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# INS(1,4,5)$P_3$ AND GLUTATHIONE INCREASE THE PASSIVE $Ca^{2+}$ LEAK IN PERMEABILIZED A7R5 CELLS

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Thapsigargin depletes intracellular $Ca^{2+}$ stores by its inhibitory effect on the $Ca^{2+}$ pumps, which unmasks an aspecific $Ca^{2+}$ leak from the stores. This aspecific $Ca^{2+}$ permeability of the stores was further investigated using $^{45}Ca^{2+}$ fluxes on intact and permeabilized A7r5 smooth-muscle cells. Stores in intact cells were found to be more leaky for $Ca^{2+}$ than those in saponin-permeabilized or *Staphylococcus aureus* $\alpha$-toxin-permeabilized cells, which suggests that a cytosolic factor may be involved. Supplementing the medium bathing the permeabilized cells with a submaximal Ins(1,4,5)$P_3$ concentration increased the leakiness of the stores. Glutathione also increased the aspecific $Ca^{2+}$ leak. This effect occurred with both the reduced and the oxidized form but reduced glutathione was more effective. Our data show that basal Ins(1,4,5)$P_3$ levels and glutathione can contribute to the relatively high $Ca^{2+}$ leak in intact cells. The washing out of these substances during permeabilization can reduce the aspecific leakiness of the stores. 

Intracellular $Ca^{2+}$ stores have to continuously resequester the $Ca^{2+}$ that passively leaks out in order to maintain their $Ca^{2+}$ content. Interrupting this cycle by blocking the intracellular $Ca^{2+}$ pumps with thapsigargin leaves this aspecific $Ca^{2+}$ leak no longer compensated, and $Ca^{2+}$ will slowly be released [1]. There is evidence that not all cells in a population display the same leak rate, because blocking the endoplasmic-reticulum $Ca^{2+}$ uptake by lowering the cytosolic [$Ca^{2+}$] in intact mast cells only depleted the stores in about half of the cells [2].

Some cellular components that affect $Ca^{2+}$ homoeostasis are present in high concentrations in intact cells. Basal Ins(1,4,5)$P_3$ (Ins$P_3$) levels often exceed 1 micromolar [3-6], and are therefore sufficiently high to activate the Ins$P_3$ receptor. Glutathione levels range between 0.5 and 10 mM [7]. The relative concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) are not static but change with the physiological condition [8].

We previously observed that the stores in permeabilized A7r5 smooth-muscle cells lost their $Ca^{2+}$ slowly during incubation in a $Ca^{2+}$-free medium containing 2 $\mu$M thapsigargin at 25°C [9]. The $t_{1/2}$ of the monoexponential decay was as long as 18 min. This rather small aspecific $Ca^{2+}$ permeability contrasts with the much higher leak observed in many intact cells (e.g. parotid acinar cells, Ref 10). We have now compared, for A7r5 cells, the rate of aspecific $Ca^{2+}$ loss from the stores in intact and permeabilized cells, and observed that

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permeabilization of the plasma membrane decreased the aspecific $Ca^{2+}$ leak of the stores. Since permeabilization results in the loss of cellular components, we have then investigated whether the readdition of some cellular substances could affect the comparatively small aspecific $Ca^{2+}$ leak in our permeabilized system. The results indicate that basal levels of $InsP_3$ as well as glutathione could contribute to the relatively large $Ca^{2+}$ leak in intact cells.

## Materials and Methods

A7r5 aortic smooth-muscle cells were cultured as described [11]. They were seeded in twelve-well clusters at a density of approximately $10^4$ $cm^{-2}$. Experiments were carried out with confluent monolayers of cells on the 7th day after plating. The average cell density was then 7.5 x $10^4$ cells $cm^{-2}$.

$^{45}Ca^{2+}$ fluxes on intact cells were performed at 25°C by exposing the cells for 15 min to a depolarizing solution containing 140.9 mM KCl, 1.2 mM $MgCl_2$, 11.6 mM Hepes (pH 7.3), 11.5 mM glucose and 0.2 mM $CaCl_2$ (46 $\mu$Ci $ml^{-1}$) and then to an efflux medium of the following composition: 135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.6 mM Hepes (pH 7.3), 11.5 mM glucose and 2 mM EGTA. This efflux medium was replaced every 2 min.

Permeabilization with saponin was achieved by incubating the cells for 10 min in a solution containing 120 mM KCl, 30 mM imidazole (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA and 20 $\mu$g $ml^{-1}$ saponin at 25°C. Permeabilization with *Staphylococcus aureus* $\alpha$-toxin (supplied by Prof. N. Blanckaert, Laboratory of Biological Chemistry, and Prof. F. Vanstapel, Biomedical N.M.R. Unit, K. U. Leuven) was achieved by incubating the cells for 10 min in a solution containing 120 mM KCl, 30 mM imidazole (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 5 % dextran-4 and 20 $\mu$g $ml^{-1}$ of $\alpha$-toxin at 37°C. Dextran-4 was added to the solutions to prevent osmotic lysis [12]. The non-mitochondrial $Ca^{2+}$ stores were then loaded with $^{45}Ca^{2+}$ for 10 min in a medium containing 120 mM KCl, 30 mM imidazole (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 0.44 mM EGTA, 10 mM $NaN_3$ and 150 nM free $Ca^{2+}$ (23 $\mu$Ci $ml^{-1}$) at 25°C. The wells were then washed twice in an efflux medium containing 120 mM KCl, 30 mM imidazole (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA and 5 mM $NaN_3$ at 25°C. This efflux medium was then replaced every 2 min.

## Results

*Higher $Ca^{2+}$ leak in permeabilized cells as compared to intact cells-* Intact A7r5 cells, loaded to steady state with $^{45}Ca^{2+}$, slowly lost their $Ca^{2+}$ during incubation in a $Ca^{2+}$-free efflux medium supplemented with 2 mM EGTA. These cells express vasopressin receptors, which are linked to $Ca^{2+}$ mobilization via $InsP_3$ production [13]. Vasopressin stimulation in $Ca^{2+}$-free solution results in a rapid release of $^{45}Ca^{2+}$ from internal stores. This $Ca^{2+}$ was then extruded across the plasma membrane, inducing a 10-fold increase in the rate of $Ca^{2+}$ efflux from the cells (Fig 1*A*).

Supplementing the $Ca^{2+}$-free efflux medium with the endoplasmic-reticulum $Ca^{2+}$-pump inhibitor thapsigargin (2 $\mu$M) also increased the rate of $Ca^{2+}$ loss from the cells (Fig 1*B*). The subsequent application of 10 $\mu$M vasopressin was almost ineffective in further releasing $Ca^{2+}$. Thapsigargin therefore unmasked a $Ca^{2+}$ leak from the stores in intact A7r5 cells, which depleted the agonist-sensitive store in about 16 min.

Saponin-permeabilized cells also lost their $Ca^{2+}$ during incubation in an unlabelled efflux medium with a free $[Ca^{2+}]$ below the threshold for $Ca^{2+}$ reaccumulation (Fig 1*C*). Stimulating the stores with the maximal dose of 100 $\mu$M $InsP_3$ strongly increased the rate of

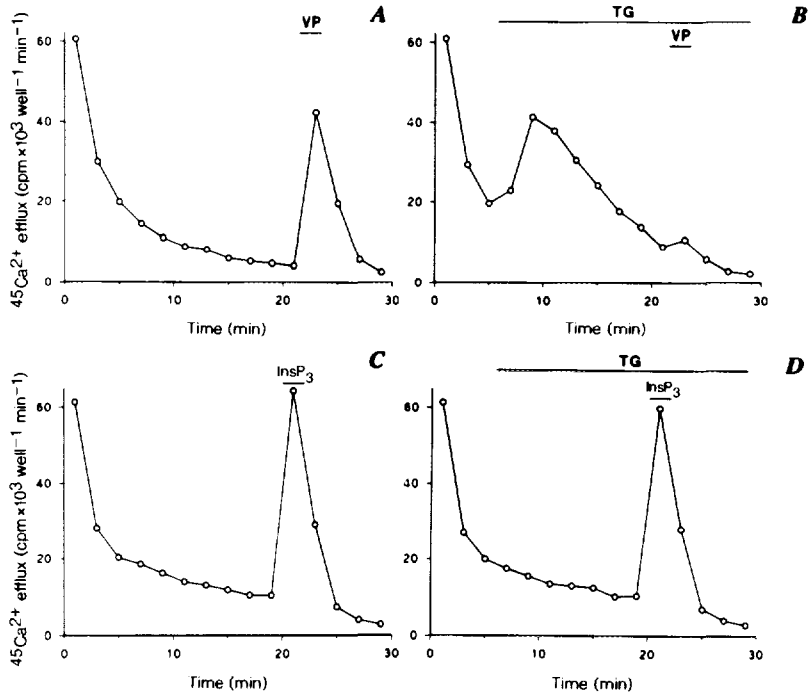


**Fig. 1. Higher passive $Ca^{2+}$ leak in intact as compared to permeabilized A7r5 cells.** In *A* and *B*, monolayers of intact A7r5 cells were loaded with $^{45}Ca^{2+}$, and from time 0 onwards incubated in a $Ca^{2+}$-free medium containing 2 mM EGTA. The cells were stimulated with 10 $\mu$M vasopressin (VP) and 2 $\mu$M thapsigargin (TG) as indicated. In *C* and *D*, the non-mitochondrial $Ca^{2+}$ stores of saponin-permeabilized cells were loaded with $^{45}Ca^{2+}$ and from time 0 onwards incubated in $Ca^{2+}$-free efflux medium containing 1 mM EGTA. 100 $\mu$M $InsP_3$ and 2 $\mu$M thapsigargin (TG) was added as indicated. The rate of $^{45}Ca^{2+}$ release from the monolayers is shown as a function of time. All experiments were done at 25°C. Typical for 4 experiments.

$Ca^{2+}$ loss. Thapsigargin (2 $\mu$M) had no effect on the basal rate of $Ca^{2+}$ efflux, nor on the $InsP_3$-induced increase in $Ca^{2+}$ efflux (Fig 1*D*), because the very low free [$Ca^{2+}$] of the efflux medium had already abolished all $Ca^{2+}$-pumping activity.

A comparison of Fig 1*B* and 1*D* reveals that a 16 min incubation in 2 $\mu$M thapsigargin depleted the vasopressin-sensitive store in intact A7r5 cells, but was unable to deplete the $InsP_3$-sensitive store when added to permeabilized cells. Since thapsigargin depletes $Ca^{2+}$ stores by unmasking an aspecific leak [1], this leak must be much higher in intact cells than in permeabilized cells.

We have compared the effect of membrane permeabilization by saponin with that by *Staphylococcus aureus* $\alpha$-toxin, because the latter toxin acts by making pores in the plasma membrane and not by a detergent effect. For making this comparison, permeabilization with $\alpha$-toxin was performed at 37°C in the presence of 5 % dextran-4. Maximal permeabilization under these conditions was achieved with 20 $\mu$g $ml^{-1}$ of the $\alpha$-toxin. The $Ca^{2+}$ leak from cells that were permeabilized with 20 $\mu$g $ml^{-1}$ $\alpha$-toxin was of the same magnitude as the $Ca^{2+}$ leak in cells permeabilized with 20 $\mu$g $ml^{-1}$ saponin (data not shown). The much smaller $Ca^{2+}$ leak in permeabilized cells as compared to intact cells (Fig 1*B* and 1*D*) was therefore not an artefact of the permeabilization procedure.

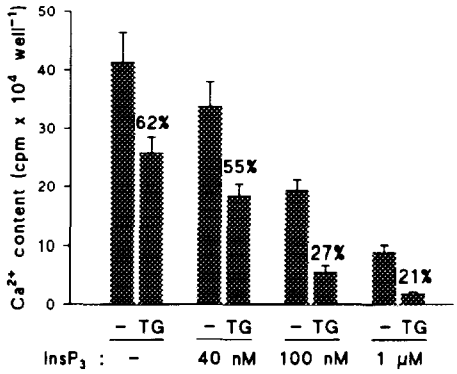


**Fig 2. The continuous presence of a submaximal $InsP_3$ concentration increases the basal $Ca^{2+}$ permeability of stores in permeabilized cells.** Stores were loaded for 20 min with $^{45}Ca^{2+}$ and then incubated for another 10 min in the same loading solution in the presence or absence of 2 μM thapsigargin (TG). All these incubations were performed either in the absence or in the presence of the indicated $InsP_3$ concentration. The $Ca^{2+}$ contents of the stores immediately after this 30-min incubation period were determined as described in Ref 11, and corrected for aspecific loading of the stores and aspecific binding to the outside of the stores. The latter values were taken as the amount of $^{45}Ca^{2+}$ bound to the cells after a 30 min incubation in a loading medium containing 2 μM thapsigargin. Means ± S.E.M. for 3 independent experiments are shown (each experiment was done in duplicate). The values indicated above the bars of the thapsigargin-treated cells express the $Ca^{2+}$ content of these cells as a percentage of the $Ca^{2+}$ content of the control cells that were not treated with thapsigargin.

*$InsP_3$ increases the basal $Ca^{2+}$ leak in permeabilized cells*- The continuous presence of low concentrations of $InsP_3$, the messenger for releasing $Ca^{2+}$ from internal stores [14], can induce a slow release mode [15-18]. It is therefore possible that the higher aspecific $Ca^{2+}$ permeability in intact cells as compared to permeabilized cells is due to a continuous submaximal activation of the $InsP_3$ receptor by basal $InsP_3$ levels. Intact cells indeed have relatively high basal $InsP_3$ levels, which often exceed 1 micromolar [3-6]. This $InsP_3$ is freely diffusible in the cytoplasm [6]. We have previously observed that the endogenous $InsP_3$ concentration was less than 1 nM in permeabilized A7r5 cells [19]. We have therefore investigated whether increasing the basal level of $InsP_3$ in our saponin-permeabilized system would have an effect on the aspecific $Ca^{2+}$ leak. The data in Fig 2 illustrate that the leakiness of the stores in permeabilized cells can indeed be increased by the continuous presence of $InsP_3$. Control stores (left two bars of Fig 2) were loaded to steady state with $^{45}Ca^{2+}$ in the absence of added $InsP_3$. The stores were subsequently incubated for 10 min in the presence or absence of 2 μM thapsigargin. This thapsigargin treatment decreased the $Ca^{2+}$ content of the control stores (i.e. in the absence of added $InsP_3$) to 62 ± 2 % (n=9) of their original value. The stores were also loaded with $^{45}Ca^{2+}$ in a medium containing 40 nM, 100 nM or 1 μM $InsP_3$. Further incubating the stores for 10 min with 2 μM thapsigargin in the continuous presence of $InsP_3$ decreased their $Ca^{2+}$ content to respectively 55, 27 and 21 % of the value obtained in the absence of thapsigargin. More $Ca^{2+}$ was therefore released by thapsigargin in the continuous presence of $InsP_3$ than in its absence, indicating that stores became more leaky in the presence of $InsP_3$. Similar results were obtained with 750 nM Ins(2,4,5)$P_3$, a poorly metabolizable inositol phosphate analogue [20] (data not shown).

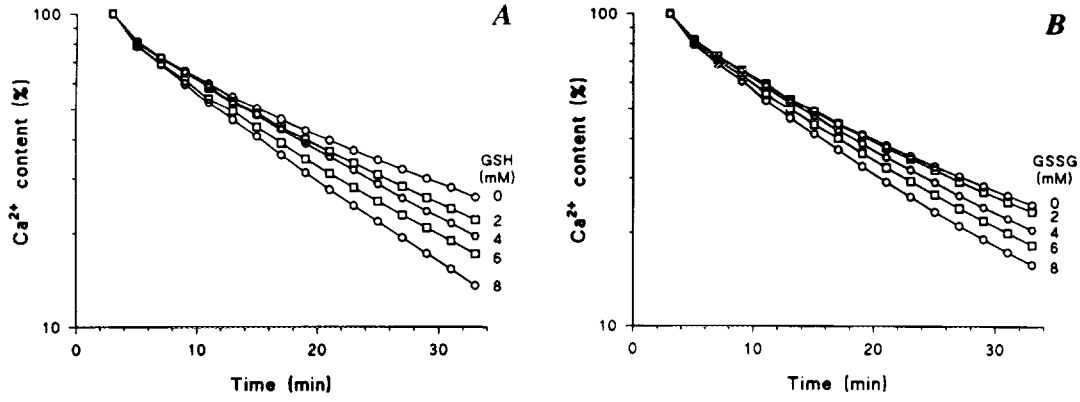


**Fig. 3. Glutathione increases the aspecific $Ca^{2+}$ leak from intracellular stores.** *A* shows how supplementing the efflux medium with the indicated GSH concentration (0-8 mM) increased the rate of aspecific $Ca^{2+}$ release from the stores. *B* shows how GSSG (0-8 mM) increased the rate of aspecific $Ca^{2+}$ release from the stores. Typical for 4 experiments.

*Glutathione increases the basal leak*- Glutathione levels in intact cells range between 0.5 and 10 mM [7], a concentration which affects $Ca^{2+}$-transport processes of internal stores [21-23]. Since endogenous glutathione will be washed out during saponin-treatment, we have studied the effect of its readdition on the aspecific $Ca^{2+}$ leak from the stores. Supplementing the efflux medium with increasing concentrations of GSH increased the aspecific $Ca^{2+}$ leak (Fig 3*A*). The addition of increasing concentrations of GSSG (Fig 3*B*) also increased the rate of aspecific $Ca^{2+}$ efflux from the stores, although GSSG was less potent than GSH. Since both GSH and GSSG exerted a rather similar effect, it seems unlikely that these effects are brought about by an interaction with critical sulfhydryl groups.

## Discussion

The main finding of this work is that the $Ca^{2+}$ stores in intact cells display a much higher inherent leakiness for $Ca^{2+}$ than those in permeabilized cells. At least two cellular components can, in our permeabilized system, increase the aspecific $Ca^{2+}$ leak: $InsP_3$ and glutathione. Based on our finding that the aspecific $Ca^{2+}$ leak in permeabilized cells can be increased by a submaximal but continuous stimulation of the $InsP_3$ receptor, we suggest that the relatively high basal levels of $InsP_3$ occurring in intact cells [3-6] could contribute to the high $Ca^{2+}$ leak in intact A7r5 cells.

We previously observed that the $EC_{50}$ for $InsP_3$-induced $Ca^{2+}$ mobilization in permeabilized A7r5 cells was much higher than in many other systems [17], at least when the assay was done in a thapsigargin-containing efflux medium with a free [$Ca^{2+}$] of 4 nM. The half maximal dose for $InsP_3$-induced $Ca^{2+}$ mobilization from stores with functionally active $Ca^{2+}$ pumps, and at a cytosolic $Ca^{2+}$ concentration similar to that in resting intact cells, was only about 100 nM (Fig 2). This concentration is similar to the value found in other systems. This higher apparent sensitivity in the present study, as compared to experiments in which $InsP_3$ was added to the $Ca^{2+}$-free efflux medium could be explained by our earlier

observations that the $InsP_3$ receptor in A7r5 cells is stimulated by cytosolic [9] as well as by luminal $Ca^{2+}$ [17].

The proposal that the $InsP_3$ receptor is contributing to the continuous $Ca^{2+}$ leak induced by thapsigargin is only valid if the $InsP_3$-sensitive store can slowly release its $Ca^{2+}$. The rapid $Ca^{2+}$ release in A7r5 cells is indeed followed by a second phase of much slower release [17]. A phase of continuous $Ca^{2+}$ release was also observed in permeabilized hepatocytes [15], basophilic leukemia cells [16] and L1210 lymphoma cells [18]. The rather slow pace-maker [$Ca^{2+}$] rise upon photoreleasing a small amount of $InsP_3$ in *Xenopus* [24] and mouse [25] oocytes indicates that a continuous release also occurs in intact cells. The $InsP_3$ receptor will only contribute to the aspecific $Ca^{2+}$ leak from stores if the basal level of $InsP_3$ is within a range which will activate the receptor. Basal $InsP_3$ levels exceeding 1 micromolar are indeed found in various cell types [3-6].

Glutathione also increased the aspecific $Ca^{2+}$ leak. These effects on the basal $Ca^{2+}$ leak occurred at physiological concentrations of glutathione [7,8]. Our experiments suggest that the redox state of glutathione is of lesser importance for this effect, since both GSH and GSSG increased the leak, although GSH seemed to be more effective. The physiological relevance of the increase of the aspecific $Ca^{2+}$ leak by glutathione needs to be further investigated.

One can only speculate about the physiological role of the aspecific $Ca^{2+}$ leak from the stores. Thapsigargin used in these experiments is an exogenous $Ca^{2+}$-pump inhibitor and could mimic, to some extent, the effects of endogenous pump inhibitors, such as unphosphorylated phospholamban [26,27] and ADP [28]. A periodic $Ca^{2+}$ release from the endoplasmic reticulum through this aspecific leak pathway related to oscillations in the ATP/ADP ratio and hence in $Ca^{2+}$-pump activity, has already been reported in permeabilized RINm5F insulinoma cells supplemented with a glycolyzing cell-free muscle extract [29].

In conclusion, we have found that stores in intact cells are more leaky for $Ca^{2+}$ than those in permeabilized cells. It is possible that the relatively high basal intracellular $InsP_3$ levels, as well as the millimolar concentrations of intracellular glutathione, contribute to this high $Ca^{2+}$ leak.

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