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Evidence that the Ca^{2+} inflow pathway in hepatocytes stimulated by thapsigargin is similar to that activated by vasopressin

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Abstract—Experiments were conducted to characterize the thapsigargin-stimulated plasma membrane Ca^{2+} inflow pathway in hepatocytes. Ca^{2+} inflow was estimated by measurement of the initial rate of activation of glycogen phosphorylase *a* following the addition of Ca^{2+} to cells previously incubated in the absence of added Ca^{2+}. Pretreatment of hepatocytes with thapsigargin caused a substantial stimulation of the rate of Ca^{2+} activation of glycogen phosphorylase *a*. This was interpreted to reflect a stimulation of plasma membrane Ca^{2+} inflow. The effect of thapsigargin on plasma membrane Ca^{2+} inflow was approximately 65% of the magnitude of the effect caused by vasopressin. When thapsigargin and vasopressin were combined as a stimulus, the degree of stimulation was similar to that caused by vasopressin alone. The thapsigargin-induced stimulation of the rate of Ca^{2+} activation of glycogen phosphorylase *a* was inhibited in a concentration-dependent manner by both Zn^{2+} and 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SK&F 96365). The concentration of each agent required for half-maximal inhibition was approximately 20 μM. It is concluded from: (i) the apparent lack of additivity in the responses of thapsigargin and vasopressin, and (ii) the sensitivity to inhibitors, that the Ca^{2+} inflow pathway in hepatocytes stimulated by thapsigargin is likely to be similar to that which is activated by vasopressin.

In hepatocytes a number of hormones including vasopressin, adrenaline, angiotensin II and epidermal growth factor stimulate the entry of Ca^{2+} across the plasma membrane (reviewed in Ref. 1). The agonist-stimulated Ca^{2+} inflow pathway is perhaps best described as a receptor-activated Ca^{2+} inflow system (RACIS*) [1]. The mechanisms by which hormones activate the hepatocyte RACIS are not well understood and evidence has been presented in support of several mechanisms [1]. These include activation by a G protein [2, 3] or by inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) [4]. Evidence has also been presented in support of the "capacitance" model in which the depletion of the $Ins(1,4,5)P_3$-sensitive Ca^{2+} store stimulates plasma membrane Ca^{2+} inflow via an unknown messenger or signal [5]. Key experimental results in support of the capacitance model are the observations that pre-treatment of hepatocytes with thapsigargin, a specific inhibitor of the (Ca^{2+} + Mg^{2+}) ATPase pump of the endoplasmic reticulum [6], stimulates the entry of Ca^{2+} [4, 7] or Mn^{2+} across the plasma membrane [8, but see 7]. The properties of the thapsigargin-stimulated Ca^{2+} inflow pathway have not been well defined. The aim of the present study was to determine whether the thapsigargin-induced stimulation of plasma membrane Ca^{2+} inflow was susceptible to inhibition by Zn^{2+} and 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SK&F 96365). Zn^{2+} is a potent inhibitor of RACIS in hepatocytes [9] while SK&F 96365 is known to inhibit receptor-activated Ca^{2+} inflow in several cell types [10, 11].

Materials and Methods

The isolation of hepatocytes from fed male rats, incubation of stirred suspensions of cells in cylindrical chambers and assessment of cell viability were performed as described previously [2]. The incubation medium contained 117 mM NaCl, 4.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 20 mM Tes/KOH pH 7.4 and hepatocytes [2.3 × 10^6 cells (35 mg wet wt)/mL]. Rates of plasma membrane Ca^{2+} inflow were estimated as described previously [2] by the measurement of the initial rate of activation of glycogen phosphorylase *a* following the addition of Ca^{2+} to cells previously incubated in the absence of added Ca^{2+}.

SK&F 96365 was dissolved in water to give a stock solution of 10 mM and stored in aliquots at −4° until required. Samples were only thawed once. SK&F 96365 was a gift from Smith Kline Beecham Pharmaceuticals (The Frythe, Welwyn, U.K.). Thapsigargin was purchased from the Calbiochem Corp. (La Jolla, CA, U.S.A.). The sources of all other reagents were as described previously [2, 9].

The results are means + SEM of the number of experiments indicated. Degrees of significance were determined by Student's *t*-test for paired samples. Values of $P > 0.05$ were considered to be not significant.

Results and Discussion

Pre-treatment of hepatocytes with 1 μM thapsigargin prior to addition of 1.3 mM Ca^{2+} resulted in a substantial increase in the rate of Ca^{2+} activation of glycogen phosphorylase *a* in comparison with cells treated with dimethyl sulphoxide (DMSO) (Fig. 1). A similar degree of stimulation was observed using either 250 nM, 500 nM or 1 μM thapsigargin (results not shown). The thapsigargin-induced stimulation of the rate of Ca^{2+} activation of glycogen phosphorylase *a* was approximately 65% of the increase induced by vasopressin (Fig. 1). The combined effect of thapsigargin and vasopressin was not substantially greater than that seen with vasopressin alone (Fig. 1).

Zn^{2+} inhibited the thapsigargin-induced stimulation of the rate of Ca^{2+} activation of glycogen phosphorylase in a concentration-dependent manner (Fig. 2). When present at 10 μM, Zn^{2+} caused only a small inhibition while 20, 50 and 100 μM caused substantial inhibition (Fig. 2). It is noteworthy that even at the highest concentrations tested, Zn^{2+} did not completely inhibit the thapsigargin effect (Fig. 2). Zn^{2+} (100 μM) had no effect on the rate of activation of glycogen phosphorylase seen in cells incubated under basal conditions (results not shown). These results

* Abbreviations: RACIS, receptor-activated Ca^{2+} inflow system; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; SK&F 96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride; DMSO, dimethyl sulphoxide.

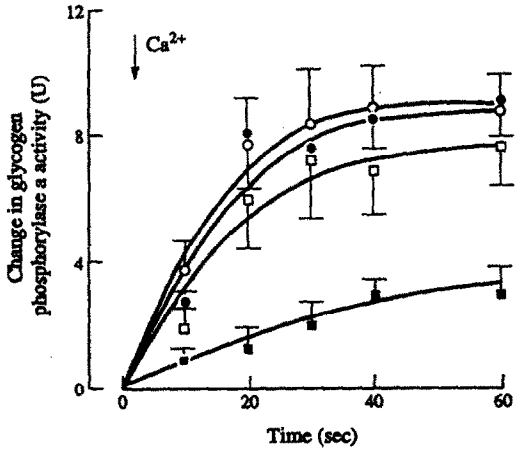

Fig. 1. Effect of thapsigargin on the rate of Ca^{2+} activation of glycogen phosphorylase. Hepatocytes were isolated and incubated in the absence of added Ca^{2+} as described in Methods and Materials. After 10 min, either 1% (v/v) DMSO (■, ●) or 1 μM thapsigargin (□, ○) was added. This was followed 30 sec later by carrier (■, □) or 30 nM vasopressin (○, ●). The incubations were continued for a further 10 min and at the completion of this period, samples were removed from each suspension to allow estimation of the glycogen phosphorylase *a* activity. After a further 1 min, 1.3 mM $CaCl_2$ was added to the suspensions (t = 0 sec on the graph) and further samples were then removed at the times indicated for estimation of glycogen phosphorylase activity. The data have been expressed as the change in glycogen phosphorylase *a* activity following Ca^{2+} addition and are the means ± SEM of four determinations, each from a separate cell preparation. The degree of significance for a comparison of the values obtained in either the presence of thapsigargin (□) or vasopressin (●) compared to those obtained in the presence of carrier (■) was $P < 0.05$ (Student's t-test for paired samples). The degree of significance for a comparison of the values obtained in the presence of thapsigargin (□) compared with those obtained in either the presence of vasopressin (●) or thapsigargin and vasopressin (○) was $P < 0.05$. The degree of significance for a comparison of the values obtained in the presence of vasopressin (●) compared to those obtained in the presence of thapsigargin and vasopressin (○) was $P > 0.05$.

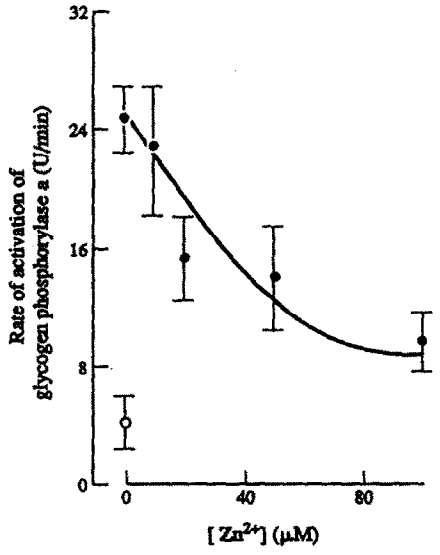

Fig. 2. Inhibition by Zn^{2+} of thapsigargin stimulation of the rate of Ca^{2+} activation of glycogen phosphorylase. Hepatocytes were isolated and incubated in the absence of added Ca^{2+} as described in Methods and Materials. Following a 10 min pre-equilibration either 1% (v/v) DMSO (○) or 1 μM thapsigargin (●) was added. After a further 10 min either water (○) or $ZnCl_2$ (●) was added and 1 min later the rate of change of glycogen phosphorylase activity following the addition of 1.3 mM $CaCl_2$ was determined. The data are presented as the rate of activation of glycogen phosphorylase *a* as a function of [Zn^{2+}] and are the means ± SEM of three determinations, each from a separate cell preparation.

provide evidence that Zn^{2+} does not inhibit Ca^{2+} inflow through the basal Ca^{2+} inflow system.

Control experiments investigating the specificity of Zn^{2+} for inhibition of Ca^{2+} inflow were performed. When cells were incubated in the absence of added Ca^{2+}, a condition under which Ca^{2+} is available only from internal stores to activate glycogen phosphorylase, Zn^{2+} (100 μM) had no effect on the ability of thapsigargin to activate glycogen phosphorylase (results not shown). When the incubation was conducted in the presence of 1.3 mM Ca^{2+} however Zn^{2+} caused a substantial decrease in the extent of thapsigargin-induced activation of glycogen phosphorylase (results not shown).

The rate of Ca^{2+} activation of glycogen phosphorylase was also inhibited in a concentration-dependent manner by SK&F 96365 (Fig. 3). When present at 10 or 25 μM, SK&F 96365 markedly inhibited the ability of thapsigargin to stimulate the rate of Ca^{2+} activation of glycogen phosphorylase (Fig. 3). Higher concentrations of SK&F 96365, i.e. 50 or 100 μM, inhibited the rate to values below that seen with DMSO alone (Fig. 3). When present at 100 μM, SK&F 96365 completely inhibited the rate of activation of glycogen phosphorylase when cells were incubated under basal conditions (results not shown). These data suggest that SK&F 96365 when present at higher concentrations also inhibits Ca^{2+} inflow through the basal Ca^{2+} inflow system. Other explanations cannot however be completely excluded.

Control experiments were conducted, testing the specificity of SK&F 96365 for inhibition of Ca^{2+} inflow. When present at 10 μM, SK&F 96365 had no effect on the ability of thapsigargin to activate glycogen phosphorylase in cells incubated in the absence of added Ca^{2+} but caused a 50% inhibition when present at 100 μM (results not shown).

The results provide further evidence that pre-treatment of hepatocytes with thapsigargin leads to a substantial stimulation of plasma membrane Ca^{2+} inflow [cf. 4, 7, 8]. In addition, the results confirm the observation of Hansen *et al.* [4] that the degree of stimulation of plasma membrane Ca^{2+} inflow caused by hormones is significantly larger than that observed following pre-treatment of hepatocytes with thapsigargin. These results support the view that the hormone-activated plasma membrane Ca^{2+} inflow pathway involves an additional component besides capacitative Ca^{2+} inflow [1, 4, 7].

The concentration of Zn^{2+} required for half maximal inhibition of the thapsigargin-induced stimulation of plasma membrane Ca^{2+} inflow, 20 μM, is similar to that which causes half maximal inhibition of vasopressin- or angiotensin II-stimulated plasma membrane Ca^{2+} inflow in hepatocytes [9]. The concentrations of SK&F 96365 required for inhibition of the thapsigargin effect are similar to

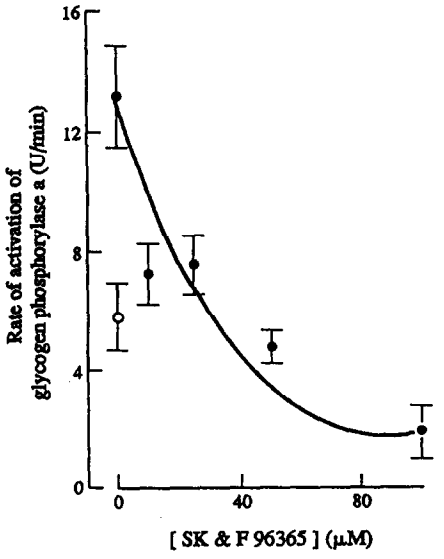

Fig. 3. Inhibition by SK&F 96365 of thapsigargin stimulation of the rate of Ca^{2+} activation of glycogen phosphorylase. Hepatocytes were isolated and incubated in the absence of added Ca^{2+} as described in Materials and Methods. Following a 10 min pre-equilibration either 1% (v/v) DMSO (○) or 1 μM thapsigargin (●) was added. After a further 10 min either water (○) or SK&F 96365 (●) was added and 1 min later the rate of change of glycogen phosphorylase activity following the addition of 1.3 mM $CaCl_2$ was determined. The data are presented as the rate of activation of glycogen phosphorylase *a* as a function of [SK&F 96365] and are the means ± SEM of three determinations, each from a separate cell preparation.

those required to inhibit vasopressin-stimulated plasma membrane Ca^{2+} inflow in hepatocytes (K. Fernando and G. J. Barritt, unpublished work) and the stimulation of plasma membrane Ca^{2+} inflow caused by a number of agonists in other cell types [10, 11]. These results, together with the apparent lack of additivity in the degree of stimulation of plasma membrane Ca^{2+} inflow observed in cells exposed to either vasopressin or to a combination of vasopressin and thapsigargin, provide some evidence that the Ca^{2+} inflow pathway in hepatocytes stimulated by thapsigargin is likely to be similar to that which is activated by vasopressin.

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