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Inositol 1,4,5-Trisphosphate and Basal Ca^{2+} Release Is Affected by the Cytoplasmic Concentration of Cl^- in Endothelial Cells

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The effects of varying Cl^- concentration in the intracellular bathing medium, on IP_3-induced $^{45}Ca^{2+}$ release from internal stores, were examined in saponin-permeabilised bovine aortic endothelial (BAE) cells. Results from this study show that the release of Ca^{2+} from the internal stores is affected by the cytoplamic concentration of Cl^- ions. Complete replacement of Cl^- with gluconate augmented IP_3 (3 μM)-induced $^{45}Ca^{2+}$ release by 33 ± 8%. Replacement of both Cl^- and K^+ with gluconate and NMG, respectively, had no significant effect on $^{45}Ca^{2+}$ release. However, resting levels of internal $^{45}Ca^{2+}$ were found to be affected by Cl^- removal. These data suggest that in BAE cells, IP_3 and also basal $^{45}Ca^{2+}$ release may be regulated by the physiological intracellular Cl^- concentration. © 1997 Academic Press

Key Words: **bovine aortic endothelial cells; inositol 1,4,5-trisphosphate; intracellular Ca^{2+} release; cytoplasmic Cl^-; Ca^{2+}-induced-Ca^{2+} release; ryanodine.**

Endothelial cells respond to a variety of agonist and physical stimuli. Receptor activation at the plasma membrane causes an elevation of intracellular Ca^{2+} and this process is known to underlie the production of vasoactive substances, which themselves affect adjacent vascular smooth muscle cells, and play a key role in the control of vascular tone (1). In most cell types studied, the Ca^{2+} required for cell activation is derived from Ca^{2+} influx across the plasma membrane and/or Ca^{2+} release from the endoplasmic reticulum (ER) (2,3). IP_3-induced Ca^{2+} release from the ER has been demonstrated to play an essential role in intracellular signalling (4). In general, agonist stimulation leads to the production of IP_3 which binds to the ER membrane causing the release of stored Ca^{2+} via the IP_3 receptor/Ca^{2+} release channel (4). In some cell types, a Ca^{2+}-induced-Ca^{2+} release (CICR) mechanism mediated by ryanodine/Ca^{2+} release channels (RyR's) also operates (2,4,5). CICR has been difficult to demonstrate in endothelial cells, although a pharmacologically novel form of CICR has been reported (6). In endothelial cells, this Ca^{2+} release can lead to the generation of complex Ca^{2+} oscillations (7) that may encode specific signalling information to different intracellular targets (8). These oscillations are thought to be controlled by complex positive and negative feedback mechanisms on the IP_3/Ca^{2+} release channel involving cytoplasmic and intra luminal Ca^{2+} (2,9,10). A number of investigations have suggested that Ca^{2+} release from the SR may also be regulated by the ionic composition of the intracellular environment and in particular by K^+ and Cl^- (for review, see 11). Mathematical models have been developed for non-excitable cells, where movements of K^+ and Cl^- across the ER membrane have been predicted to play a critical role in dampening Ca^{2+} oscillations (12).

In skeletal and cardiac muscle, ionic movements associated with the uptake and release of stored Ca^{2+} have been examined. Cl^- and K^+ have been shown to affect the release of stored Ca^{2+} in a number of different cell types (11). Early proposals have mainly focussed on K^+ and Cl^- being involved with maintaining electroneutrality across the internal store membranes during Ca^{2+} release. It has been suggested that Ca^{2+} release from the SR is accompanied by ionic currents whose function may be to dissipate the potential difference that would be expected to occur when positively

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Abbreviations used: BAE, bovine aortic endothelial; IP_3, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; CICR, Ca^{2+}-induced-Ca^{2+} release; DMEM, Dulbecco's modified Eagles medium; gluconate, D-gluconic acid (2,3,4,5,6-Pentahydroxy-caproic acid); NMG, N-methyl glucosamine; SDS, sodium dodecyl sulphate; cpm, counts per min; SERCA, sarco/endoplasmic reticulum ATPase; RyR, ryanodine receptor/Ca^{2+} release channel.

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charged Ca^{2+} vacated the internal stores (13-16). Alternatively, K^+ and or Cl^- may interact with the internal Ca^{2+} release channels on the ER and directly affect Ca^{2+} release.

We have recently shown that K^+ movements play a role in regulating Ca^{2+} release in BAE cells (17,18). However, the role of Cl^- in regulating IP_3-induced Ca^{2+} release in endothelial cells, has not been addressed. This report concerns the measurement of IP_3-induced $^{45}Ca^{2+}$ release from internal Ca^{2+} stores in saponin-permeabilised endothelial cells. The permeabilised cell approach allows precise manipulation of the intracellular solution surrounding the ER without the possibility of altering ER proteins during cell fractionation and channel purification. If Cl^- affects intracellular Ca^{2+} release mechanisms, then by substituting Cl^- with a large impermeant anion such as gluconate, IP_3-induced Ca^{2+} release from the internal stores should be affected. The possibility that the intracellular Cl^- concentration regulates Ca^{2+} release in endothelial cells is discussed.

METHODS

Tissue preparation and cell culture techniques were carried out as previously described [12]. Culture medium was removed and cells washed in a balanced salt solution (BSS): (mM) 135 NaCl, 5.9 KCl, 12 Hepes, 1.5 $CaCl_2$, 1.2 $MgCl_2$, and 10 D-glucose (pH 7.3), before being placed on a mechanical shaker. Cells were permeabilised at room temperature (22°C) with 40μg/ml saponin for 10 min in 'skinning solution' (mM); 120 KCl, 10 Hepes, 2 $MgCl_2$, 1 ATP and 1 EGTA (pH 7.0). The efficiency of permeabilisation was routinely checked using trypan blue. Permeabilised cells were then loaded with $^{45}Ca^{2+}$ (10μCi ml^{-1}) in 'loading buffer' (mM) 120 KCl, 10 Hepes, 5 $MgCl_2$, 5 ATP, 0.44 EGTA, 5 NaN_3 and 0.12 $CaCl_2$ (pH 6.88) for 12 min at 22°C and subsequently washed 3 times in isotope free ice cold 'loading buffer' to terminate loading. $^{45}Ca^{2+}$ efflux was into a solution containing (mM): 120 KCl (substituted with gluconate or NMG where appropriate), 10 Hepes, 3 EGTA, 5 NaN_3, 1 ATP (pH 6.88). When adjusting pH, care was taken to avoid introducing contaminating ions to the solutions. NaN_3 was used to inhibit the uptake of $^{45}Ca^{2+}$ into mitochondrial stores.

Concentrations of $CaCl_2$ and $MgCl_2$ required to give a free $[Ca^{2+}]$ of 100nM were calculated using the REACT programme (19). Solutions were exchanged every 2 minutes and bi-directional $^{45}Ca^{2+}$ flux (i.e. in the absence of the Ca^{2+}-ATPase inhibitor, thapsigargin (20)) during that interval was measured by liquid scintillation counting. $^{45}Ca^{2+}$ remaining in the cells at the end of the experiment was determined by solubilising the cells with 2% SDS in distilled water. The loss of $^{45}Ca^{2+}$ at each time point (i.e. fractional loss) was calculated from the cpm as a fraction of the total $^{45}Ca^{2+}$ remaining (including the SDS fraction) within the cells at that time point (9,21). Changes in fractional loss (Δ) were calculated as the difference between the fractional loss at the peak of the stimulus response and the fractional loss at the point immediately preceeding the stimulus. IP_3-dependent $^{45}Ca^{2+}$ release was induced by addition of a 2 min pulse of IP_3 after a steady baseline efflux was obtained. Experiments were performed in the continuous presence of modified solutions at 22°C as semi-intact cell preparations tend to deteriorate more rapidly at higher temperatures (22).

All chemicals used were obtained from Sigma, UK. Tissue culture materials were from Gibco Ltd., UK. $^{45}Ca^{2+}$ was purchased from ICN Biomedicals Inc., California.

For each experiment the control response was taken to be that obtained in 120 mM KCl. Data are expressed as mean ± standard error of the mean (S.E.M.). Where appropriate, raw data were analysed using paired t tests. A p value < 0.05 was considered significant and denoted by *.

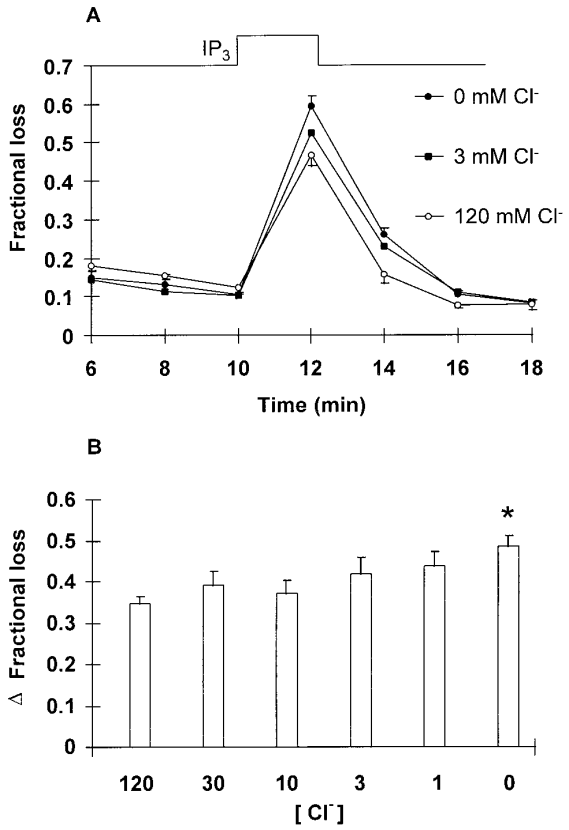

FIG. 1. (A) IP_3-induced $^{45}Ca^{2+}$ release from saponin-permeabilised BAE cells. Mean data showing the fractional loss of $^{45}Ca^{2+}$ in different bathing solutions where the concentration of Cl^- was changed whilst maintaining a constant K^+ concentration. Where indicated (by the horizontal bar), a 2 min pulse of IP_3 (3 μM) was applied (n = 8). (B) Mean data following the protocol described in (A) where Δ fractional loss was determined for solutions containing the Cl^- concentrations indicated. The response in 120 K^+ was taken as the control value (n = 8).

RESULTS

Cl^- sensitivity of IP_3-induced $^{45}Ca^{2+}$ release. Figure 1A shows typical data from $^{45}Ca^{2+}$ efflux experiments in which cells were bathed in a standard intracellular solution containing either 0, 3 or 120 mM Cl^- and where indicated, the cells were exposed to a 2 min pulse of 3 μM IP_3. If the intracellular concentration of Cl^- regulates the release of Ca^{2+} during stimulation, then removal of Cl^- from the bathing solution should affect $^{45}Ca^{2+}$ release. Complete replacement of Cl^- with gluconate significantly increased mean IP_3-evoked $^{45}Ca^{2+}$ release by 30% (●) compared with the control response (○) ($p < 0.005$, paired t test, n = 8). The sensitivity of the release process to a range of Cl^- concentrations

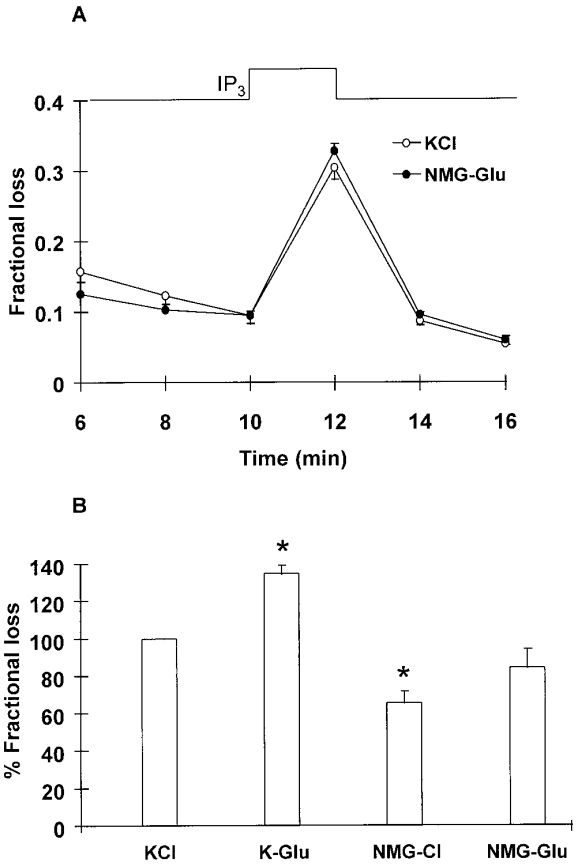

FIG. 2. (A) IP_3-induced $^{45}Ca^{2+}$ release from saponin-permeabilised BAE cells. Mean data showing the fractional loss of $^{45}Ca^{2+}$ in a solution containing either 120 mM KCl^- (○) or NMG gluconate (●). Where indicated (by the horizontal bar), a 2 min pulse of IP_3 (3 μM) was applied (n = 4). (B) Data obtained following the same protocol and used to construct a histogram showing how removal of K^+, Cl^-, or both K^+ and Cl^- together affects Δ fractional loss of $^{45}Ca^{2+}$ (n = 8).

was determined by varying Cl^- in the efflux buffer from 0 to 120 mM and measuring the $^{45}Ca^{2+}$ released by a standard dose of IP_3. Figure 1B shows that as the concentration of Cl^- is reduced there is a progressive increase in IP_3-induced $^{45}Ca^{2+}$ loss (n = 8). These observations show that IP_3-induced $^{45}Ca^{2+}$ release is dependent on the intracellular Cl^- concentration bathing the internal stores.

We have reported that, in BAE cells, complete substitution of K^+ with the large cation substitute NMG, causes a significant reduction in the measured IP_3 response (17). Therefore, the effects of replacing both Cl^- and K^+ with gluconate and NMG in the same experiment, were investigated and the raw data presented in Figure 2A (n = 4). Figure 2B shows mean data summarising the effects of intracellular ion substitutions on IP_3-induced $^{45}Ca^{2+}$ release. Ordinate shows the peak change (△) in fractional loss as a percentage of the KCl control value of 100%. Substituting Cl^- with gluconate caused a significant increase in the response size (33%, $p < 0.05$) and substituting K^+ with NMG caused a significant decrease of a similar magnitude (30%, $p < 0.05$). Removal of both K^+ and Cl^- was found to have no significant affect on the IP_3-induced fractional loss of $^{45}Ca^{2+}$ (n = 8) because the two equal and opposite affects of removing K^+ and Cl^-, effectively cancel each other out.

Assuming that a movement of Cl^- from the internal stores is required to balance the loss of Ca^{2+} during stimulation, the positive effect of Cl^- removal on $^{45}Ca^{2+}$ loss could be due to Cl^- depletion from the internal stores. If this were the case, depleting the internal stores of Cl^- would be expected to alter $^{45}Ca^{2+}$ efflux. To test this hypothesis, prior to applying a standard pulse of IP_3, the cells were pre-incubated for varying times in Cl^- free intracellular bathing solution. Prolonged exposure to a Cl^- free intracellular environment would be expected to empty the intracellular stores of Cl^- by increasing the chemical gradient for Cl^- movement from the internal stores. If Cl^- does act as a co-ion in the Ca^{2+} release process, this protocol may be expected to reduce the measured IP_3-induced $^{45}Ca^{2+}$ release.

Figure 3 shows combined data from 3 experiments where cells were incubated in Cl^- free solution for either 0, 2, 6 or 10 min before being challenged with a standard pulse of IP_3. The IP_3 was given in the control solution containing 120 mM K^+ and Cl^-. Pre-incubation for varying amounts of time in Cl^- free solution had no affect on the measured IP_3 response. Assuming effective emptying of the intracellular Cl^- stores, this observation suggests that the Cl^- effects reported are not due to Cl^- store depletion., Alternatively, the K^+ counter-current may be able to compensate for the absence of intracellular Cl^- movements.

Cl^- sensitivity of basal $^{45}Ca^{2+}$ release. To establish whether the intracellular Cl^- concentration plays any part in maintaining the resting cytoplasmic Ca^{2+} con-

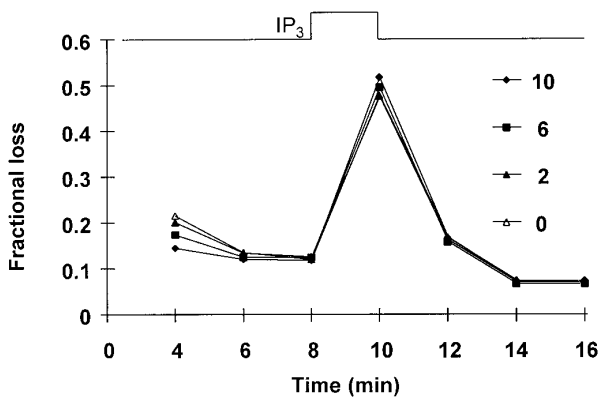

FIG. 3. Combined data showing the effect of pre-incubation in a Cl^--free solution for 0, 2, 6, or 10 min prior to being challenged with a standard pulse of IP_3 at the point indicated by the horizontal bar (n = 3).

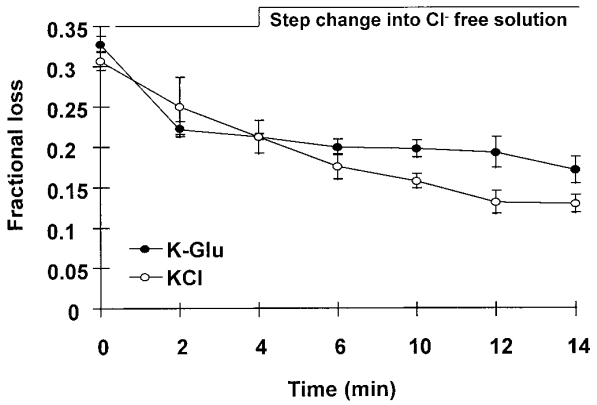

FIG. 4. Mean data showing how a step change into Cl^--free solution (●) (at the time indicated by the horizontal bar) significantly affects the basal efflux of $^{45}Ca^{2+}$ compared to control (○) (n = 3).

centration, Cl^- was removed from the bathing solution at the point indicated by the horizontal bar and and basal $^{45}Ca^{2+}$ efflux was measured (Figure 4). Removal of Cl^- led to a sustained and significant increase in basal $^{45}Ca^{2+}$ leak over a period of 10 min ($p < 0.05$, n = 3). This observation suggests, that in BAE cells, the intracellular Cl^- concentration may also influence resting Ca^{2+} concentrations and therefore indirectly regulate the basal production of vasoactive substances.

DISCUSSION

Ca^{2+} release from the internal stores in BAE cells is a complex process that may involve counter-ion movements. The data presented show that IP_3-induced Ca^{2+} release in BAE cells is influenced by the intracellular concentration of Cl^-. Additionally, it is shown that the intracellular Cl^- concentration can influence basal $^{45}Ca^{2+}$ release in BAE cells.

It is possible that Cl^- serves as a co-ion to stabalise ER membrane potential during large transmembrane Ca^{2+} movements induced by IP_3. Alternatively, Cl^- may regulate the IP_3/Ca^{2+} release channel directly, by functioning as a 'co-factor' during channel activation. The possibility should also be considered that gluconate, the anion substitute used in these experiments, may itself block the IP_3/Ca^{2+} release channel or have other pharmacological properties.

A number of investigations, on skeletal muscle, have identified Cl^- channels on the SR membranes. For example Kourie et al (1996) found a novel type of Cl^- channel from rabbit SR that showed voltage dependent activation and inactivation characteristics (23). Planar lipid bilayer techniques have been used to obtain single channel recordings of Cl^- channels derived from skeletal (24,25) and cardiac muscle SR (26). It has generally been assumed that these proteins are involved in dissipating potential differences that occur across the internal Ca^{2+} store membranes. Such ionic redistributions have also been proposed in a variety of non-excitable cells such as hepatocytes (27), brain microsomes (28,29) and platelet membrane vesicles (30).

The experiments described in this paper are consistent with Cl^- regulating the release of stored $^{45}Ca^{2+}$ from the internal stores in BAE cells. However, at present, it is not possible to say whether the Cl^- effects observed are due the disruption of a co-current mechanism or via a direct effect of Cl^- removal on the Ca^{2+} release channels in the internal store membranes. Recent reports have shown that $[^3H]IP_3$ binding to cerebellar microsomes is dependent on the ionic species present and have questioned the role of K^+ as a counter-ion required for Ca^{2+} release (31). These authors have postulated the existence of putative K^+ ion binding sites on the IP_3/Ca^{2+} release channel that are occupied by intracellular K^+ ions and enhance Ca^{2+} release channel activity. Since IP_3 receptors can be purified and reconstituted into vesicles whilst retaining their Ca^{2+} releasing properties (32), there is clearly no absolute requirement for Cl^- or K^+ channels to be present in order to sustain IP_3-induced Ca^{2+} release (31). Here we have shown that depleting intracellular Cl^- stores by pre-incubation in a Cl^--free bathing solution, has no discernable affect on subsequent $^{45}Ca^{2+}$ release in response to IP_3. This observation, in conjunction with the published literature, argues against Cl^- acting as a co-ion and leads us to speculate on a putative role for Cl^- as an additional 'co-factor' in IP_3 channel modulation.

Our results indicate that in BAE cells, not only IP_3, but also basal $^{45}Ca^{2+}$ release is regulated by the concentration of Cl^- in the bathing medium. Cl^- has long been known to elicit the release of stored Ca^{2+} from various types of purified skeletal muscle SR (33-36). It has generally been assumed that the mechanism by which Cl^- is able to cause the release of stored Ca^{2+} is linked to ionic shifts across the internal store membranes.

In muscle, the RyR provides a CICR mechanism whereby Ca^{2+} feeds forward to release further stored Ca^{2+} in an amplification process (2,3). Recently a close correlation between RyR activity and Cl^- concentration has been demonstrated (37). Cl^- has been shown to sensitise the RyR to Ca^{2+} and thus provide an explanation for the phenomena of Cl^--induced Ca^{2+} release from SR membranes (38). However, results from the present study show the opposite effect to that obtained in skeletal muscle. In BAE cells, Ca^{2+} removal *increases* basal $^{45}Ca^{2+}$ release. Reasons for this discrepancy are, at present, unclear but there are at least two possible explanations. Firstly, RyR's do exist in BAE cells but are regulated by Cl^- in an entirely opposite way to muscle RyR's, and secondly, primary cultures of BAE cells do not express RyR's and the effects observed are mediated entirely by IP_3 receptors. The first option seems implausable. Furthermore, functional

RyR's have yet to be convincingly demonstrated in freshly isolated endothelial cells of any type. This leads us to favour the second option and propose that IP_3/Ca^{2+} release channels, in BAE cells, are partially inactivated by physiological concentrations of intracellular Cl^-. It is noteworthy that RyR channels (and probably IP_3 channels as they share considerable homology (39)) are non-specifically sensitised to Ca^{2+} when reconstituted into liposomes or planar lipid bilayers (38). These effects previously descibed, could be attributed to disturbances of the putative Cl^- binding sites during receptor purification and reconstitution.

In summary, the present study shows, that in endothelial cells, the Cl^- concentration in the cytoplasm may be able to influence the basal and IP_3-induced release of stored Ca^{2+}. Thus intracellular Cl^- concentration could indirectly affect cell activation and the production of vasoactive substances.

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