* 1998).

## METHODS

### Preparation of acinar cells

A suspension of single cells and small acini was prepared from mouse pancreas, after dislocation of the neck, by enzymatic dispersion as previously described (González *et al.* 1997). Briefly, the pancreas was injected with a small volume (1 ml) of collagenase solution (Worthington, 200 U $ml^{-1}$) and incubated at 37 °C under gentle agitation for 6–12 min. Finally, the cells were released by vigorous manual agitation. Throughout the preparation procedure, as well as during the loading and perfusion, we used a physiological solution containing (mM): 140 NaCl, 4·7 KCl, 2 $CaCl_2$, 1·1 $MgCl_2$, 10 glucose, 10 Hepes and 0·01% trypsin inhibitor (soybean); pH 7·4. $Ca^{2+}$-free solutions had a similar composition but $Ca^{2+}$ was omitted and 1 mM EGTA was added.

### Cell loading and $[Ca^{2+}]_i$ determination

After isolation the cells were suspended in physiological solution and loaded with the fluorescent ratiometric calcium indicator fura-2 AM (1–2 $\mu$M, 30 min, room temperature, 20–25 °C). Once loaded, the cells were washed and used within 2–4 h.

For experiments, a small volume of cell suspension was placed on a thin glass coverslip attached to a Perspex perfusion chamber. Perfusion (approximately 1 ml $min^{-1}$) at room temperature was started after a 2 min period to allow spontaneous attachment of the cells to the coverslip. No coating treatment was necessary to immobilize the cells. The chamber was placed on the stage of an inverted fluorescence-equipped microscope (Nikon Diaphot). Cells were excited at 340 and 380 nm by a computer-controlled filter wheel, and the emitted images were captured by a cooled digital CCD camera (C-6790, Hamamatsu Photonics) and recorded using dedicated software (Argus-HisCa, Hamamatsu Photonics). After the calculation of the 340 nm/380 nm ratio pixel by pixel, the intracellular free calcium concentration ($[Ca^{2+}]_i$) was determined using standard methods (Grynkiewicz *et al.* 1985). The calibration parameters $R_{max}$ (3·2), $R_{min}$ (0·15) and $S_f/S_b$ (4) were determined *in vivo* using 10 $\mu$M ionomycin in $Ca^{2+}$-free and 10 mM $Ca^{2+}$ solutions. We used a $K_d$ value of 250 nM for fura-2 (Grynkiewicz *et al.* 1985).

### Determination of manganese entry

To study $Mn^{2+}$ influx and the effects of this cation on $Ca^{2+}$ entry, we used pulses of 100 $\mu$M $MnCl_2$ added to the normal $Ca^{2+}$-containing physiological solution. In these experiments we examined the fluorescence emitted by fura-2 under 340 and 380 nm excitation wavelengths ($F_{340}$ and $F_{380}$). $Mn^{2+}$ entry was assessed following the procedure described by Alonso-Torre *et al.* (1993) and modified by Shuttleworth (1995). Briefly, $F_{340}$ and $F_{380}$ are added after factorization in such a way that total corrected fluorescence

($F_{tot} = (F_{340} + F_{380}) \times \delta$) is independent of $[Ca^{2+}]_i$, but decreases when fura-2 is quenched by manganese. The correction factor $\delta$ was calculated separately for each individual cell by observing the effect of the initial ACh-induced large $[Ca^{2+}]_i$ peak applied at the beginning of these experiments (see Fig. 9), in the absence of $Mn^{2+}$. In our experimental conditions, $\delta$ is close to the resting fura-2 ratio divided by the peak evoked by ACh. This allows simultaneous determination of ratiometric $[Ca^{2+}]_i$ and $Mn^{2+}$ entry (Alonso-Torre *et al.* 1993; Suttleworth, 1995). To estimate the rate of $Mn^{2+}$ entry, we calculated the decline in $F_{tot}$ in response to $Mn^{2+}$ quench as the fold increase of the slope with respect to the immediately preceding 60 s (Shuttleworth, 1995).

### Materials

Chemicals were purchased from Sigma (Spain), except collagenase CLSPA, which was obtained from Worthington Biochemical Corporation (USA), fura-2, which was from Molecular Probes Europe (The Netherlands), and GF109293X and PP-1, which were from Calbiochem (USA).

## RESULTS

To study CCE in fura-2-loaded mouse pancreatic acinar cells we have used commonly accepted protocols based on depletion of intracellular $Ca^{2+}$ stores. Perfusion of pancreatic acinar cells with a $Ca^{2+}$-free medium containing thapsigargin (1 $\mu$M), a specific inhibitor of the $Ca^{2+}$ pump of internal stores (Thastrup *et al.* 1989), resulted in a transient $[Ca^{2+}]_i$ increase due to release of $Ca^{2+}$ from intracellular pools. Subsequent treatment with a $Ca^{2+}$-containing solution induced a sustained $[Ca^{2+}]_i$ increase indicative of CCE, as shown by its dependence on the presence of extracellular $Ca^{2+}$ (Fig. 1*A*).

Since inhibition by divalent cations and $La^{3+}$ is a key figure in the original description of the store-operated $Ca^{2+}$ channel $I_{CRAC}$ (Hoth & Penner, 1993) we tested the effects of $La^{3+}$ and $Zn^{2+}$. As shown in Fig. 1*B*, CCE evoked by thapsigargin was clearly inhibited by $La^{3+}$ in all the cells tested. This inhibition was also present when CCE was achieved using a physiological extracellular $Ca^{2+}$ level (2 mM; data not shown) instead of 5 mM. The inhibition was fast and dose dependent (not shown), and was reproduced by $Zn^{2+}$ (Fig. 1*C*).

Another tool to inhibit CCE is flufenamic acid, an inhibitor of non-selective cation channels (Schumann *et al.* 1994; Weiser & Wienrich, 1996), which is a potential secondary route for $Ca^{2+}$ in rodent pancreatic acinar cells given the

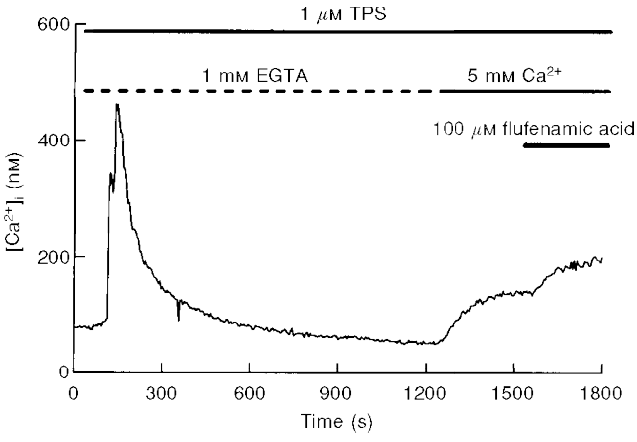


**Figure 2. Effect of flufenamic acid on thapsigargin-evoked CCE**

Once $Ca^{2+}$ influx was established in $Ca^{2+}$-depleted cells, application of flufenamic acid (100 $\mu$M) did not reduce the entry of extracellular calcium. The trace is representative of 4 experiments.

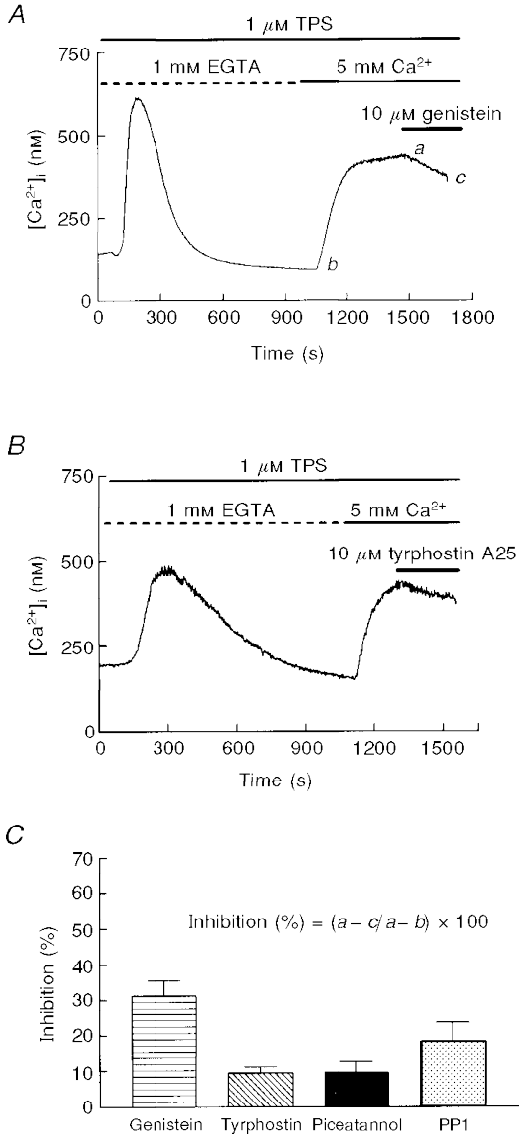


**Figure 3. Effect of application of genistein or tyrphostin A25 during CCE in mouse pancreatic acinar cells**

Genistein (*A*; 10 $\mu$M) and tyrphostin A25 (*B*; 10 $\mu$M), were applied during the plateau phase of $Ca^{2+}$ entry. Data are typical of 5 (*A*) or 3 (*B*) separate experiments. *C*, the effects of different tyrosine kinase inhibitors following the protocol shown in *A* and *B*. Percentage inhibition is calculated by substracting the $[Ca^{2+}]_i$ decrease evoked by the inhibitor ($a - c$ in *A*) from the previous $[Ca^{2+}]_i$ increase induced by $Ca^{2+}$ readmission ($a - b$ in *A*). Data (means ± S.E.M.) are from 4 separate experiments, except for genistein (5 experiments).

existence of non-selective cation channels in these cells (Thorn & Petersen, 1992). As can be observed in Fig. 2, flufenamic acid did not reduce entry of extracellular calcium after depletion of intracellular $Ca^{2+}$ pools, indicating that the non-selective cation channel of rodent pancreas is not the main entry pathway involved in CCE in $Ca^{2+}$-depleted cells. Moreover, the sustained $[Ca^{2+}]_i$ during calcium entry was enhanced in the presence of flufenamic acid, suggesting that a non-specific cation channel reduces the electrochemical gradient for calcium entry.

It has previously been reported that, in pancreatic acinar cells, tyrosine kinase inhibitors such as genistein and tyrphostins acutely block CCE (Yule *et al.* 1994; Pfeiffer *et al.* 1995). To test this possibility in thapsigargin-treated cells we used 10 $\mu$M genistein (Fig. 3*A*), tyrphostin A25 (Fig. 3*B*), another broad-range tyrosine kinase inhibitor, and PP-1 and piceatannol, inhibitors specific for several non-receptor tyrosine kinases (Oliver *et al.* 1994; Hanke *et al.* 1996), during the $Ca^{2+}$ entry-induced plateau (Fig. 3) or several minutes before readmission of extracellular $Ca^{2+}$ (Fig. 4). As shown in Figs 3 and 4, in the two experimental conditions the effect was residual or not significant. The only exception was the acute application of genistein, which induced a 30% inhibition of CCE; however, this result is not consistent with the lack of effect in the case of pretreatment, a condition in which the effect was expected to be larger (Figs 3*C* and 4*B*).

To check a possible involvement of PKC in CCE we used the exogenous activator of PKC phorbol 12-myristate, 13-acetate (PMA) and the specific inhibitor, GF109293X (Toullec *et al.* 1991). After depletion of internal pools with thapsigargin, pancreatic acinar cells were perifused with either PMA or GF109293X (3 $\mu$M) 5 min before initiation of CCE (Fig. 5). Calcium influx was significantly enhanced in PMA-treated cells, while GF109293X induced a significant impairment of calcium entry. Moreover, a similar finding was attained when PMA or GF109293X was applied during the sustained plateau of CCE (data not shown), showing that PKC can promote $Ca^{2+}$ influx.

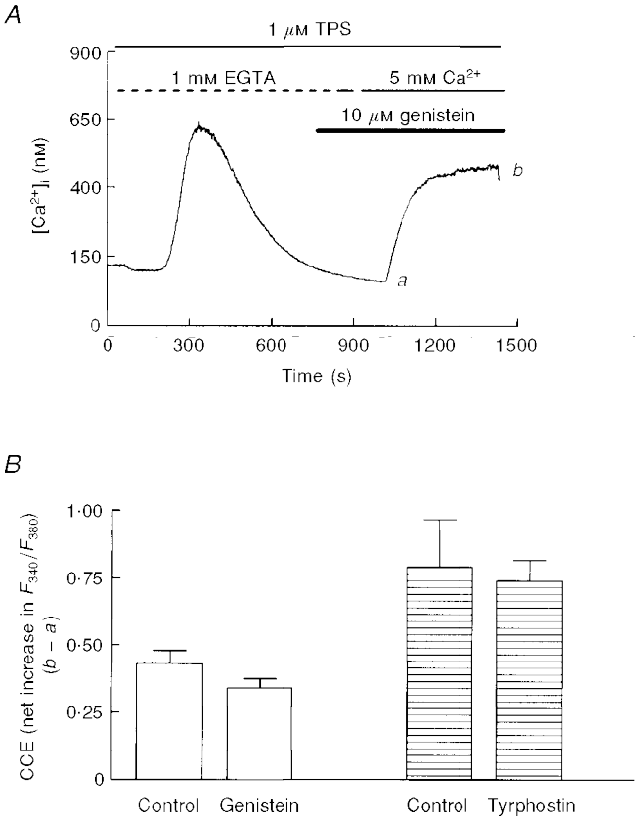


**Figure 4. Effect of pretreatment with tyrosine kinase inhibitors on thapsigargin-evoked CCE**

*A*, following a protocol similar to that in Fig. 3, mouse pancreatic acinar cells were $Ca^{2+}$ depleted before activation of CCE by admission of extracellular $Ca^{2+}$. Genistein (10 $\mu$M) was applied from the beginning of the experiment. *B*, effects of genistein and tyrphostin A25 following the protocol described in *A*. Bars represent means ± S.E.M. of the net increment of $F_{340}/F_{380}$ (taken as $b - a$ in *A*) during the CCE-evoked plateau in control and pretreated cells from the same preparations (5 independent experiments).

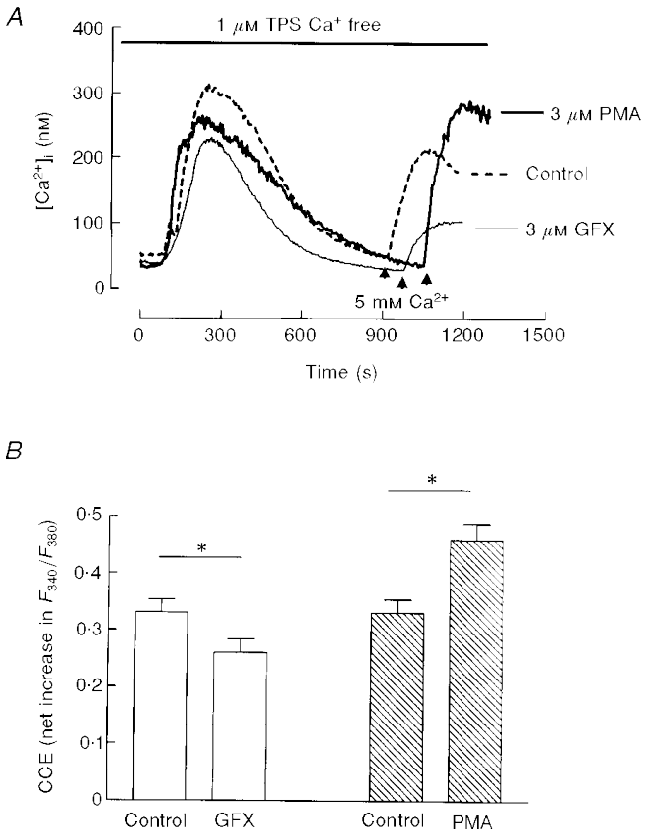


**Figure 5. PKC modulates CCE in pancreatic acinar cells**

*A*, the PKC inhibitor GF109293X (bisindolylmaleimide I; 3 $\mu$M) and the exogenous activator of PKC PMA (3 $\mu$M) were applied 5 min before readmission of extracellular calcium, indicated by the arrows. *B*, histogram depicting means ± S.E.M. of CCE (net increase in $F_{340}/F_{380}$) from 5 independent experiments following the protocol shown in *A*. * $P < 0{\cdot}05$, *t* test.

Another index of capacitative calcium entry is the plateau phase of the $Ca^{2+}$ response to calcium-mediated agonists: during $Ca^{2+}$ mobilization by physiological agonists of pancreatic acinar cells, such as acetylcholine (ACh) and cholecystokinin (CCK), activation of CCE contributes to the maintenance of the $Ca^{2+}$ signal (Yule & Gallacher, 1988; Hurley & Brinck, 1990). As shown in Fig. 6*A*, impairment of $Ca^{2+}$ influx during the sustained phase of the ACh-evoked response by removal of extracellular calcium induced an accelerated decay of $[Ca^{2+}]_i$. Upon application of $Ca^{2+}$-free solution, the rate of decay was enhanced about 2-fold (slopes of decay $0{\cdot}0032 \pm 0{\cdot}0008$ before calcium removal *vs.* $0{\cdot}0061 \pm 0{\cdot}001$ after removal, means ± S.E.M., $P < 0{\cdot}01$, *t* test, 7 experiments). Also, the whole time course of the response to ACh was partially dependent on calcium entry, as shown in the inset of Fig. 6*A*. Therefore, in agreement with previous studies, we used maintenance of this decay as an index of calcium influx (Muallem *et al.* 1989, 1990). When $La^{3+}$ was applied in the presence of ACh we obtained a small inhibition in $[Ca^{2+}]_i$ (Fig. 6*B*). This pattern was observed in 72% of 67 studied cells, without appreciable effect in the remaining 28%. A similar inhibition was obtained when cells were perfused with $Zn^{2+}$ during the response to ACh (Fig. 6*C*) (64% of 50 cells, 36% without effect). This is in contrast with the clear effect of the two cations in $Ca^{2+}$-depleted cells shown in Fig. 1.

When flufenamic acid was applied during the decay of the sustained response to ACh there was a clear reduction in $[Ca^{2+}]_i$ (Fig. 7) (the rate of decay expressed as slope of the signal before and after addition of flufenamic acid was $0{\cdot}0044 \pm 0{\cdot}001$ *vs.* $0{\cdot}0099 \pm 0{\cdot}002$, $P < 0{\cdot}05$, 5 experiments), while this drug was without effect when applied during $Ca^{2+}$ entry in $Ca^{2+}$-depleted cells (see Fig. 2). Thus the pattern of inhibition of CCE by flufenamic acid (i.e. inhibition during ACh decay but not during the $Ca^{2+}$ readmission plateau) is opposite to that of $La^{3+}$ and $Zn^{2+}$ (weak reduction during decay and strong inhibition of late entry).

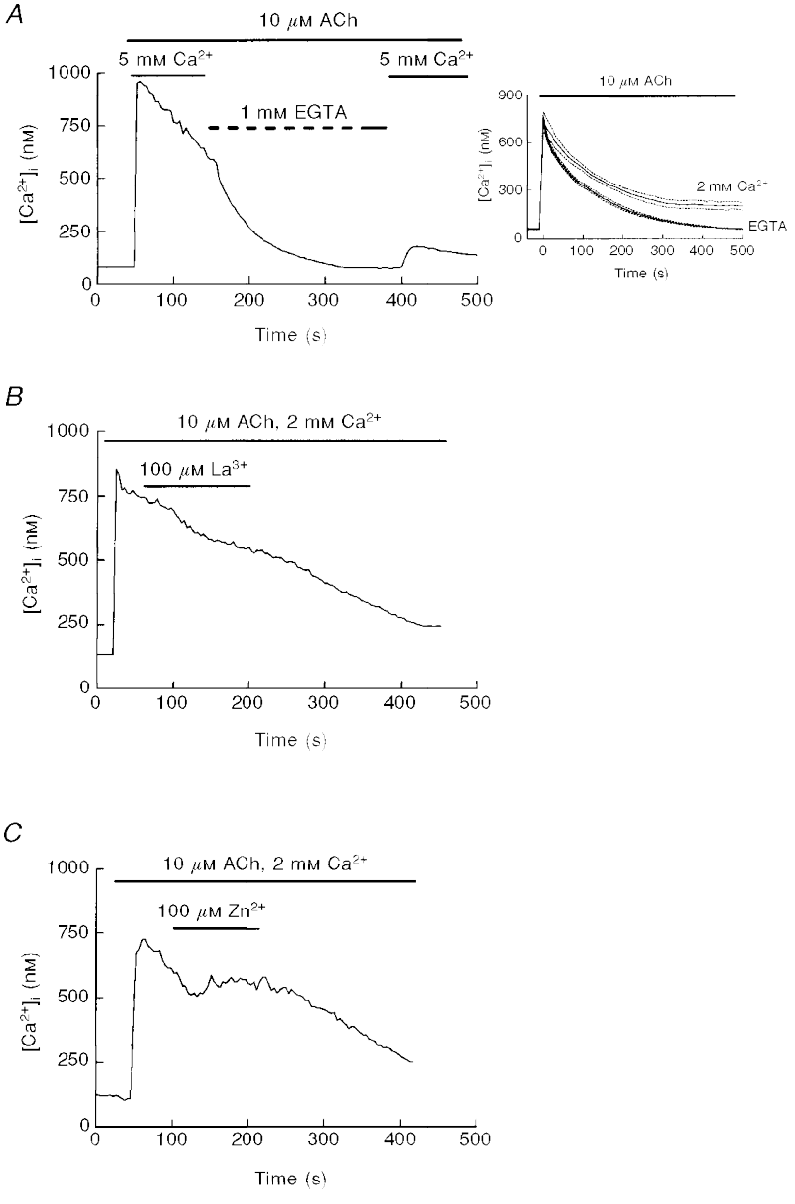


**Figure 6. Effect of extracellular calcium, $La^{3+}$ and $Zn^{2+}$ on the ACh-evoked $[Ca^{2+}]_i$ decay**

*A*, treatment of mouse pancreatic acinar cells with 10 μM ACh in 2 mM $Ca^{2+}$-containing medium results in a $[Ca^{2+}]_i$ transient with a sustained plateau phase dependent on influx of extracellular $Ca^{2+}$, as shown by the effect of a $Ca^{2+}$-free solution. The inset shows the averaged responses (means ± S.E.M.) to ACh in the presence (2 mM $Ca^{2+}$) and the absence (1 mM EGTA) of extracellular calcium. All the responses were previously aligned at the onset of the response. *B* and *C*, addition of 100 μM $La^{3+}$ (*B*) or $Zn^{2+}$ (*C*) during the plateau phase of the $Ca^{2+}$ response to ACh caused only a slight reduction in $Ca^{2+}$ entry. Traces are typical of 7 (*A*), 8 (*B*) or 4 experiments (*C*).

We also investigated the effects of tyrosine kinase inhibitors on the ACh-evoked $[Ca^{2+}]_i$ decay. Unlike their scarce or non-existent effects during calcium influx in $Ca^{2+}$-depleted cells (Figs 3 and 4), when genistein and piceatannol were applied during the sustained response to ACh we observed a clear reduction in $[Ca^{2+}]_i$, indicative of a reduction of $Ca^{2+}$ influx (Fig. 8) (rate of decay before genistein $0{\cdot}0033 \pm 0{\cdot}001$ *vs.* $0{\cdot}0080 \pm 0{\cdot}0007$ after genistein, $P < 0{\cdot}05$, 5 experiments; decay before piceatannol, $0{\cdot}0023 \pm 0{\cdot}0006$ *vs.* $0{\cdot}0047 \pm 0{\cdot}0004$, $P < 0{\cdot}05$, 5 experiments). Therefore their pattern of effect on CCE was similar to that of flufenamic acid.

The differences in the sensitivity of the ACh-evoked decay and the $Ca^{2+}$ readmission-induced plateau to several experimental conditions suggested the existence of two different $Ca^{2+}$ entry pathways in mouse pancreatic acinar cells: while the initial $Ca^{2+}$ entry could operate through a non-specific channel, in $Ca^{2+}$-depleted cells influx could be explained by the presence of $I_{CRAC}$-like conductance. Therefore, we wanted to explore this possibility further in experiments designed to evaluate in the same individual cell the two phases of calcium entry, i.e. ACh-induced decay and the $Ca^{2+}$ readmission-evoked plateau. As shown in Fig. 9*A*, in the presence of extracellular $Ca^{2+}$, 10 $\mu$M ACh induced a $[Ca^{2+}]_i$ peak followed by a decay that is sensitive to removal of external calcium and eventually reaches a steady level slightly higher than resting values. If extracellular calcium is removed after stabilization (to complete $Ca^{2+}$ depletion), perfusion with a $Ca^{2+}$-containing solution induces a plateau in $[Ca^{2+}]_i$ due to CCE. As can be seen in Fig. 9*A*, the two stages of calcium influx (i.e. early phase of ACh decay and the $Ca^{2+}$ readmission-induced plateau) are sensitive to removal of external calcium.

If our hypothesis was correct the two phases should display a different behaviour in the presence of $Mn^{2+}$, since this cation, provided it penetrates the cells, can be used as a surrogate for calcium entry given its quenching effect on fura-2 (e.g. Muallem *et al.* 1990; Jacob, 1992), and at the same time is an inhibitor of $I_{CRAC}$ (Hoth & Penner, 1993). To assess $Mn^{2+}$ entry we examined its effect on the recorded fura-2 fluorescence under 340 and 380 nm excitation wavelengths with simultaneous ratiometric $[Ca^{2+}]_i$ determination (see Methods).

We expected $Mn^{2+}$ to enter the cell during the ACh decay, while the final $Ca^{2+}$ reintroduction-induced plateau should display inhibition by manganese and a small permeation of this cation. As seen in Fig. 9*B*, this was the case. $Mn^{2+}$ influx was much more patent during the initial $[Ca^{2+}]_i$ decay than during the $Ca^{2+}$ readmission phase: the rate of $Mn^{2+}$ quench, expressed as the fold increase in the slope of $F_{tot}$ evoked by $Mn^{2+}$ application with respect to the immediately preceding 60 s, was $22{\cdot}58 \pm 2{\cdot}9$ during the ACh decay compared with $1{\cdot}76 \pm 0{\cdot}36$ during the plateau phase (6 experiments, $P < 0{\cdot}05$). On the other hand, manganese only inhibited CCE when applied during $Ca^{2+}$ readmission (note the lack of effect of $Mn^{2+}$ on the ACh decay, Fig. 9*B*). This behaviour was present in most of the cells examined

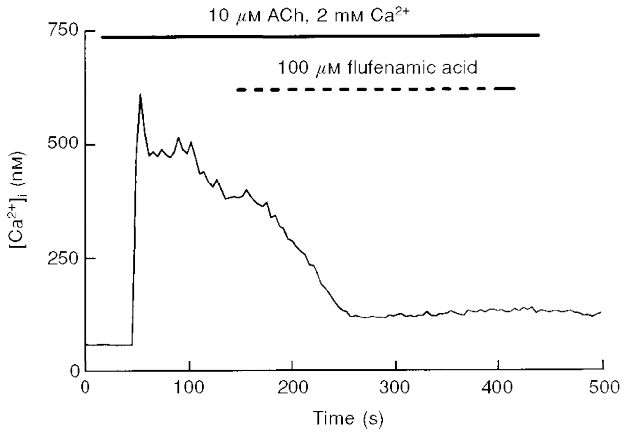


**Figure 7. Inhibition of ACh-evoked $[Ca^{2+}]_i$ decay by flufenamic acid**

Pancreatic acinar cells were perifused with 100 $\mu$M flufenamic acid during the plateau phase of the $[Ca^{2+}]_i$ response induced by 10 $\mu$M ACh. Data are representative of 5 experiments.

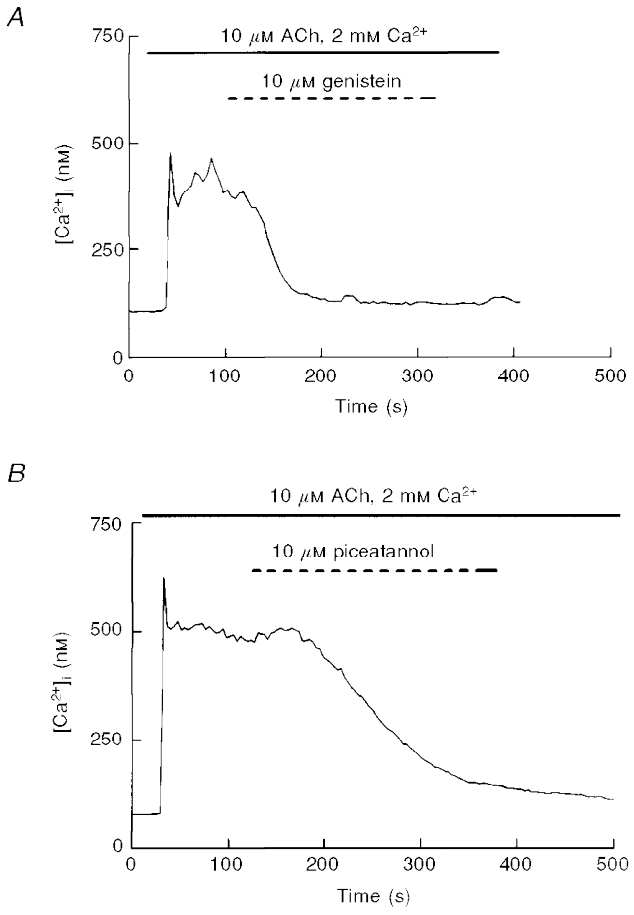


**Figure 8. Effects of tyrosine kinase inhibitors on ACh-induced $[Ca^{2+}]_i$ decay**

ACh (10 $\mu$M)-stimulated pancreatic acinar cells were perfused with either genistein (*A*, 10 $\mu$M) or piceatannol (*B*, 10 $\mu$M) during the decay phase of the ACh response. Traces are typical of 6 (*A*) and 5 (*B*) experiments.

(78% of 61 studied cells, 6 independent experiments), and indicates different selectivity and sensitivity during the two phases of calcium entry. This result supports the hypothesis that at least two $Ca^{2+}$ conductances (or one conductance with two different stages) are present in mouse pancreatic acinar cells.

The presented data cannot rule out the possibility that the initial phase of influx is not CCE, but is elicited by cholinergic activation of a non-capacitative mechanism, independent of $Ca^{2+}$ depletion. If this is true one would expect a $[Ca^{2+}]_i$ increase when ACh is applied in the presence of extracellular calcium after depletion of the stores using thapsigargin, since ACh would open the non-capacitative conductance. However, as shown in Fig. 10*A*, ACh *reduces* $[Ca^{2+}]_i$, probably by enhancement of $Ca^{2+}$ extrusion. Another indication that store depletion can activate the two phases of calcium entry is presented in Fig. 10*B*. The response to thapsigargin in the presence of extracellular $Ca^{2+}$ has a low sensitivity to $La^{3+}$ during the initial phase, which is still inhibited by a $Ca^{2+}$-free solution, while the late phase is clearly inhibited by $La^{3+}$. This pattern was present in 85% of 34 studied cells (4 independent experiments).

## DISCUSSION

Similar to other non-excitable tissues, influx of extracellular calcium in pancreatic acinar cells is mediated by a voltage-independent channel, but other features are poorly understood. Our data show that, upon cholinergic stimulation, two different conductances are sequentially activated: an initial non-specific cation channel, inhibited by genistein and flufenamic acid and scarcely sensitive to $La^{3+}$ and $Zn^{2+}$, followed by a more specific $Ca^{2+}$ entry pathway, inhibited by $Mn^{2+}$, $La^{3+}$ and $Zn^{2+}$. Other authors have previously reported the coexistence of different $Ca^{2+}$ entry pathways in other cell types (Estacion & Mordan, 1993; Ross & Cahalan, 1995; Hug *et al.* 1996), although the

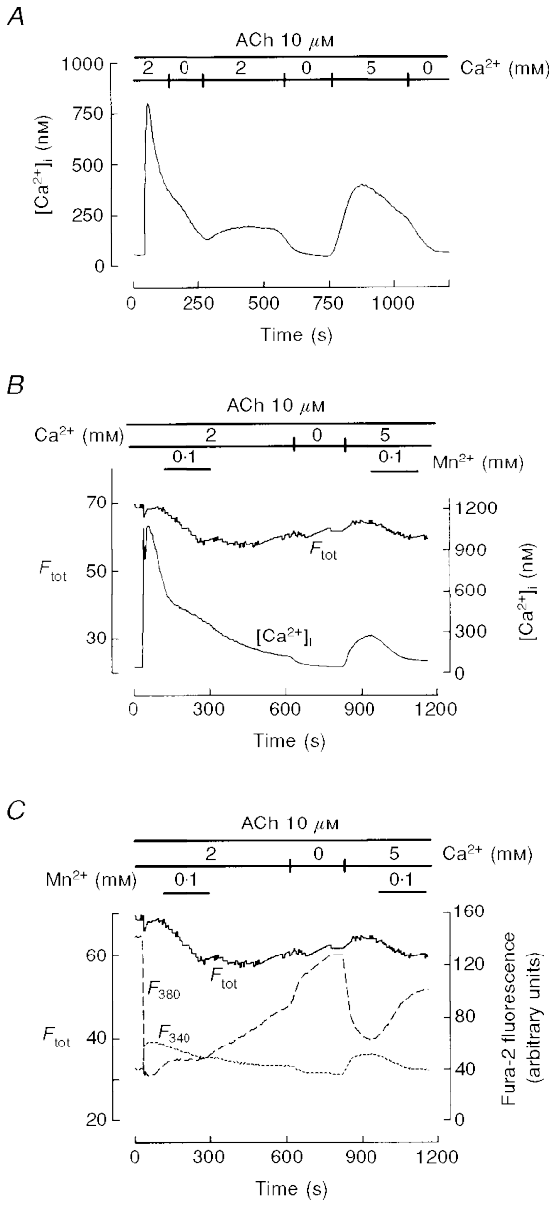


**Figure 9. Effect and entry of extracellular $Mn^{2+}$ during $Ca^{2+}$ mobilization in response to cholinergic stimulation of mouse pancreatic acinar cells**

*A*, single cells were treated with 10 $\mu$M ACh in 2 mM $Ca^{2+}$-containing solution and exposed to pulses of $Ca^{2+}$-free solution during the early phase of decay and after $[Ca^{2+}]_i$ stabilization close to resting levels. After the second exposure to $Ca^{2+}$-free medium (to achieve complete $Ca^{2+}$ depletion), 5 mM $Ca^{2+}$ was introduced to allow calcium influx. The effect of $Ca^{2+}$ removal shows the presence of $Ca^{2+}$ entry during both the ACh-induced decay and the final CCE. *B*, cells were perifused with 100 $\mu$M $Mn^{2+}$ in the presence of 2 mM extracellular $Ca^{2+}$ during the decay of the ACh response and during application of 5 mM external calcium. Traces represent $[Ca^{2+}]_i$ and the corrected total fluorescence ($F_{tot}$), which serves to detect $Mn^{2+}$ entry. *C*, original $F_{340}$ and $F_{380}$ traces from the same cell recorded in *B*. $Mn^{2+}$ entry into the cytosol during the ACh decay, as indicated by quenching of $F_{340}$ and delay in $F_{380}$ recovery (*C*) and by a decrease in $F_{tot}$ (*B* and *C*), is not accompanied by inhibition of $Ca^{2+}$ influx (*B*). In comparison, during the $Ca^{2+}$ readmission-evoked plateau, $Mn^{2+}$ entry is weaker but CCE inhibition is evident. This record is representative of 6 experiments.

temporal evolution of the agonist-evoked $Ca^{2+}$ entry is, to our knowledge, a novel feature.

The inhibitory effect of flufenamic acid and genistein, as well as the permeation of $Mn^{2+}$, indicates early activation of the non-selective cation channel in response to cholinergic stimulation. This is in agreement with a recent paper describing $I_{NCRAC}$, a non-selective cation current inhibited by genistein, as the main route for CCE in mouse pancreas (Krause *et al.* 1996). In addition, other authors have also proposed that non-specific cation channels can support $Ca^{2+}$ influx in several cell types (Schumann *et al.* 1994; Weiser & Wienrich, 1996). However, Krause *et al.* (1996) concluded that $Ca^{2+}$ influx was insensitive to $La^{3+}$, which differs from our results. In our opinion this can be explained by differences in the sensitivity of the methods (we used fura-2, which is more sensitive than fluo-3 used by Krause *et al.*) and/or by the fact that at that stage the second $Ca^{2+}$ entry phase is already slightly active in our experiments (see below).

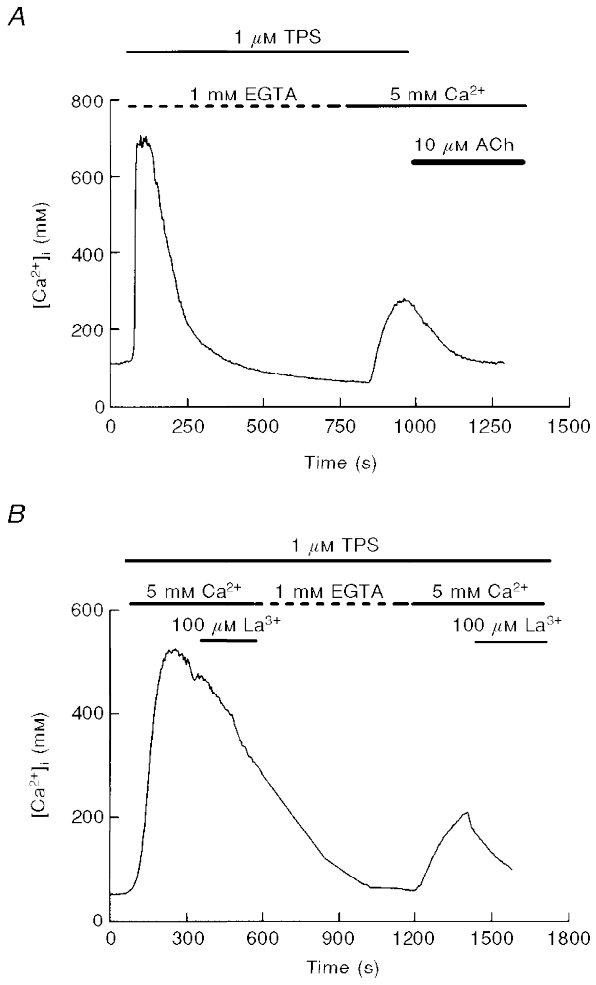


**Figure 10. Effects of lanthanum and ACh on capacitative calcium entry evoked by thapsigargin**

*A*, effect of 10 $\mu$M ACh application during the sustained plateau evoked by $Ca^{2+}$ influx in cells pretreated with thapsigargin (1 $\mu$M). The decrease in $[Ca^{2+}]_i$ was observed in 4 experiments. *B*, CCE evoked by depletion of the stores shows temporal change in sensitivity to lanthanum. Cells were treated initially with 1 $\mu$M thapsigargin in the presence of external calcium, and subsequentially perifused with 100 $\mu$M $La^{3+}$- and EGTA-containing solutions. After posterior readmission of extracellular calcium a second application of lanthanum was given. The trace, showing a differential effect of lanthanum on the initial and late phases of CCE, is representative of 4 separate experiments.

The temporal change in the sensitivity of CCE to different experimental manipulations clearly reveals that, after the initial non-specific conductance discussed above, the $Ca^{2+}$ entry pathway behaves as a relatively specific calcium channel, in keeping with the report of the presence of $I_{CRAC}$ in exocrine pancreas by Bahnson *et al.* (1993), who showed a low permeation for $Mn^{2+}$. In this particular aspect our data are clearly different from Krause *et al.* (1996), the discrepancies being probably due to differences in the timing of the experimental protocol, since we have used a period of $Ca^{2+}$ depletion greater than 10 min, while Krause *et al.* studied the behaviour of the $Ca^{2+}$ entry pathway after shorter periods.

The inhibition of store depletion-evoked CCE by lanthanum and divalent cations is in keeping with numerous reports describing this cation as an efficient tool for blocking $Ca^{2+}$ influx (Pandol *et al.* 1987; Muallem *et al.* 1989; Estacion & Mordan, 1993). This feature is characteristic of the $Ca^{2+}$-selective $I_{CRAC}$ channels (Hoth & Penner, 1993). Although previous authors have used permeation of $Mn^{2+}$ to study CCE in exocrine pancreas (e.g. Muallem *et al.* 1990), our data clearly demonstrate that $Mn^{2+}$ can inhibit influx (see Fig. 9) in $Ca^{2+}$-depleted cells. Experimental conditions could account, at least in part, for this difference. Extracellular calcium concentration is usually reduced while using $Mn^{2+}$ as a surrogate for $Ca^{2+}$ influx (Jacob, 1990; Muallem *et al.* 1990; Yule *et al.* 1994; Shuttleworth, 1995), which could lead to reduction of selectivity of $Ca^{2+}$ channels (Hess & Tsien, 1984). In fact we have found that activation of $Mn^{2+}$ entry upon cholinergic stimulation is higher when extracellular calcium is removed (data not shown). In any case, a previous report also describes inhibition of $Ca^{2+}$ fluxes by $Mn^{2+}$ in pancreas (Rorsman & Hellman, 1983).

Our results allow us to propose the hypothesis that, once a $Ca^{2+}$-mobilizing agonist binds to its receptors in pancreatic acinar cells, it activates sequentially two conductances for $Ca^{2+}$ influx. In an early phase a non-selective cation channel is activated, and after some time it is replaced by a more selective $Ca^{2+}$ channel, similar to the $I_{CRAC}$ channel. At any given moment, the detectable $Ca^{2+}$ entry is the result of the overall opening of the two channels, and the experimental behaviour will fit to the predominant conductance. This would explain the increase in sensitivity to $La^{3+}$ and divalent cations with time, as well as the apparent decrease of $Mn^{2+}$ permeability and the loss of effect of flufenamic acid and tyrosine kinase inhibitors. Of course it is not

possible to rule out the possibility that a single channel with two stages of different permeability is the cause of our observations. In any case, this explanation introduces temporal evolution as a new aspect in the physiology of calcium entry pathways.

Whether the two calcium entry mechanisms are strictly capacitative (i.e. activated by depletion of stores) or not, cannot be deduced from our results, but the results in Fig. 10 strongly support this concept.

Although speculative, there is a potential relationship between our results and the presence of *trp-* and *trp-l*-encoded $Ca^{2+}$ channels in mammals. TRP forms a $Ca^{2+}$-selective channel, sensitive to $La^{3+}$ and activated by store depletion, while TRP-L is poorly selective for $Ca^{2+}$ and relatively insensitive to inhibition by $La^{3+}$ (for a review, see Montell, 1997). This pattern closely resembles our finding of two different conductances. Since coexpression of TRP and TRP-L produces a functional CCE pathway forming heteromultimers (Niemeyer *et al.* 1996), it is possible that in pancreatic acinar cells CCE is driven by these proteins, and one single complex with two different states of permeability could account for the apparent diversity of $Ca^{2+}$ influx channels.

The putative mechanism allowing temporal co-ordination of these two pathways is not known, but current data suggest that protein kinases are involved in the process. The effect of PKC on CCE depends on the experimental conditions and the cell type: PKC inactivates $I_{CRAC}$ in RBL-2H3 cells (Parekh & Penner, 1995) as well as $Ca^{2+}$ influx in other cell types (Montero *et al.* 1993; Törnquist, 1993), whilst activating influx in NIH 3T3 (Pedrosa Ribeiro & Putney, 1996) and RINm5F cells (Bode & Goke, 1994), in line with our results. Interestingly, in *Drosophila* PKC forms a supramolecular complex with TRP, TRP-L and other proteins involved in $Ca^{2+}$ mobilization (Huber *et al.* 1996). This complex has been postulated as a regulatory unit for $Ca^{2+}$ influx, and could convey complex inhibitory and/or activating signals to modulate the status of the channels (Huber *et al.* 1998). In mouse pancreatic acinar cells, PKC could serve as a signal to switch from the initial $Ca^{2+}$ conductance to a more specific $Ca^{2+}$ channel.

Regarding the role of tyrosine kinases in the control of CCE, the situation is controversial. Although these enzymes were proposed by Vostal as the signal activating CCE (Vostal *et al.* 1991), and despite the observation that CCE is blocked by tyrosine kinase inhibitors in rat pancreatic acinar cells (Yule *et al.* 1994), our results show a residual role in the control of the late CCE, while the initial $Ca^{2+}$ influx is more sensitive to tyrosine kinase inhibitors in agreement with Krause *et al.* (1996). Vostal (Vostal & Shafer, 1996) recently corrected his original hypothesis and report that genistein inhibition of calcium influx is not associated with a decrease in tyrosine kinase activity. The discrepancies in the effect of genistein depending on the experimental conditions (Figs 3 and 4) reinforces the conclusion that the effects of tyrosine kinase inhibitors on CCE must be evaluated with caution. However, the putative link between tyrosine kinase and CCE cannot be ruled out. In fact, our finding that piceatannol, an inhibitor structurally different from genistein, inhibits $Ca^{2+}$ entry during ACh decay supports the hypothesis CCE channels have some relationship with its target, the non-receptor tyrosine kinase $p72^{Syk}$, a protein apparently involved in $Ca^{2+}$ transport mechanisms (Jenner *et al.* 1997).

In conclusion, our results show that cholinergic stimulation of mouse pancreatic acinar cells activates an initial phase of CCE relatively insensitive to $La^{3+}$, inhibited by tyrosine kinase inhibitors and carried by a non-selective $Ca^{2+}$ channel, followed by a more selective $Ca^{2+}$ conductance inhibited by $La^{3+}$, $Mn^{2+}$ and $Zn^{2+}$. In addition, PKC seems to be involved in the control of CCE in pancreatic acinar cells. The identity of the channels underlying this pattern and its regulatory mechanisms deserve further investigation.


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