Role of endothelial Ca\(^{2+}\) stores in the regulation of hydraulic conductivity of Rana microvessels in vivo

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Submitted 11 July 2002; accepted in final form 10 December 2002

Glass, C. A. and D. O. Bates. Role of endothelial Ca\(^{2+}\) stores in the regulation of hydraulic conductivity of Rana microvessels in vivo. Am J Physiol Heart Circ Physiol 284: H1468–H1478, 2003. First published January 2, 2003; 10.1152/ajpheart.00585.2002.—Vascular permeability is regulated by endothelial cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)). To determine whether vascular permeability is dependent on extracellular Ca\(^{2+}\) influx or release of Ca\(^{2+}\) from stores, hydraulic conductivity (L\(_{p}\)) was measured in single perfused frog mesenteric microvessels in the presence and absence of Ca\(^{2+}\) influx and store depletion. Prevention of Ca\(^{2+}\) uptake into stores by sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibition increased L\(_{p}\) in the absence of extracellular Ca\(^{2+}\) influx. L\(_{p}\) was further increased when Ca\(^{2+}\) influx was restored. Depletion of the Ca\(^{2+}\) stores with ionomycin and SERCA inhibition increased L\(_{p}\) in the presence and the absence of extracellular Ca\(^{2+}\) influx. However, store depletion in itself did not significantly increase L\(_{p}\) in the absence of active Ca\(^{2+}\) release from stores into the cytoplasm. There was a significant positive correlation between baseline permeability and the magnitude of the responses to both Ca\(^{2+}\) store release and Ca\(^{2+}\) influx, indicating that the Ca\(^{2+}\) regulating properties of the endothelial cells may regulate the baseline L\(_{p}\). To investigate the role of Ca\(^{2+}\) stores in regulation of L\(_{p}\), the relationship between SERCA inhibition and store release was studied. The magnitude of the L\(_{p}\) increase during SERCA inhibition significantly and inversely correlated with that during store release by Ca\(^{2+}\) ionophore, implying that the degree of store depletion regulates the size of the increase on L\(_{p}\). These data show that microvascular permeability in vivo can be increased by agents that release Ca\(^{2+}\) from stores in the absence of Ca\(^{2+}\) influx. They also show that capacitative Ca\(^{2+}\) entry results in increased L\(_{p}\) and that the size of the permeability increase can be regulated by the degree of Ca\(^{2+}\) release.

calcium influx; store calcium release; endothelium
Calculation of $L_p$ was calculated from the Starling equation, where $\Delta P$ is the hydrostatic pressure difference, $\Delta \pi$ is the oncotic pressure difference between the capillary lumen and the interstitium, and $\sigma$ is the oncotic reflection coefficient. The effective oncotic pressure ($\sigma \Delta \pi$) of 1% BSA is 3.6 cmH$_2$O.

$$L_p = \frac{(Jv/A)}{(\Delta P - \sigma \Delta \pi)}$$  \hspace{1cm} (2)

**Statistics**

Baseline values are expressed as the mean $L_p$ during the time perfused. All other perfusion treatments are expressed as the peak (highest) $L_p$ value. Peaks and baselines were compared on the basis of repeated-sample measures analysis (2). Where there is more than one transient increase in $L_p$, the peak of the largest transient increase from the baseline was used. Pooled data are expressed as means $\pm$ SE. A nonparametric paired Wilcoxon test was used to compare $L_p$ measurements and the nonparametric Spearman rank test to look for correlations between data groups unless otherwise stated.

**RESULTS**

**Does Inhibition of Ca$^{2+}$ Reuptake Into ER Increase $L_p$ in Absence of Extracellular Ca$^{2+}$ Influx and Does CCE Increase $L_p$?**

**Effects of TG.** To determine whether Ca$^{2+}$ store release was sufficient to increase $L_p$ in the absence of Ca$^{2+}$ influx, the irreversible SERCA inhibitor 100 nM TG was perfused with the nonspecific cation channel blocker 5 mM NiCl$_2$ (Ni$_{2+}$). TG (100 nM) has previously been shown in this laboratory to transiently increase $L_p$ in the presence of Ca$^{2+}$ influx (25). Vessels were perfused with 1% BSA in FR and rat erythrocytes for a variable time period until the baseline $L_p$ stabilized and then perfused with TG and Ni$_{2+}$ for 20 min. During all Ni$_{2+}$ perfusions, 5 mM Ni$_{2+}$ was also added to the superfusate. To determine whether CCE induced by depleted Ca$^{2+}$ stores could stimulate increased $L_p$, the vessels were then perfused with 1% BSA in normal FR (and normal FR superfusion) to wash out the Ni$_{2+}$ and allow Ca$^{2+}$ influx for a further 20 min (with continued SERCA inhibition).

Figure 1A shows an example of the effects of TG perfusion on $L_p$ in the presence and absence of Ca$^{2+}$ influx in a single microvessel. The $L_p$ transiently increased during perfusion with TG in the presence of Ni$_{2+}$ and reached a maximum (peaked) at 10 min, returning to baseline after 15 min. $L_p$ increased again when Ni$_{2+}$ was removed to allow CCE. Approximately 10 min after the removal of Ni$_{2+}$, the $L_p$ peaked and then recovered to baseline over a further 10 min. In 11 vessels, when perfused with TG and Ni$_{2+}$, the $L_p$ increased from a means $\pm$ SE 0.9 $\pm$ 0.2 $\times$ 10$^{-7}$ to 3.6 $\pm$ 1.0 $\times$ 10$^{-7}$ cm$\cdot$s$^{-1}$·cmH$_2$O$^{-1}$ ($P < 0.05$, $n = 11$). When Ni$_{2+}$ was washed out in these vessels, the mean value of the highest (peak) $\pm$ SE $L_p$ was 6.3 $\pm$ 1.9 $\times$ 10$^{-7}$ cm$\cdot$s$^{-1}$·cmH$_2$O$^{-1}$ ($P < 0.005$, $n = 10$), as summarized in Fig. 1B. No $L_p$ increase was measured over 60 min when vessels were perfused with either 1% BSA (baseline solution) or 0.05% DMSO vehicle in baseline solution. Interestingly, the baseline $L_p$ significantly corre-
Fig. 1. Permeability can be increased both by store depletion and capacitative Ca\(^{2+}\) entry (CCE). A: hydraulic conductivity (L\(_p\)) measurements from a single microvessel perfused with 100 nM thapsigargin (TG) and 5 mM Ni\(^{2+}\) for 20 min, and then TG alone for at least 10 min. The peak values taken for comparison between vessels are indicated. A transient L\(_p\) increase was measured in the presence of TG and Ni\(^{2+}\) and a second larger transient increase in L\(_p\) in the presence of TG alone. B: mean baseline L\(_p\) [bovine serum albumin (BSA)] and peak L\(_p\) with TG/Ni\(^{2+}\) and TG alone. Values are means \(\pm\) SE. *P < 0.05, ***P < 0.005. Only significant differences are shown. C: correlation between BSA baseline L\(_p\) and peak TG/Ni\(^{2+}\) L\(_p\) (r = 0.73, P < 0.005, n = 12). Inset: relationship between baseline and peak when baseline was low. D: correlation between BSA baseline L\(_p\) and peak TG L\(_p\) (r = 0.87, P = 0.0005, n = 10). Inset: relationship between baseline and peak when baseline was low.

lataed with peak L\(_p\) during perfusion with TG and Ni\(^{2+}\) (r = 0.73, P < 0.005, n = 12, Spearman rank correlation coefficient, Fig. 1C), suggesting that the greater the leak of Ca\(^{2+}\) from intracellular stores, the greater the permeability. This was still true if the extreme data point was removed (r = 0.61, P < 0.05, n = 11, Fig. 1C, inset). This correlation appeared to be more specific for vessels with baseline L\(_p\) of >1. Furthermore, the baseline L\(_p\) also correlated with the peak L\(_p\) during perfusion with TG in the absence of Ni\(^{2+}\) (r = 0.87, P = 0.0005, n = 10, Fig. 1D). Again this was true if the extreme data point is removed (r = 0.76, n = 9, Fig. 1D, inset). This suggests that CCE stimulated a greater increase in vessels with a higher baseline L\(_p\).

There was no significant correlation found between the peak L\(_p\) during TG and Ni\(^{2+}\) perfusion and TG alone.

Effects of CPA. The same protocol was used with the reversible SERCA inhibitor CPA (30 \(\mu\)M) to confirm the findings in Fig. 1. Vessels were perfused with 1% BSA in FR and rat erythrocytes until the baseline L\(_p\) stabilized and were then perfused with CPA and 5 mM Ni\(^{2+}\) for 20 min. The vessels were then perfused with CPA and superfused with normal FR for a further 20 min to wash out the Ni\(^{2+}\) and allow Ca\(^{2+}\) influx.

Figure 2A shows a typical example of the L\(_p\) responses to CPA in a single microvessel. During perfusion with CPA and Ni\(^{2+}\) a small transient increase in L\(_p\) was measured during the first 2–3 min. There was a second, smaller increase in L\(_p\) after ~12 min. When Ni\(^{2+}\) was removed, the L\(_p\) immediately increased but recovered toward baseline by 20 min. Figure 2B summarizes data from six vessels. During CPA and Ni\(^{2+}\) perfusion, the L\(_p\) increased from 2.2 ± 0.9 \times 10^{-7} to 20.8 ± 13.8 \times 10^{-7} (cm\(^{-2}\)) \cdot (cm\(\cdot\)H\(_2\)O\(^{-1}\)) (P < 0.05, n = 6). This confirms that inhibition of Ca\(^{2+}\) store reuptake can increase L\(_p\) in the absence of Ca\(^{2+}\) influx. On Ni\(^{2+}\) removal the L\(_p\) again increased to 21.8 ± 7.7 \times 10^{-7} (cm\(^{-2}\)) \cdot (cm\(\cdot\)H\(_2\)O\(^{-1}\)) (P < 0.05, n = 6), again showing that CCE increased vascular permeability. One different finding from the TG data was that this time, there was no significant correlation between either the peak L\(_p\) with CPA and Ni\(^{2+}\) or the peak L\(_p\) with CPA alone compared with the baseline L\(_p\). However, there was a significant correlation between the peak response with CPA and Ni\(^{2+}\) and the peak with CPA alone (CCE) (r = 0.94, P < 0.05, n = 6) (Fig. 2C). This relationship appeared to be best fit by an exponential (see Fig. 2C), indicating that a more significant store depletion (i.e., a greater L\(_p\) increase) resulted in a greater L\(_p\) response to CCE. Although further experiments may be necessary to define the actual relationship between permeability due to CCE and because of store depletion, an estimate is given in Fig. 2C. The possible reasons for the differences between this and the TG experiments are discussed later.

To exclude the effect of Ni\(^{2+}\) on other mechanisms of Ca\(^{2+}\) regulation a specific cation channel blocker, 100 \(\mu\)M SKF (17, 28), was used instead of Ni\(^{2+}\). Vessels were perfused with TG and SKF for 20 min once a baseline with 1% BSA had been established. SKF was washed out by a further 10 min perfusion with 1% BSA.
Figure 3A shows the $L_p$ measured in two vessels. The top trace shows an experimental vessel treated as described by the protocol above (solid diamond), and the bottom trace shows the $L_p$ measurements taken with a control vessel treated with only SKF (solid square). The $L_p$ increased transiently and peaked after 5-min perfusion with SKF and TG in the experimental vessel, and on SKF removal the $L_p$ transiently increased for a second time returning to basal levels within 5 min. There was no difference between the time to peak in the presence of CPA and Ni$^{2+}$ and an immediate increase in $L_p$ when the Ni$^{2+}$ was removed that returned toward basal levels after 20 min. A second example of a single vessel is shown in Fig. 3B. This vessel perfusion with TG and SKF resulted in a cyclic alteration in $L_p$. This type of response increased for a second time returning to basal levels within 5 min. There was no difference between the time to peak in the presence of TG/SKF compared with TG/Ni. The $L_p$ in the control vessel does not significantly change during a 60-min perfusion (40 min shown).

Fig. 2. The size of the increase in $L_p$ due to CCE correlates with that due to store depletion. A: $L_p$ measurements from a single microvessel perfused with 30 μM cyclopiazonic acid (CPA) and 5 mM Ni$^{2+}$ for 20 min, followed by perfusion with CPA alone for 20 min. There was an immediate transient increase in $L_p$ in the presence of CPA and Ni$^{2+}$ and an immediate increase in $L_p$ when the Ni$^{2+}$ was removed that returned toward basal levels after 20 min. B: mean baseline $L_p$ (BSA) and peak $L_p$ with CPA/Ni$^{2+}$ and CPA alone. Values are means ± SE. *$P < 0.05$. C: correlation between the peak CPA/Ni$^{2+}$ $L_p$ and peak CPA $L_p$ ($r = 0.94$, $P < 0.05$, $n = 6$). Only significant differences are shown.

Fig. 3. Store depletion also increases $L_p$ in the presence of a pharmacological Ca$^{2+}$ channel inhibitor. A: $L_p$ measurements taken from a single microvessel perfused with 100 μM SKF-96365 (SKF) and 100 nM TG for 20 min, followed by TG alone for at least 10 min (●). A transient $L_p$ increase was measured when perfused with TG and SKF that returned to baseline after 20 min. A second transient $L_p$ increase occurred on removal of SKF. The lower trace shows a different microvessel perfused with SKF alone for 40 min (●) that did not significantly change throughout this time. B: another example of measurements taken from a single microvessel. Four of ten vessels tested displayed cyclic behavior when subjected to this protocol. C: mean BSA baseline $L_p$, peak SKF/TG $L_p$, and peak TG $L_p$. Values are means ± SE. *$P < 0.05$, **$P < 0.01$; only significant differences are shown.
was seen in 4 of 10 vessels tested with this protocol (two vessels were excluded due to a high-baseline \( L_p \)). In eight vessels in which these responses were measured, \( L_p \) significantly increased from 2.4 ± 0.6 \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1} \) to 15.1 ± 6.5 \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1} (P < 0.01, n = 8) while perfused with SKF and TG. When the SKF was washed out, \( L_p \) peaked at 12.5 ± 2.7 \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1} \) in the presence of TG alone (P < 0.01, n = 8) as shown in Fig. 3C. This was not significantly different to the peak \( L_p \) during perfusion with SKF and TG (P > 0.1, n = 8). In general, therefore, the effects of store depletion in the absence of Ca\(^{2+}\) entry were broadly similar irrespective of the combination of agonist used (TG/Ni, TG/SKF, and CPA/Ni). However, subtle changes in the responses are interpreted in the discussion.

**Does Store Depletion Increase \( L_p \) in Absence of Extracellular Ca\(^{2+}\) Influx?**

After it was determined that SERCA inhibition was sufficient to increase \( L_p \) in the absence and presence of extracellular Ca\(^{2+}\) influx, 5 \( \mu \text{M} \) IM was used to determine whether the stores were fully depleted by SERCA inhibition after a 20-min perfusion. If the stores were not fully depleted, an increase in \( L_p \) would be expected in the presence of IM. Vessels were perfused with 1% BSA until the baseline \( L_p \) stabilized and then with Ni\(^{2+}\) for at least 10 min to establish a new baseline. The pipette was refilled with Ni\(^{2+}\) and 30 \( \mu \text{M} \) CPA and perfused for another 20 min, followed by perfusion for 20 min with Ni\(^{2+}\), CPA, and IM. To determine whether CCE was still able to stimulate \( L_p \) increases, the vessels were perfused with CPA and IM in the absence of Ni\(^{2+}\) to allow Ca\(^{2+}\) influx.

Figure 4A shows an example of the effect of store depletion on vascular permeability in the absence of Ca\(^{2+}\) influx in a single vessel. During perfusion with CPA and Ni\(^{2+}\), the \( L_p \) increased slightly and returned to baseline in 3 min (point i). When IM was coperfused with CPA and Ni\(^{2+}\), the \( L_p \) increased after ~10 min (point ii). The \( L_p \) increased further when the Ni\(^{2+}\) was washed out (points iii and iv). This contrasted strongly with vessels that were perfused with IM alone (an example is shown in Fig. 4B). In this case perfusion with IM resulted in a transient increase in \( L_p \) that peaked between 3 and 5 min and returned to baseline within 10 min and was maintained at a low \( L_p \). He and Curry (12) have previously performed experiments using the same technique looking at the effect of IM on \( L_p \) in the presence of Ni\(^{2+}\). They measured an attenuated response to IM that recovered fully in the presence of Ni\(^{2+}\).

When the effects of IM, CPA, and Ni\(^{2+}\) were examined in eight vessels, the baseline did not significantly change with Ni\(^{2+}\) perfusion alone (from 6.6 ± 2.3 \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1} \) to 2.9 ± 0.5 \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1} \) \( P > 0.1 \), n = 8, Fig. 4C). CPA and Ni\(^{2+}\) coperfusion slightly, but significantly, increased \( L_p \) to 5.4 ± 0.9 \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1} \) \( P < 0.01 \) vs. Ni\(^{2+}\) baseline, \( n = 8 \). Coperfusion of Ni\(^{2+}\), CPA, and IM maintained an increase in \( L_p \) to \( \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1} \) \( P < 0.01 \) vs. Ni\(^{2+}\) baseline, \( n = 8 \) 7.4 ± 2.1 but this was not significantly greater than perfusion with Ni\(^{2+}\) and CPA alone \( P > 0.5 \), \( n = 8 \), implying that the stores

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**Fig. 4. Store release increases permeability; combined Ca\(^{2+}\) release, depletion, and entry most potently increases permeability; and the size of the increase in \( L_p \) induced by store depletion correlates with the baseline \( L_p \). A: \( L_p \) measurements taken from a single microvessel perfused with 5 mM Ni\(^{2+}\) for 10 min, Ni\(^{2+}\) and 30 \( \mu \text{M} \) CPA for 20 min, Ni\(^{2+}\), CPA, and 5 \( \mu \text{M} \) ionomycin (IM) for 20 min, then CPA and IM for 10 min. During perfusion with CPA and Ni\(^{2+}\), the \( L_p \) increased slightly and returned to baseline (point i). When perfused with CPA, Ni\(^{2+}\), and IM, \( L_p \) increased after 10 min (point ii). When Ni\(^{2+}\) was removed the \( L_p \) increased further (points iii to iv). B: \( L_p \) measurements taken from a single microvessel during perfusion of 5 \( \mu \text{M} \) IM for 15 min demonstrating that IM induces a transient \( L_p \) response. C: mean Ni\(^{2+}\) baseline \( L_p \), peak Ni\(^{2+}\)/CPA \( L_p \), peak Ni\(^{2+}\)/CPA/IM \( L_p \), and peak CPA/IM \( L_p \). Values are means ± SE. *\( P < 0.05 \), **\( P < 0.01 \); only significant differences are shown. D: correlation between mean Ni\(^{2+}\) \( L_p \) (baseline) and peak Ni\(^{2+}\)/CPA \( L_p \) \((r = 0.83, P < 0.02, n = 8)\).**
were depleted during a 20-min perfusion. However, Ni2+ removal during perfusion with CPA and IM dramatically increased $L_p$ to $27.4 \pm 18.8 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$ ($P < 0.05$ vs. Ni$^{2+}$ baseline, $n = 6$), indicating that CCE could result in a significant increase in permeability. Interestingly, there was again a significant positive correlation between perfusion with Ni$^{2+}$ (baseline, no Ca$^{2+}$ influx) and perfusion with CPA and Ni$^{2+}$ (store depletion, $r = 0.83$, $P < 0.02$, $n = 8$, see Fig. 4D).

**Does Active Store Release Increase $L_p$ in Absence of Extracellular Ca$^{2+}$ Influx?**

To determine the effect of actively releasing Ca$^{2+}$ from stores, we measured the permeability when the IM was added at the same time as SERCA inhibition in the absence of Ca$^{2+}$ influx (this time using TG and SKF). After the $L_p$ stabilized with perfusion with 1% BSA, SKF was perfused for 10 min to establish a new baseline. SKF, TG, and IM were co-perfused for 20 min to ensure the Ca$^{2+}$ stores were released. This was followed by a 10-min perfusion with IM to wash out the SKF and allow Ca$^{2+}$ influx.

Figure 5A shows an example of the effect of Ca$^{2+}$ store release in the absence of Ca$^{2+}$ influx and the effect of depleted Ca$^{2+}$ stores in the presence of Ca$^{2+}$ influx in a single vessel. When SKF, TG, and IM were co-perfused, there was an immediate large transient increase in $L_p$ that returned to basal levels within 5 min. When the SKF was removed and Ca$^{2+}$ influx allowed, there was a second smaller transient increase in $L_p$ between 4–8 min.

This experiment was carried out in eight vessels, and the mean data are shown in Fig. 5B. In these vessels, perfusion of SKF did not significantly change the baseline $L_p$ (from $3.1 \pm 0.8 \times 10^{-7}$ to $2.1 \pm 0.8 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$, $P > 0.1$, $n = 8$). The presence of SKF, TG, and IM significantly and transiently increased $L_p$ to $17.2 \pm 9.4 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$ ($P < 0.02$ vs. SKF baseline, $n = 8$). When SKF was washed out to allow Ca$^{2+}$ entry, a second transient increase in $L_p$ to $14.3 \pm 3.5 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$ was measured ($P < 0.05$ vs. SKF baseline, $n = 7$). There was no correlation between the time to peak and the baseline $L_p$ in the presence of either of the influx inhibitors.

**Does SERCA Inhibition with CPA Deplete Intracellular Ca$^{2+}$ Stores in Presence of Ca$^{2+}$ Influx?**

To determine whether SERCA inhibition with CPA resulted in depletion of intracellular stores, and hence an inhibition of store release-mediated increase in $L_p$, we investigated the relationship between the permeability increase stimulated by store depletion (SERCA inhibition, reduced-store Ca$^{2+}$) and that stimulated by active release of Ca$^{2+}$ from the store (stimulated by IM, defined as store release). If the permeability increase in the presence of Ca$^{2+}$ influx was dependent on the amount or rate of Ca$^{2+}$ released from stores, then we would predict an inverse relationship between the permeability increase caused by CPA perfusion (store depletion) and that caused by CPA and IM perfusion (store release). Thus if CPA causes store depletion and release, then CPA will increase permeability, but subsequent IM will not (i.e., there will be little or no Ca$^{2+}$ in the stores for IM to release). However, if CPA results in SERCA inhibition but not store depletion, then there will be a small effect of CPA but a large effect of ionomycin (i.e., the stores will be full before IM is added and empty afterward).

To test this prediction, 22 vessels were perfused with CPA for 20 min after baseline measurement. In 11 of these, this was followed by perfusion with CPA and IM for at least 10 min. Figure 6A–C, gives examples of $L_p$ measurements in three different vessels. Figure 6A shows a small transient increase in $L_p$ in the first 2–5 min of CPA perfusion and a large immediate transient increase in $L_p$ when perfused with CPA and IM that declined toward baseline within 10 min. Figure 6B shows a vessel that responded transiently with both CPA perfusion and CPA and IM coperfusion to a similar magnitude. Figure 6C shows a vessel that responded with a larger transient $L_p$ increase with CPA perfusion and a smaller transient $L_p$ increase in the presence of CPA and IM. Taking all 22 vessels, CPA perfusion transiently increased $L_p$ from a mean of $2.0 \pm 0.4 \times 10^{-7}$ to $10.9 \pm 2.0 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$ ($P < 0.0001$, $n = 22$, Fig. 6D). In the 11 vessels, in which this was followed by co-perfusion
of CPA and IM, \( L_p \) transiently increased to 30.4 ± 9.9 × 10^{-7} cm·s^{-1}·cmH_2O^{-1} (\( P = 0.002, n = 11 \), Fig. 6D).

Interestingly, as predicted, there was a highly significant negative correlation between the \( L_p \) increase with CPA perfusion (fold increase from BSA) and that with CPA and IM perfusion (fold increase from recovery after CPA) \( (r = -1.0, \text{ Spearman rank correlation coefficient (nonparametric), } P < 0.005, r = -0.95, \text{ Pearson correlation coefficient (parametric), } P < 0.005, \text{ see Fig. 6E} ) \). This showed that the degree of store emptying was variable across vessels and is consistent with the hypothesis that the size of the IM response was proportional to degree of filling of the \( \text{Ca}^{2+} \) stores.

Furthermore, there was again a significant correlation between the baseline \( L_p \) and peak CPA \( L_p \) during perfusion with CPA \( (r = 0.55, P < 0.01, \text{ Fig. 6F} ) \). This provides further evidence for the hypothesis that the baseline \( L_p \) is determined by the leak of \( \text{Ca}^{2+} \) from the stores. On closer examination two populations of vessels were found, those with a basal \( L_p < 3.6 \times 10^{-7} \) cm·s⁻¹·cmH₂O⁻¹ and those with a basal \( L_p > 3.6 \times 10^{-7} \) cm·s⁻¹·cmH₂O⁻¹.
10⁻⁷ cm·s⁻¹·cmH₂O⁻¹ as demonstrated in Fig. 6G. There is a slight but not significant correlation between the basal \( L_p \) and the peak CPA \( L_p \) when the baseline is less than 3.6 × 10⁻⁷ cm·s⁻¹·cmH₂O⁻¹ (\( r = 0.45, P = 0.056, n = 19 \)). However, when the baseline \( L_p \) is greater than 10 × 10⁻⁷ cm·s⁻¹·cmH₂O⁻¹ (including one vessel with a baseline \( L_p \) greater than 10 × 10⁻⁷ cm·s⁻¹·cmH₂O⁻¹), the correlation became highly significant (\( r = 1.0, \) Spearman rank, \( r = 0.995, \) Pearson, \( P < 0.005, n = 4 \)). This implies that some vessels have a high store leak of Ca²⁺, and this endows them with a high permeability.

**DISCUSSION**

*Release of Ca²⁺ from Intracellular Stores is Sufficient to Increase Vascular Permeability*

Vascular permeability is significantly increased by SERCA inhibition using either TG or CPA in the absence of Ca²⁺ influx (by inhibition of plasmalemmal cation channels by Ni²⁺ or SKF). TG has been shown to irreversibly block all isoforms of SERCA in endothelial cells in culture (8). This SERCA inhibition appears to be sufficient to significantly increase \( L_p \) in the absence of Ca²⁺ influx (Figs. 1–3). These data support the observations of He and Curry (12), who previously showed that Ni²⁺ significantly attenuates but does not completely block the permeability response to release of Ca²⁺ from stores by IM. However, they state that the permeability responses remained attenuated even after Ni²⁺ was washed out in their experiments, although no data were shown. In our experiments a large increase in \( L_p \) was demonstrated on removal of Ni²⁺ (Figs. 1 and 2) or SKF (Fig. 3). The main difference between our experiments and those performed by He and Curry (12) was that we used TG or CPA to inhibit SERCA, whereas He and Curry used IM to release Ca²⁺ from the stores. Therefore it is likely that the stores were still depleted when the Ni²⁺ or SKF were removed in the experiments described here, whereas Ca²⁺ release during the IM-mediated increase in permeability could be reuptaken as SERCA was unaffected.

To date, the signaling mechanism for store-dependent Ca²⁺ influx or “capacitative Ca²⁺ entry” as described by Putney (26) remains unknown, as does the identity of the cation channel(s) involved (22). However, it is known that IM stimulates the release of Ca²⁺ from intracellular stores in endothelial cells, rather than by a direct action on plasma membrane Ca²⁺ channels, (21) at least in vitro. In cultured bovine pulmonary artery endothelial cells Ca²⁺ influx was a graded response determined by the degree of Ca²⁺ store depletion and that maximal Ca²⁺ influx did not require complete Ca²⁺ store depletion (29). Furthermore, Ca²⁺ entry has been shown to be regulated by the degree of store filling in endothelial cells in culture (16). It is possible that a small amount of Ca²⁺ in the ER is required to signal extracellular Ca²⁺ influx. This theory is supported by the differences seen between our experiments and those of He and Curry (12) when Ni²⁺ is removed. Interestingly, the H₂ receptor agonist dimaprit transiently increased the permeability of occluded rat brain venular capillaries in the presence of SKF or zero extracellular Ca²⁺ supporting the hypothesis that permeability increases can be brought about by store-mediated Ca²⁺ release (27).

It is interesting to note that the data described here showed an increase in permeability in response to both CPA and TG that took some minutes to occur. We have assumed that this is due to a delayed increase in [Ca²⁺], in response to these agonists, because previous work (25), where [Ca²⁺], and permeability were measured in the same model (although in different vessels), showed that there was a distinct time lag between the application of TG to the cells and the increase in both [Ca²⁺] and permeability. This is in contrast to many studies of endothelial cells in culture where TG results in an immediate transient increase in intracellular Ca²⁺, followed by a sustained increased [Ca²⁺]; (8). It is clear that the ability of endothelial cells to regulate their [Ca²⁺], in response to such agonists is different in vitro than it is in vivo.

**Degree of Store Release Regulates Permeability**

He et al. (13) demonstrated a positive correlation between the peak \( L_p \) response to IM and the [Ca²⁺]i, when the [Ca²⁺]i was greater than 130 nM, which lies just outside of the range of an unstimulated endothelial cell (60–110 nM). They suggest that 130 nM may be the threshold level for Ca²⁺-dependent processes (13). It has therefore been assumed that the size of the \( L_p \) increase is proportional to the size of the Ca²⁺ increase. In our experiments there was a significant correlation between baseline \( L_p \) and peak \( L_p \) during store depletion (TG and Ni²⁺, \( r = 0.73 \)). Furthermore, there was a correlation between baseline \( L_p \) and peak \( L_p \) with CCE, and this was greater when inhibition of Ca²⁺ influx by Ni²⁺ (\( r = 0.87 \)) was removed than when SKF was removed (\( r = 0.52 \)). These findings support the hypothesis that, assuming [Ca²⁺]i, and permeability are correlated, the degree of leak of Ca²⁺ from intracellular stores regulates the baseline permeability, in the same manner that the rate (or amount) of release or influx regulates the agonist-stimulated increase in permeability (12). In the absence of Ca²⁺ influx, SERCA inhibition would be expected to result in a greater increase in [Ca²⁺]i, if the leak from stores was greater. The correlation with Ni²⁺ but not SKF would be explained if Ni²⁺ blocks other means of Ca²⁺ regulation such as the plasma membrane Ca²⁺/ATPase (PMCA) as well as the Na⁺/Ca²⁺ exchanger (15). A high-base-line leak from stores would result in an increase in the signal for CCE to a greater extent with Ni²⁺ because Ca²⁺ buffering or extrusion would be limited. This would then result in increased Ca²⁺ entry on removal of the block to influx. This extrusion mechanism has not been extensively studied in endothelial cells in vivo, and its importance may well have been underestimated. With SKF moreover, the Ca²⁺ regulation would not be affected, and extrusion of Ca²⁺ by the
PMCA should reset the Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry signal.

If the baseline \(L_p\) was determined by the degree of Ca\(^{2+}\) store leak, it might follow that with a higher baseline, a larger response would be expected when SERCA and Ca\(^{2+}\) influx are inhibited. The measured significant correlation between baseline \(L_p\) and the peak with TG and Ni\(^{2+}\) (Fig. 1C) suggests that the magnitude of Ca\(^{2+}\) release and Ca\(^{2+}\) influx is indeed determined by the degree of Ca\(^{2+}\) leak from the ER under baseline (nonstimulated) conditions. If the response was large enough to deplete the stores, there would be a stronger signal to stimulate capacitative Ca\(^{2+}\) influx and hence a greater response would be observed when the Ca\(^{2+}\) influx inhibitor was removed.

The responses were heterogeneous, as has been previously described both within a vessel (23) and between vessels (4, 10), which would be expected if responses were dependent on the degree of Ca\(^{2+}\) store leak in each endothelial cell and that leak was different from cell to cell or vessel to vessel.

**Does Store Emptying or Active Release Increase \(L_p\) in Absence of Extracellular Ca\(^{2+}\)?**

SERCA inhibition by CPA was able to significantly increase the \(L_p\) in the absence of Ca\(^{2+}\) influx and also correlated to the baseline \(L_p\) (Fig. 4). When IM was also added (still in the absence of Ca\(^{2+}\) influx), the \(L_p\) only increased slightly compared with Ni\(^{2+}\) and CPA treatment, and although significantly higher than the baseline, it was not significantly different to Ni\(^{2+}\) and CPA treatment. This suggests that either CPA was able to empty the stores or that the leak from the stores had sufficiently run down the store in 20 min to prevent a larger response with IM. A run down of stores has previously been shown to occur in endothelial cells in culture when stimulated with an agonist such as histamine (16).

To further investigate the effect of emptying stores on the permeability, a similar protocol to that in Fig. 4 was used with SKF and TG as the inhibitors. This time IM was added to the perfusate at the same time point as TG to ensure the stores were not depleted (Fig. 5). A large significant increase in the \(L_p\) was measured with SKF, TG, and IM treatment, implying that the reason why there was no increase in \(L_p\) with IM after CPA treatment was because CPA reduced the degree of Ca\(^{2+}\) filling of the store, as expected (20). Once Ca\(^{2+}\) influx was permitted the \(L_p\) increased again. Interestingly, both responses were transient when SKF was used but sustained when Ni\(^{2+}\) was used (Fig. 4A vs. Fig. 5A). It appeared that Ni\(^{2+}\) had an effect on the inactivation of the permeability response (the recovery to baseline). One interpretation of this is that Ni\(^{2+}\) may have nonspecifically blocked the PMCA analogous to its inhibition of the Na/Ca exchanger (15), preventing Ca\(^{2+}\) efflux as well as influx across the plasma membrane. Therefore it appears that active release of Ca\(^{2+}\) from stores increases permeability, in the absence of influx, more effectively than the lack of Ca\(^{2+}\) in the store itself.

**CPA Increased \(L_p\) Without Completely Depleting Intracellular Ca\(^{2+}\) Stores in Presence of Ca\(^{2+}\) Influx**

CPA transiently increased \(L_p\) in the presence of Ca\(^{2+}\) influx and showed a correlation to the baseline \(L_p\) (Fig. 6). When IM was subsequently added to the perfusate, the \(L_p\) response on average increased above that seen with CPA alone indicating that CPA did not deplete the stores entirely in the presence of Ca\(^{2+}\) influx (despite doing so in the absence of influx). However, the relative responses to CPA and CPA co-perfusion with IM differed between vessels as shown by the three examples (Fig. 6, A–C). Some vessels responded to a larger extent with CPA (store depletion) than they did to CPA and IM perfusion (store release) (Fig. 6C) and some responded more during the CPA and IM perfusion than they did to CPA perfusion alone (Fig. 6A), whereas others responded moderately to both treatments (Fig. 6B). When the data were pooled and averaged, they showed that store release (with CPA and IM coperfusion) increased the \(L_p\) twice as much as store depletion (with CPA alone).

The leak of Ca\(^{2+}\) from the stores has been suggested to have a positive feedback on the inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)] receptor as the local [Ca\(^{2+}\)] rises during SERCA inhibition due to phospholipase C activation (11). As the stores begin to run down, CCE is triggered and the global [Ca\(^{2+}\)] in the cytoplasm will increase (26). When the global cytosolic [Ca\(^{2+}\)] increases, Ca\(^{2+}\) itself will inhibit the Ins(1,4,5)P\(_3\) receptor with an IC\(_{50}\) of 300 nM preventing further loss of Ca\(^{2+}\) from the stores (1, 7). Therefore, when IM was included in the perfusate, we propose that there was sufficient release of store Ca\(^{2+}\) to further increase the \(L_p\) probably by signaling Ca\(^{2+}\) influx.

A highly significant inverse correlation between CPA perfusion (SERCA inhibition) and CPA and IM coperfusion (store release) was shown. This again demonstrated that CPA did not completely empty the stores and that the size of the increase in \(L_p\) was proportional to the intracellular Ca\(^{2+}\) store depletion. Alternately, IM could be releasing Ca\(^{2+}\) from organelles other than the ER, such as the golgi (19) and mitochondria, which have been shown to be up to 25% of the Ca\(^{2+}\) store in vitro (31).

A significant correlation between baseline \(L_p\) and the \(L_p\) during CPA perfusion (with Ca\(^{2+}\) influx) was demonstrated (Fig. 6F). However, on closer examination, the data can be interpreted as two populations of vessels as shown in Fig. 6G. The first population of vessels has been defined as having a baseline \(L_p < 3.6 \times 10^{-7}\) cm\(^{-1}\)cm\(_{H2O}^{-1}\) and the second population having a baseline >3.6 \(\times 10^{-7}\) cm\(^{-1}\)cm\(_{H2O}^{-1}\). Interestingly, the slope of the line is the same for both populations but is shifted to the right when the baseline is above 3.6 \(\times 10^{-7}\) cm\(^{-1}\)cm\(_{H2O}^{-1}\). He et al. (13) have demonstrated a positive correlation between the peak \(L_p\) response to IM and the [Ca\(^{2+}\)]\(_i\) when the [Ca\(^{2+}\)]\(_i\) was...
>130 nM (13). There did not appear to be any correlation between the baseline permeability and [Ca\(^{2+}\)] in their experiments. However, they did not test whether there was a relationship between the baseline \(L_p\) and the Ca\(^{2+}\) loading of the stores, and this relationship, suggested by the experiments above, awaits further study.

**Permeability is Increased by CCE and CRAC**

CCE is the stimulation of Ca\(^{2+}\) influx due to a depletion of Ca\(^{2+}\) from the intracellular store. We tested the hypothesis that CCE could stimulate permeability increases by measuring the effect of removing inhibitors of Ca\(^{2+}\) entry once the stores had been depleted. This resulted in a significant, transient increase in \(L_p\), suggesting that CCE was able to stimulate increased permeability (Fig. 5A). In addition, we also tested the hypothesis that CRAC entry could stimulate permeability increases by perfusing vessels with a Ca\(^{2+}\) ionophore under conditions of varying store filling. As predicted, there was a significant negative correlation between store filling and the size of the response to IM (see Fig. 6E). Therefore, it appears that it is not the mechanism of increased Ca\(^{2+}\) influx that determines the permeability of the vessel, but either the [Ca\(^{2+}\)]\(_i\) or the rate of increase of [Ca\(^{2+}\)]\(_i\) in the cytoplasm.

**Is Permeability Regulated by Rate of Ca\(^{2+}\) Increase or Endothelial [Ca\(^{2+}\)]\(_i\)?**

We have shown that microvascular permeability in vivo can be increased in the absence of extracellular Ca\(^{2+}\) influx although not to the same magnitude as when Ca\(^{2+}\) influx is present. Our findings are therefore consistent with those of He and Curry (12), although we show a more significant increase in permeability in the absence of Ca\(^{2+}\) influx. Because there are several sources of Ca\(^{2+}\), it is likely that they have differing rates of flux into the cytosol. This has been demonstrated using Ca\(^{2+}\) fluorescent dyes in HeLa cell cultures (6). It is possible that these different rates of flux are responsible for the differing degrees of permeability while Ca\(^{2+}\) is continuously recycled across the membranes. Interestingly, baseline \(L_p\) is not determined by the absolute [Ca\(^{2+}\)]\(_i\) (13), despite the fact that during stimulation the permeability measurements closely track the [Ca\(^{2+}\)]\(_i\). Because we found that the magnitude of the permeability responses correlated with the baseline permeability, we propose that this is linked to the degree of store filling because the rate of Ca\(^{2+}\) leak into the cytosol from stores would be expected to be greater if the stores had a higher [Ca\(^{2+}\)]\(_i\) (a higher concentration gradient would exist between the ER store and the cytosol).

The net Ca\(^{2+}\) leak, possibly through the Ins(3)P receptor, which is itself determined by the [Ca\(^{2+}\)]\(_i\) gradient between the ER store and the cytosol, is continually compensated for by Ca\(^{2+}\) reuptake through SERCA or Ca\(^{2+}\) flux across the plasmalemma (30) and extrusion by the PMCA. We propose that it is the balance between these Ca\(^{2+}\) movements that regulates \(L_p\) as the plasma and store membranes are in close proximity to each other. The expected order for rate of flux would be the following: untreated Ca\(^{2+}\) leak < SERCA inhibition < Ins(1,4,5)P\(_3\) receptor activation < capacitative Ca\(^{2+}\) influx < agonist-mediated Ca\(^{2+}\) influx. We predict therefore that this would also be the order for the magnitude of the permeability changes in a single vessel. However, it has not yet been possible to determine the rate of change of cytosolic [Ca\(^{2+}\)] and the permeability in the same vessel at the same time.

In summary, we have shown that permeability is increased by release of Ca\(^{2+}\) from intracellular stores in the absence of Ca\(^{2+}\) influx, and by CCE in the presence of Ca\(^{2+}\) influx, and provided evidence supporting the hypothesis that the baseline permeability is set by the degree of leak of Ca\(^{2+}\) from stores.

The authors thank Rebecca Foster and Rachel Perrin for technical assistance and support.

This study was supported by British Heart Foundation Grants FS88027 and BB2000003 (to D. O. Bates) and FS2000057 (to C. A. Glass).

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