

TYROSINE KINASE INHIBITORS ATTENUATE "CAPACITATIVE" Ca^{2+} INFLUX IN RAT PANCREATIC ACINAR CELLS

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The effect of several tyrosine kinase inhibitors was tested on Ca^{2+} influx mediated by thapsigargin- and CCh-induced intracellular store depletion. Genestein inhibited Ca^{2+} influx in a concentration dependent manner without affecting Ca^{2+} release or Ca^{2+} pumping activity. A measureable effect was observed at 3 μM with total inhibition of influx seen at 100 μM . Tyrphostin A25 (300 μM ; 78% inhibition) and methyl 2,5 dihydroxycinnamate (10 μM ; 51 % inhibition) also inhibited Ca^{2+} influx. The degree of attenuation was not markedly altered by preincubation of the inhibitors. Genestein also inhibited Ca^{2+} influx induced by CCh. These data indicate that inhibition of Ca^{2+} influx could in part underlie the previously reported inhibition of enzyme secretion by these agents.

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The primary function of rat pancreatic acinar cells is to synthesize, store and then undergo regulated secretion of digestive enzymes (1). An important early signal in the stimulation of secretion by phosphoinositide-linked agonists, such as cholecystokinin (CCK) and acetylcholine, is an increase in $[\text{Ca}^{2+}]_i$. It is well established that the increase in $[\text{Ca}^{2+}]_i$ is an aggregate of two general processes; initially $1,4,5 \text{ IP}_3$ induces the release of Ca^{2+} from intracellular stores, an event which is followed temporally by the influx of Ca^{2+} across the plasma membrane (1,2). Recently, a possible importance of tyrosine phosphorylation in the secretory mechanism stimulated by CCK or the muscarinic agonist carbachol (CCh) has been suggested, even though the receptors for these ligands possess no intrinsic tyrosine kinase activity (3-5). Evidence for this proposal includes the observation that changes in tyrosine phosphorylation occur on several proteins upon stimulation with CCK or carbachol (4,5). Furthermore, tyrosine kinase inhibitors have been reported to attenuate agonist stimulated enzyme secretion from rat pancreatic acinar cells (4,5). A potential site of action of these inhibitors is at the level of Ca^{2+} homeostasis; indeed these inhibitors have been shown in fibroblasts and platelets

to attenuate Ca^{2+} influx but to be without effect on Ca^{2+} release from intracellular stores (6,7). In a recent study we reported only marginal effects of tyrosine kinase inhibitors on CCK-induced changes in acinar $[\text{Ca}^{2+}]_i$ (5). However, these experiments were designed primarily to investigate potential effects on Ca^{2+} mobilization from intracellular stores. Therefore, we have reevaluated the effects of these agents specifically on Ca^{2+} influx with a view that this may be a potential site of action for the reported inhibition of enzyme secretion mediated by these agents.

MATERIALS AND METHODS

Chemicals: fura-2/AM and fura-2 free acid were purchased from Molecular Probes Eugene, OR); genestein, methyl 2,5 dihydroxycinnamate, tyrphostin A25 and thapsigargin were obtained from LC Services (Boston, MA); collagenase from Worthington Biochemicals (Freehold, NJ); bovine serum albumin (fraction V) from ICN Immunobiologicals (Lisle, IL); minimal essential amino acids from GIBCO (Grand Island, NY). All other chemicals were obtained from Sigma Chemical (St Louis, MO.)

Preparation of pancreatic acini: acini were prepared by methods as previously described (8,9). In brief, pancreata were excised from fed adult male Sprague-Dawley rats (200-250 g) and acini prepared by enzymatic digestion with purified collagenase. Acini were then suspended in a physiological salt solution (PSS) containing an essential amino acid mixture, 10 mg/ml BSA, 0.1 mg/ml soybean trypsin inhibitor (SBTI) and (in mM); 137 NaCl, 4.7 KCl, 0.56 MgCl_2 , 1.28 CaCl_2 , 1.0 Na_2HPO_4 , 10 HEPES, 2 L-glutamine, 5.5 D-glucose. The pH was adjusted to 7.4 and equilibrated with 100% oxygen. In experiments where Ca^{2+} free medium is indicated Ca^{2+} was omitted and 1 mM EGTA was added. In experiments where barium substitution is indicated the medium was rendered Ca^{2+} free and 30 mM barium chloride was added to medium containing 107 mM NaCl.

Measurement of intracellular Ca^{2+} : isolated acini were incubated with 1 μM fura-2/AM at ambient temperature for 30 min and then washed and resuspended in fresh PSS, without BSA. For measurement of intracellular Ca^{2+} , fura-2 loaded acini were transferred to a closed chamber, mounted on the stage of a Nikon Diaphot microscope, and continuously superfused at 1 ml/min with PSS at 37° C. Solution changes were rapidly accomplished by means of a valve attached to an 8 chambered reservoir. Individual cells protruding from an acinus were optically isolated by means of a pin-hole diaphragm. Cells were alternately excited at 340 and 380 nm (SPEX fluorolog system, SPEX industries inc, Edison, NJ.) and the resultant emission at 505 nm was stored and subsequently analyzed by DM3000cm software (SPEX). Calibration of fluorescent signals was accomplished as previously described (9) according to the equation of Grynkiewicz *et al.* (10). In Ba^{2+} influx experiments fluorescence was measured using digital imaging microscopy with an ATTOFLUOR (Rockville, MD.) ratiovision system as previously described (11). Briefly, excitation at 340/380 nm was alternately supplied by a computer controlled filter and shutter system and the resultant emission at 505 nm was recorded by an intensified CCD camera and subsequently digitized. Mean gray values at 340 and 380 nm, in user defined areas of interest were used to compute 340/380 ratios.

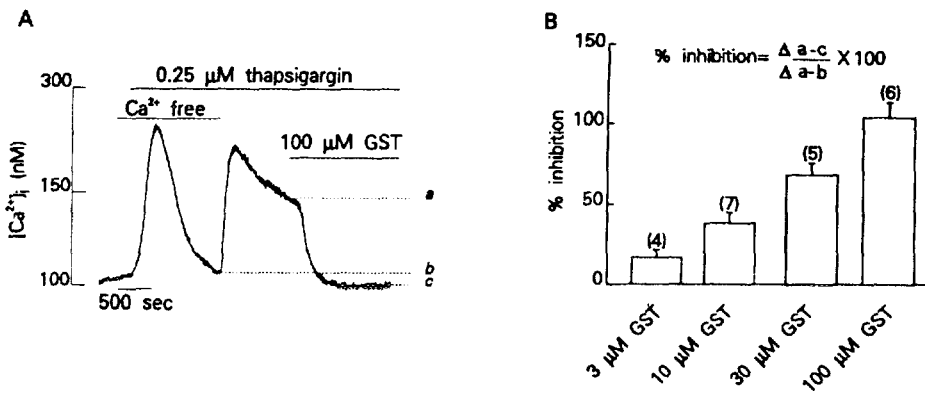


Figure 1. *Genestein inhibits Ca^{2+} influx in a concentration dependent manner.* In fig. 1B: Ca^{2+} influx induced by store depletion was stimulated by thapsigargin ($0.25 \mu M$). Initially, cells were stimulated in Ca^{2+} free conditions, subsequently the $[Ca^{2+}]_i$ levels returned to near basal values. After Ca^{2+} was readmitted to the superfusion medium a new elevated level was established. $100 \mu M$ GST totally inhibited this increase in $[Ca^{2+}]_i$. The degree of inhibition was calculated as shown in the figure. Fig. 1B shows the concentration dependence of the inhibition with mean \pm S.E.M calculated for the number of experiments in parentheses.

RESULTS AND DISCUSSION

Genestein inhibits thapsigargin-induced Ca^{2+} influx: Although the mechanism which underlies Ca^{2+} influx is poorly understood, evidence suggests that depletion of the intracellular stores is a primary signal to gate Ca^{2+} influx. This model has been termed the "capacitative" model for Ca^{2+} influx (12). Primary evidence for this hypothesis has been derived from using agents such as thapsigargin, which induce Ca^{2+} influx apparently by depletion of Ca^{2+} pools as a result of inhibiting SERCA type Ca^{2+} pumps, thus allowing Ca^{2+} to leak from the pools (13). The nature of the signal which conveys information from the depleted pool to the plasma membrane is unknown. Recent reports have suggested a soluble factor termed CIF (calcium influx factor) is stored and subsequently released on depletion of the pool (14). In order to assess the effect of agents on capacitative type influx, a paradigm was utilized which isolated Ca^{2+} influx stimulated by store depletion. As shown in fig. 1A application of thapsigargin in Ca^{2+} free conditions resulted in a transient increase in $[Ca^{2+}]_i$ as a result of release and depletion of Ca^{2+} from intracellular stores. On readmission of Ca^{2+} to the superfusion medium a rapid increase in $[Ca^{2+}]_i$ was observed; this elevated level persisted provided extracellular Ca^{2+} was present and was attributed to Ca^{2+} influx. The effect of the tyrosine kinase inhibitor genestein (GST) was assessed under these conditions. GST at concentrations as low as $3 \mu M$ resulted in a measurable inhibition of Ca^{2+} influx, resulting in 16.8 ± 5.0 % inhibition. The degree of inhibition was calculated as shown in

fig.1. This inhibition was concentration dependent; at 100 μM GST a complete inhibition of the $[\text{Ca}^{2+}]_i$ signal was observed. Similar concentrations of GST have been shown to inhibit tyrosine phosphorylation induced by CCK in pancreatic acini (5). Other chemically unrelated tyrosine kinase inhibitors such as tyrphostin A25 (Ty A25; $78.0 \pm 6.0\%$ inhibition at 300 μM) and methyl 2,5-hydroxycinnamate (DHC; $51.2 \pm 11.8\%$ inhibition at 10 μM) also inhibited Ca^{2+} influx utilizing this protocol, however to lesser extents than GST. In the case of DHC, a maximal concentration of agent was not utilized since concentrations above 10 μM , which are optimal in other studies (6) resulted in contaminating fluorescence, interfering with the fura-2 signal. The observation that this inhibitory effect was common to several tyrosine kinase inhibitors makes it most likely that their site of action is indeed to inhibit tyrosine phosphorylation required at some step in the influx pathway. Since similar effects of tyrosine kinase inhibitors have been noted in fibroblasts and platelets (6,7) it may indicate that a common step involving tyrosine phosphorylation may be present in cell types which exhibit this form of Ca^{2+} influx.

Preincubation with tyrosine kinase inhibitors attenuates Ca^{2+} influx: To determine that the inhibition of $[\text{Ca}^{2+}]_i$ signals by tyrosine kinase inhibitors was specific for the Ca^{2+} influx phase of the response and to assess whether a more potent effect of DHC and Tyr A25 would be seen after preincubation, the effects of the agents added prior to the incubation of thapsigargin was appraised. This protocol is shown in fig. 2A/B. Incubation of GST (100 μM), DHC (10 μM) or TY A25 (300 μM) for 5 min prior to addition of thapsigargin was without effect on the release phase of the response as measured by the magnitude of the thapsigargin-induced peak (fig. 2C). In contrast, all three agents markedly inhibited the influx phase of the response as measured by the magnitude of $[\text{Ca}^{2+}]_i$ signal on readmission of extracellular Ca^{2+} (fig. 2D). Since the inhibition of Ca^{2+} influx observed with DHC and TY A25 was not greater than that seen without preincubation it seems likely that the attenuation of Ca^{2+} influx is due to the inhibition of a relatively rapid event and that the different degrees of inhibition observed with these agents are a result of differential potency of the agents directed against the particular kinase involved in the modulation of Ca^{2+} influx.

Genestein attenuates CCh stimulated Ca^{2+} influx: In a previous study (5) little or no effect of preincubated GST was observed on the plateau phase of Ca^{2+} signalling induced by cholecystokinin (CCK). In order to reevaluate if Ca^{2+} influx activated by a physiological stimulus was similarly inhibited by tyrosine kinase inhibitors the effect of

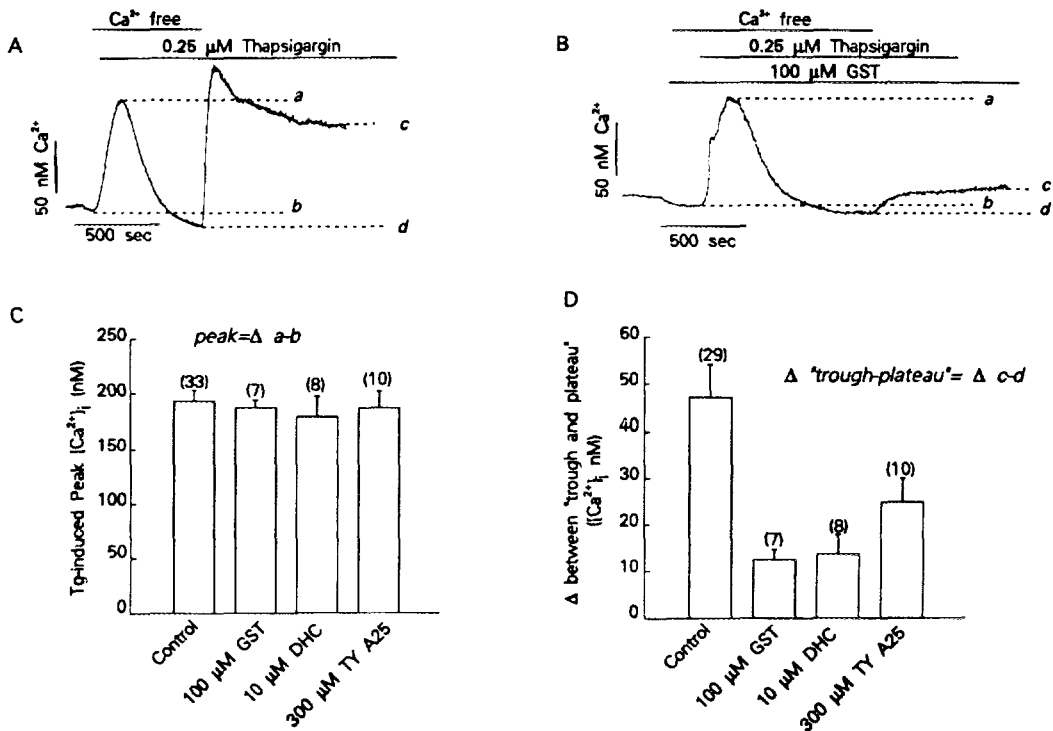


Figure 2. *Preincubation of tyrosine kinase inhibitors attenuates influx but not release.* The effects of preincubated tyrosine kinase inhibitors was tested on Ca^{2+} release and Ca^{2+} influx. Where inhibitors were added they were added approximately 5 min prior to administration of thapsigargin ($0.25 \mu M$) as shown in fig. 2 A/B. The pooled data (fig. 2 C/D) show that these agents did not alter the release phase of the response but markedly affected Ca^{2+} influx.

GST was assessed on influx promoted by CCh, which induces a more marked phase of Ca^{2+} entry into rat acini than does CCK. To isolate, and to augment the influx phase of the response a similar protocol to that utilized with thapsigargin was applied. Cells were stimulated in Ca^{2+} free conditions by addition of a maximal concentration of CCh ($100 \mu M$). On readmission of Ca^{2+} to the superfusion medium an increase in the $[Ca^{2+}]_i$ signal was observed to a new plateau level, which is attributed to depletion-activated Ca^{2+} influx (fig. 3A). GST at a maximal concentration ($100 \mu M$) markedly inhibited this $[Ca^{2+}]_i$ signal on average by $80.4 \pm 7.2\%$. When GST was included in the superfusion medium prior to addition of CCh a marked inhibition of the influx phase of the response was observed together with no effect on the initial release of Ca^{2+} (fig. 3B). To ensure that influx stimulated by depletion paradigms represents an augmentation of a normally occurring Ca^{2+} influx pathway the effect of GST was assessed on the plateau phase of responses to CCh in normal calcium containing medium. When GST was superfused concurrently with CCh a marked, acute inhibition of the plateau phase was observed

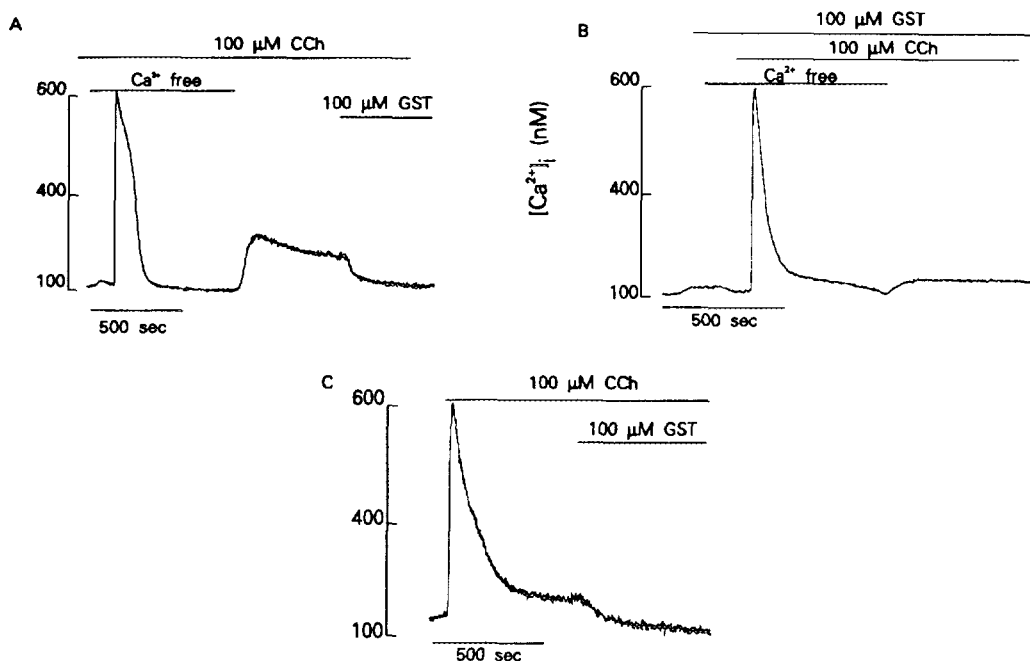


Figure 3. Genestein inhibits CCh stimulated Ca^{2+} influx. In fig. 3A, a similar protocol was utilized with CCh to isolate Ca^{2+} influx. After stimulation with CCh ($100\ \mu\text{M}$) in Ca^{2+} free conditions, influx was initiated by readmission of Ca^{2+} to the superfusion medium. GST ($100\ \mu\text{M}$) markedly attenuated Ca^{2+} influx stimulated by this protocol. In 3B preaddition of GST) did not alter release of Ca^{2+} stimulated by CCh but markedly attenuated Ca^{2+} influx. Fig. 3C, GST inhibits the plateau phase of the Ca^{2+} signal stimulated by CCh in normal Ca^{2+} containing media. All traces are representative of at least 4 individual experiments.

(fig. 3C). Since both agonist- and thapsigargin-induced influx were affected similarly by tyrosine kinase inhibitors it is likely that both agents induce influx through common mechanisms. However, as Ca^{2+} influx stimulated by thapsigargin was inhibited by GST to a greater degree than influx stimulated by CCh (> 100 % vs 80 %; thapsigargin and CCh respectively) there remains a possibility that CCh is able to induce influx by a mechanism not identical to thapsigargin, or sensitive to inhibition by genestein. The identity of a potential tyrosine kinase, together with its' substrate remains unclear. It is known however, that neither the m3 muscarinic receptor or the endoplasmic reticulum Ca^{2+} -ATPase possesses intrinsic tyrosine kinase activity making it likely that the role of any tyrosine kinase is downstream from the formation of second messengers and the store depletion event. Possible substrates include proteins involved in the formation or activation of a CIF-type molecule or the plasma membrane Ca^{2+} influx channel itself. In support of the latter possible site of action, tyrosine kinase inhibitors have been observed to affect the gating of other membrane ion channels which conduct Ca^{2+} , such

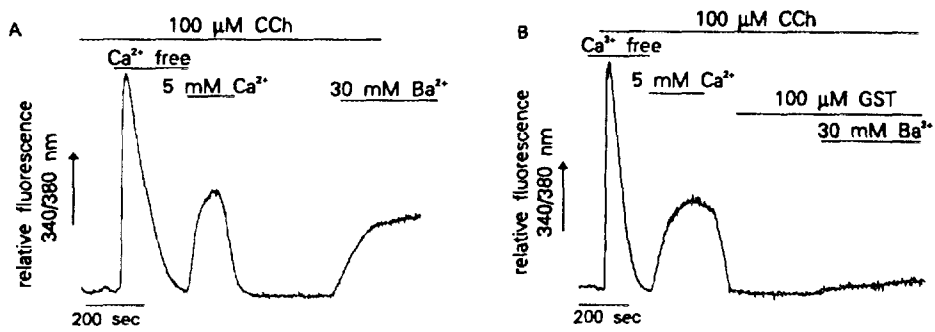


Figure 4. *Genestein* inhibits Ba^{2+} influx. In fig. 4a cells were first stimulated with CCh (100 μ M) in Ca^{2+} free conditions. Ca^{2+} influx was tested by utilizing a control pulse of Ca^{2+} (5 mM). After removal of Ca^{2+} from the superfusion medium, the cells were superfused with a medium containing BaCl (30 mM). An increase in the fluorescent signal is observed which is the result of unidirectional Ba^{2+} influx. In fig. 4B, the Ba^{2+} influx is markedly inhibited by preincubation with GST (100 μ M). Results are the mean fluorescent signals from >10 cells in a single experiment and are representative of at least 4 individual experiments.

as the NMDA receptor/cation channel (15). It has also been reported that an increase in cellular levels of cGMP is an important signal in the gating of Ca^{2+} influx in rat pancreatic acinar cells (16,17), therefore tyrosine phosphorylation may play an as yet undocumented role in this pathway.

***Genestein* inhibits depletion-stimulated Ba^{2+} influx:** The increase in fura-2 fluorescence as a result of Ca^{2+} binding in these experiments reflects the net appearance of Ca^{2+} in the cytoplasm. The signal therefore represents the compound effect of Ca^{2+} pumping activity and Ca^{2+} influx, the possibility exists that these effects of tyrosine kinase inhibitors could be on Ca^{2+} pumps present on the plasma membrane (and on the Ca^{2+} pool in the case of CCh experiments). In subsequent experiments to isolate unidirectional influx, 30 mM Ba^{2+} was substituted for Ca^{2+} in the extracellular bathing medium as indicated in fig. 4 A/B. This protocol exploits the fact that Ba^{2+} binds to fura-2 resulting in similar fluorescence properties as compared to Ca^{2+} but is not subject to removal from the cytoplasm by Ca^{2+} -ATPases and thus will continue to accumulate in the cytoplasm (18). This result is illustrated in fig. 4A; after a control pulse of 5 mM Ca^{2+} addition of 30 mM Ba^{2+} to the superfusion medium result in an increase in fluorescence. Concomitant superfusion of atropine (100 μ M) did not affect the increase in fluorescence (data not shown), indicating that Ba^{2+} was not subject to removal from the cytoplasm by Ca^{2+} -ATPase pumping activity. This increase in fluorescence was markedly inhibited by 3 min prior incubation with 100 μ M GST (fig. 4B), indicating that the major effect of GST is on the influx process and not on Ca^{2+} pumping activity.

In summary, the present study reports that a number of tyrosine kinase inhibitors, with unrelated chemical structures, have profound inhibitory effects on Ca^{2+} influx stimulated by thapsigargin-induced store depletion, and by depletion mediated by CCh, a physiological agonist on this cell type. The inhibition appeared specific for Ca^{2+} influx since no effects of these agents were observed on the processes of Ca^{2+} release or Ca^{2+} pumping activity. This data may indicate a role for tyrosine phosphorylation in the gating of depletion activated or capacitative type Ca^{2+} entry in rat pancreatic acinar cells.

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