RESEARCH COMMUNICATION

Reciprocal regulation of capacitative and non-capacitative Ca^{2+} entry in A7r5 vascular smooth muscle cells: only the latter operates during receptor activation

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In A7r5 vascular smooth muscle cells, Arg^8 -vasopressin (AVP) stimulates phospholipase C leading to activation of two distinct Ca^{2+} entry pathways. The capacitative Ca^{2+} entry (CCE) pathway is activated by depletion of Ca^{2+} stores, is permeable to Mn^{2+} , Ba^{2+} and Ca^{2+} , and is selectively blocked by Gd^{3+} (1 μM). A7r5 cells also express a non-capacitative Ca^{2+} entry (NCCE) pathway, which is activated by arachidonic acid that is released by the sequential activities of phospholipase C and diacylglycerol lipase. This pathway is permeable to Sr^{2+} , Ba^{2+} and Ca^{2+} and selectively blocked by (*R,S*)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-*N,N*-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate ('LOE-908'). We use these selective tools to show that AVP, via the same signalling pathway that leads to activation of NCCE, also inhibits CCE and that the inhibition is not due to depolarization of the plasma membrane. Using the selective

inhibitors to resolve the contributions of each Ca^{2+} entry pathway during stimulation with AVP, we establish that reciprocal regulation of CCE and NCCE by arachidonic acid ensures that only NCCE is active in the presence of AVP, whereas CCE is active only after its removal. NCCE and CCE are therefore activated in a strict temporal sequence: NCCE first and then CCE. Because Ca^{2+} passing through different Ca^{2+} entry pathways can selectively regulate different responses, reciprocal regulation of CCE and NCCE may allow a stimulus to first evoke a response and then recruit actively a different response when the stimulus is removed.

Key words: arachidonic acid, A7r5 cell, vascular smooth muscle, vasopressin.

INTRODUCTION

Receptors that stimulate the formation of inositol 1,4,5-trisphosphate (IP_3) typically evoke both release of Ca^{2+} from intracellular stores and Ca^{2+} entry across the plasma membrane [1]. Capacitative Ca^{2+} entry (CCE) channels, which open in response to a signal generated by depletion of intracellular Ca^{2+} stores, are widely assumed to provide a major route for such Ca^{2+} entry [2]. Experiments with thapsigargin, which allow intracellular Ca^{2+} stores to be emptied without receptor activation, provide the most persuasive evidence in favour of a significant role for CCE. Such experiments have often shown that empty Ca^{2+} stores can evoke Ca^{2+} entry that is at least as great as that evoked by activation of receptors that stimulate IP_3 formation [2]. However, many cells also express non-capacitative Ca^{2+} entry (NCCE) pathways [3–5], some of which are activated by arachidonic acid [6,7]. The physiological roles of these different Ca^{2+} entry pathways are unclear [3,8,9].

A7r5 vascular smooth muscle cells, in common with most other cells [10], express a CCE pathway that is activated after depletion of intracellular Ca^{2+} stores [11]. In A7r5 cells, this CCE pathway is permeable to Mn^{2+} , Ba^{2+} and Ca^{2+} , but not to Sr^{2+} , and it is blocked selectively and essentially irreversibly by 1 μM Gd^{3+} [5]. Our previous work [5,11] established that Arg^8 -vasopressin (AVP), via its interaction with vasopressin $\text{V}_{1\text{A}}$ receptors [12], also stimulates NCCE. The NCCE pathway is permeable to Sr^{2+} , Ca^{2+} and Ba^{2+} , but not to Mn^{2+} , and it is

reversibly blocked by a high concentration (100 μM) of Gd^{3+} . NCCE is activated, without the need for emptying of intracellular Ca^{2+} stores, by arachidonic acid released by the sequential activities of phospholipase C and diacylglycerol (DAG) lipase [5], the path by which most DAG is metabolized in vascular smooth muscle [13]. Activation of NCCE by AVP is therefore prevented by RHC-80267, a selective inhibitor of DAG lipase [5]. In A7r5 cells, therefore, both limbs of the phosphoinositide pathway regulate distinct Ca^{2+} entry pathways: IP_3 activates CCE by emptying intracellular Ca^{2+} stores, and DAG provides the arachidonic acid that causes activation of NCCE (Figure 1a). In the present study, we address the interplay between CCE and NCCE in A7r5 cells.

MATERIALS AND METHODS

Fura 2 measurements of bivalent cation entry in A7r5 cells

A7r5 cells were cultured on glass coverslips, loaded with fura 2 and mounted in an optical cuvette in the light path of a fluorescence spectrometer (F-4500; Hitachi), as described previously [5]. The cells were excited with light of appropriate wavelength (340 nm and 380 nm for Ca^{2+} recordings; 360 nm for Mn^{2+} and Ba^{2+} recordings), and emitted light was collected at a wavelength of 510 nm. After correction for autofluorescence, calibrations of fluorescence ratios ($R_{340/380}$) to cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) were performed using look-up tables

Abbreviations used: AVP, Arg^8 -vasopressin; $[\text{Ca}^{2+}]_c$, cytosolic free Ca^{2+} concentration; CCE, capacitative Ca^{2+} entry; DAG, diacylglycerol; HBS, Hepes-buffered saline; IP_3 , inositol 1,4,5-trisphosphate; I_{ARCC} , arachidonic acid-activated Ca^{2+} entry channel; LOE-908, (*R,S*)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-*N,N*-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate; NCCE, non-capacitative Ca^{2+} entry; RHC-80267, 1,6-bis(cyclohexyloximinocarbonylamino)hexane; U73122, 1-[6-[[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione.

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Figure 1 CCE and NCCE in A7r5 cells

(a) AVP stimulates formation of IP₃ and DAG. IP₃ stimulates release of Ca²⁺ from intracellular stores and therefore activation of CCE, whereas DAG can be metabolized to arachidonic acid (arachidonate), which activates NCCE. Selective inhibitors of steps in the signalling pathway are shown by hammerheads. (b) Extracellular Ca²⁺ was restored to cells pretreated with thapsigargin/ionomycin (TG) to activate CCE before and after addition of 1 μM Gd³⁺. Subsequent activation of NCCE by addition of AVP (100 nM) is also shown. (c) CCE was first recorded after restoration of extracellular Ca²⁺ to TG-treated cells in the presence of LOE-908 (30 μM). After addition of 1 μM Gd³⁺ to block CCE, AVP (100 nM) was added. (d) The effects of Gd³⁺ (1 μM, closed bars; control, open bars) on Ca²⁺ entry via the CCE pathway and AVP-evoked Sr²⁺ entry via the NCCE pathway are summarized. (e) The effects of 30 μM LOE-908 (closed bars) on Ca²⁺ entry via the CCE and NCCE pathways are shown. Results are expressed as the means ± S.E.M. for at least three independent experiments.

created from Ca²⁺-standard solutions (Molecular Probes, Europe BV, Leiden, The Netherlands) [5]. Cells were continuously perfused (17 ml/min) at 20 °C with HEPES-buffered saline [HBS: 135 mM NaCl/5.9 mM KCl/1.2 mM MgCl₂/1.5 mM CaCl₂/11.6 mM HEPES/11.5 mM glucose (pH 7.3)]. Where appropriate, CaCl₂ was either omitted (Ca²⁺-free HBS; EGTA was not included, because it would chelate Mn²⁺ and Gd³⁺) or replaced by other bivalent cations. Other changes to the composition of the HBS are described below. The half-time for exchange of media within the cuvette was 9.6 ± 0.3 s.

Because the voltage-gated L-type Ca²⁺ channels of A7r5 cells are often spontaneously active [14] and are regulated by AVP

[15], we included verapamil (10 μM) in all media to inhibit completely L-type Ca²⁺ channels. The effectiveness of this inhibition was verified by demonstrating that the increase in [Ca²⁺]_e (220 ± 13 nM, *n* = 3), which was evoked by addition of HBS containing 53 mM KCl (to depolarize the plasma membrane), was abolished by 10 μM verapamil (5 ± 1 nM).

Where appropriate, CCE was maximally activated by pre-treating cells (10 min) with ionomycin (1 μM) and thapsigargin (1 μM). This procedure completely emptied the hormone-sensitive intracellular Ca²⁺ stores: the increase in [Ca²⁺]_e evoked by AVP (100 nM) in Ca²⁺-free HBS was 431 ± 24 nM (*n* = 3) in normal cells, and 5 ± 2 nM in cells after thapsigargin/ionomycin

Figure 2 CCE and NCCE do not operate independently

(a) Restoration of extracellular Ca^{2+} to cells in which the intracellular Ca^{2+} stores have been emptied by treatment with ionomycin/thapsigargin evokes similar CCE Ca^{2+} signals in normal cells and in cells lacking DAG lipase. After blocking CCE with $1 \mu\text{M}$ Gd^{3+} , AVP (100 nM) activates NCCE only in normal cells (i). However, the Ca^{2+} entry evoked by combining empty stores with AVP in normal cells is less than in variant cells (ii), and less than that evoked by empty stores alone (i). Results are summarized in (b), where closed bars denote normal cells, and open bars denote cells lacking DAG lipase. Results are expressed as the means $\pm$ S.E.M. for at least three independent experiments. CCE, Ca^{2+} entry evoked by depletion of intracellular stores; NCCE, Ca^{2+} entry evoked by addition of AVP to cells with empty stores, but with CCE blocked by Gd^{3+} ; and CCE + AVP, Ca^{2+} entry recorded by addition of AVP to cells with empty Ca^{2+} stores and neither pathway blocked. (c) Rates of Ba^{2+} entry into normal A7r5 cells are shown for control cells (basal) and for thapsigargin/ionomycin-treated cells in which only CCE was active (30 μM LOE-908 present), after addition of AVP with CCE blocked (NCCE only, 1 μM Gd^{3+} present), or after addition of AVP with neither pathway blocked (CCE + AVP). The results are summarized in (d) and are expressed as the means $\pm$ S.E.M. for at least three independent experiments.

treatment. Ionomycin was used in these experiments because thapsigargin alone could only empty the stores slowly. Subsequent experiments (Figure 1) established that the only bivalent cation entry activated by this treatment has the properties of CCE, suggesting that pretreatment with ionomycin does not directly lead to it transporting bivalent cations across the plasma membrane; other studies have reached a similar conclusion [16].

For measurements of Mn^{2+} entry via CCE, we established that the rate of quench of fura 2 fluorescence in the presence of Mn^{2+} (50 μM) before emptying the intracellular stores ($Q1$) was indistinguishable from that recorded after thapsigargin/ionomycin treatment with Ca^{2+} entry pathways blocked by 100 μM Gd^{3+} ($Q2$; $Q2/Q1 = 91 \pm 12\%$, $n = 3$). In subsequent experiments, the basal rate of Mn^{2+} entry was therefore determined at the end of each experiment in the presence of 100 μM Gd^{3+} . The effect of AVP on Mn^{2+} entry via CCE was measured by perfusing thapsigargin/ionomycin-treated cells with Ca^{2+} -free HBS containing 50 μM MnCl_2 for 120 s; AVP (or other additions) was present for the final 60 s. Least-squares linear regression was then used to define the rate of fluorescence quench during the final 20 s of the first 60 s interval ($R1$, i.e. control rate of Mn^{2+} entry via CCE) and then during the final 20 s of the second 60 s interval ($R2$, i.e. AVP-modulated rate of Mn^{2+} entry via CCE) (see Figure 4a for a typical protocol). Under control conditions, rates of Mn^{2+} entry via CCE were similar during the first and second recording intervals ($R2/R1 = 96 \pm 9\%$, $n = 5$). After subtracting the basal rate of Mn^{2+} entry from all recordings, the ratio of the rates of Mn^{2+} entry during the two intervals ($R2/R1$) was used to quantify the effects of AVP (or other drugs) (see Figure 4c).

To reverse rapidly the effects of AVP (see Figure 7), removal of AVP from the perfusing medium was accompanied by addition

of a high concentration (100 nM) of a peptide antagonist of the vasopressin V_{1A} receptor [17] (see below).

All traces are typical of at least three experiments, and results are expressed as the means $\pm$ S.E.M for at least three independent experiments.

Materials

A7r5 cells were originally from the A.T.C.C. Arachidonic acid, 1,6-bis(cyclohexyloximinocarbonylamino)hexane (RHC-80267), 1-{6[(17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1H-pyrrole-2,5-dione (U73122) and verapamil were from Calbiochem–Novabiochem (Nottingham, U.K.). 2-Aminoethyl diphenylborane ('2-APB'), AVP and the selective peptide vasopressin V_{1A} receptor antagonist ([1-mercaptopyclohexyl] acetyl-Tyr[O-ethyl]-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂) [17] were from Sigma (Poole, Dorset, U.K.). (*R,S*)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-*N,N*-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate (LOE-908) was generously given by Boehringer Ingelheim (Biberach an der Riss, Germany). The targets of the drugs used are shown in Figure 1(a). All other manufacturers have been reported previously [5].

RESULTS AND DISCUSSION

CCE and NCCE do not operate independently

Our previous attempts [5] to resolve the relative roles of the CCE and NCCE pathways to the Ca^{2+} signals evoked by AVP were hampered by lack of a selective inhibitor of the NCCE pathway. The results shown in Figures 1(b) and 1(d) agree with previous

work [5], demonstrating that $1 \mu\text{M}$ Gd^{3+} selectively blocks CCE without affecting NCCE; 2-aminoethyl diphenylborane ($100 \mu\text{M}$) also selectively blocks CCE, without affecting NCCE (results not shown), in accordance with results from other cell types [8]. Conversely, LOE-908 ($30 \mu\text{M}$), which has been used to inhibit non-selective cation currents [18], blocks NCCE without affecting CCE (Figures 1c and 1e). In subsequent experiments, we used $1 \mu\text{M}$ Gd^{3+} to selectively block CCE, and LOE-908 ($30 \mu\text{M}$) to selectively block NCCE.

Maximal activation of CCE (using thapsigargin/ionomycin) evokes a 5-fold larger increase in $[\text{Ca}^{2+}]_c$ ($459 \pm 32 \text{ nM}$) compared with maximal activation of NCCE ($90 \pm 10 \text{ nM}$) (Figure 2b). Yet paradoxically, in both A7r5 [5,18] and other cell types [8,19], it has been suggested that CCE is unlikely to provide the major route for the Ca^{2+} entry evoked by physiological stimuli. The experiments described below attempt to resolve this apparent paradox.

We established previously [5] a variant of the A7r5 cell line in which most responses to AVP were normal, but AVP failed to stimulate NCCE (Figure 2a). Because exogenous arachidonic acid activated NCCE in these cells, we suggested that these variant cells lack DAG lipase [5]. The variant cells therefore afford the opportunity to address the role of DAG lipase in regulating AVP-evoked Ca^{2+} entry. We expected the Ca^{2+} entry evoked by AVP to be greater in normal A7r5 cells (where both CCE and NCCE could be activated) compared with in the variant cells (where only CCE could be activated). Instead, although the Ca^{2+} entry evoked by depletion of intracellular Ca^{2+} stores was similar in the two cell lines (Figure 2a), the Ca^{2+} entry recorded in thapsigargin-treated cells in the presence of AVP was much larger in cells lacking DAG lipase than in normal cells (Figures 2a and 2b).

Ba^{2+} permeates both pathways, but it is not transported by Ca^{2+} pumps [11]; therefore Ba^{2+} allows the activities of the Ca^{2+} entry pathways to be measured free of any effects of AVP on Ca^{2+} extrusion [20]. Figure 2(c) demonstrates that maximal activation of either CCE (using thapsigargin/ionomycin) or NCCE alone (by AVP after blockade of CCE with $1 \mu\text{M}$ Gd^{3+}) stimulates Ba^{2+} entry. Furthermore, the rate of Ba^{2+} entry through CCE is approx. 5-fold greater than that through NCCE (Figure 2d), consistent with the results obtained by measuring Ca^{2+} signals, where the peak increases in $[\text{Ca}^{2+}]_c$ evoked by the two pathways also differed by approx. 5-fold (Figure 2b). However, the Ba^{2+} entry evoked by AVP after thapsigargin treatment is much smaller than that evoked by thapsigargin alone (Figure 2c). Hence, irrespective of whether Ca^{2+} (Figure 2b) or Ba^{2+} (Figure 2d) entry is used to assess the activities of the two pathways, our results concur in demonstrating that the CCE and NCCE pathways do not operate independently. Instead AVP both stimulates NCCE, as reported previously [5,11], and inhibits CCE. Our results with the variant cell line (Figure 2b) suggest that DAG lipase is required for both effects of AVP.

In the experiments described so far, CCE was activated pharmacologically by pretreating cells with thapsigargin/ionomycin, but it was important to resolve whether AVP was capable of activating CCE when the inhibitory effect of AVP was prevented. To address this issue, A7r5 cells were stimulated with AVP in the presence of RHC-80267 ($50 \mu\text{M}$) to prevent activation of DAG lipase (Figure 3a). Under these conditions, a maximally effective concentration of AVP (100 nM) stimulated Ca^{2+} entry (increase in $[\text{Ca}^{2+}]_c = 335 \pm 22 \text{ nM}$, $n = 3$), which is comparable with that evoked by thapsigargin/ionomycin ($349 \pm 7 \text{ nM}$), and in both cases the Ca^{2+} signal was reversed by addition of $1 \mu\text{M}$ Gd^{3+} (Figure 3b). The concentration of AVP that caused half-maximal release of the intracellular Ca^{2+} stores ($\text{EC}_{50} = 22 \pm$

Figure 3 AVP activates CCE when DAG lipase is inhibited

(a) Cytosolic Ca^{2+} signals were recorded from cells in the presence of RHC-80267 ($50 \mu\text{M}$, to inhibit DAG lipase) after addition of vasopressin (100 nM) in the absence (upper trace) or presence (lower trace) of $1 \mu\text{M}$ Gd^{3+} to inhibit CCE. The CCE in the upper trace is inhibited after subsequent addition of Gd^{3+} . (b) The increases in $[\text{Ca}^{2+}]_c$ recorded 200 s after addition of AVP (20 nM or 100 nM) or restoration of extracellular Ca^{2+} to thapsigargin/ionomycin (TG/I)-treated cells are shown by the closed bars and the responses recorded in the presence of $1 \mu\text{M}$ Gd^{3+} by the open bars. Results are expressed as the means $\pm$ S.E.M. for at least three independent experiments. (c) Concentration-dependent effect of AVP on Ca^{2+} release from intracellular stores.

10 nM ; Figure 3c) was similar to that required for half-maximal activation of CCE: the CCE signal evoked by 20 nM AVP was $44 \pm 7\%$ of that evoked by 100 nM AVP (Figure 3b). These results are important because they demonstrate that, when DAG lipase is inhibited, the emptying of intracellular Ca^{2+} stores by AVP is closely coupled to activation of CCE.

Arachidonic acid co-ordinates the two Ca^{2+} entry pathways by activating NCCE and inhibiting CCE

Pretreatment of cells with thapsigargin/ionomycin emptied completely the AVP-sensitive intracellular Ca^{2+} stores (see the Materials and methods section), and maximally activated Mn^{2+} entry via the CCE pathway. AVP (100 nM) inhibited this capacitative Mn^{2+} entry (Figures 4a and 4b), and the sensitivity of this inhibition to AVP ($\text{EC}_{50} = 19 \pm 2 \text{ nM}$) was the same as that for the effect of AVP on release of Ca^{2+} from intracellular stores ($\text{EC}_{50} = 22 \pm 10 \text{ nM}$; Figure 3c) and Ca^{2+} entry via NCCE ($\text{EC}_{50} = 18 \pm 2 \text{ nM}$; Figure 4d).

Inhibition of Mn^{2+} entry via CCE by AVP was reversed by U73122 ($30 \mu\text{M}$, an inhibitor of phospholipase C) and by RHC-80267 (Figures 4b and 4c), the latter with the same sensitivity ($\text{IC}_{50} = 24 \pm 4 \mu\text{M}$) as inhibition of Ca^{2+} entry via NCCE (IC_{50} of approx. $25 \mu\text{M}$). Furthermore in the A7r5 cells lacking DAG

Figure 4 DAG-lipase-dependent inhibition of CCE by AVP

(a) Rates of Mn^{2+} ($50 \mu\text{M}$) entry are shown before emptying of the intracellular Ca^{2+} stores (i), after addition of thapsigargin/ionomycin (during the break in the trace) to maximally activate CCE (ii), followed by addition of AVP (100 nM , iii) and finally the basal rate was recorded after addition of $100 \mu\text{M}$ Gd^{3+} (iv). Ionomycin and an increased $[\text{Mn}^{2+}]$ (1 mM) are added at the end of the experiment to quench all the fura 2 fluorescence. (b) Rates of Mn^{2+} entry (measured by recording fura-2 fluorescence at $\lambda_{\text{excitation}} = 360 \text{ nm}$) are shown for cells in which CCE had been maximally activated. AVP (100 nM) was present, as shown for the lower traces, alone or in combination with RHC-80267 ($50 \mu\text{M}$) or U73122 ($30 \mu\text{M}$). (c) Stimulated rates of Mn^{2+} entry via CCE were calculated from paired comparisons (i.e. stimulated rate during treatment with AVP as a percentage of stimulated rate during the immediately preceding interval) before and after addition of the indicated drugs or after replacement of extracellular Na^+ by *N*-methyl-D-glucamine (NMDG). The open bar shows results from cells lacking DAG lipase. Results are expressed as the means $\pm$ S.E.M. for at least three independent experiments. (d) Concentration-dependent effects of AVP on Ca^{2+} entry via NCCE (□) and inhibition of Mn^{2+} entry via CCE (●); for clarity error bars are shown only for the latter.

lipase, AVP had no effect on Mn^{2+} entry via the CCE pathway (Figure 4c). Hence, the results with both selective pharmacological inhibitors and the mutant cell line are consistent in suggesting that both phospholipase C and DAG lipase are required for AVP to inhibit CCE.

AVP still inhibited completely Mn^{2+} entry via the CCE pathway when extracellular Na^+ was replaced with the impermeant cation *N*-methyl-D-glucamine (Figure 4c), indicating that inhibition of CCE by AVP was not due to the depolarization of the plasma membrane caused by Na^+ entry via the NCCE pathway. The lack of detectable CCE in cells stimulated with AVP in the presence of LOE-908 to block the NCCE pathway (see Figure 7) further substantiates this conclusion.

We were also concerned that inhibition of a K^+ channel (possibly by arachidonic acid [21]) might have depolarized the plasma membrane, and thereby diminished the electrochemical gradient for Ca^{2+} entry. To address this possibility, CCE was examined under conditions where the K^+ gradient across the plasma membrane was collapsed by reversing the normal extracellular concentrations of KCl (to 135 mM) and NaCl (to 5.9 mM). As expected, these conditions reduced greatly the amplitude of the CCE Ca^{2+} signal (results not shown), but by increasing the extracellular CaCl_2 concentration (to 10 mM) the CCE signal was restored to a level similar to that recorded in normal HBS (Figures 5a and 5b). Despite the absence of an inwardly directed K^+ gradient across the plasma membrane and a complete block

Figure 5 Inhibition of CCE by AVP is not caused by membrane depolarization

(a) CCE was measured in cells pretreated with thapsigargin/ionomycin in HBS with 135 mM KCl replacing NaCl, with LOE-908 (30 μ M) present throughout, and with the $[Ca^{2+}]_e$ of the medium increased to 10 mM. Addition of AVP (100 nM) rapidly inhibited CCE under these conditions. (b) The increase in $[Ca^{2+}]_i$ recorded 150 s after restoration of extracellular Ca^{2+} is shown for cells stimulated in normal HBS (open bar) or in high- K^+ HBS with LOE-908 (closed bar). The effect of AVP (100 nM, hatched bar) on the Ca^{2+} signal recorded 450 s after restoration of extracellular Ca^{2+} in high- K^+ HBS with LOE-908 is compared with the control response in the same medium (closed bar). Results are expressed as the means $\pm$ S.E.M. for at least three independent experiments. (c) AVP inhibits Mn^{2+} entry under similar conditions (high- K^+ , LOE-908 present, and with the $[Mn^{2+}]_e$ increased to 200 μ M). The trace is typical of three similar experiments (see the text).

of the NCCE pathway by LOE-908, addition of AVP (100 nM) rapidly inhibited Ca^{2+} entry via CCE (Figures 5a and 5b). Finally, when we used the Mn^{2+} -evoked quench of fura 2 to record the activity of the CCE pathway, AVP inhibited completely the Mn^{2+} entry evoked by store depletion, despite the presence of LOE-908 and with the K^+ gradient across the plasma membrane collapsed (Figure 5c). Under these conditions, emptying of intracellular Ca^{2+} stores caused a 5.8 ± 0.9 -fold ($n = 3$) increase in the rate of Mn^{2+} entry, but AVP then caused the stimulated rate of Mn^{2+} entry to fall to only $3 \pm 4\%$ of the rate recorded before AVP addition.

Figure 6 Reciprocal regulation of CCE and NCCE by arachidonic acid

(a) In thapsigargin/ionomycin-treated cells with LOE-908 (30 μ M) present to inhibit NCCE, arachidonic acid (arachidonate, 50 μ M) inhibits CCE (i), but with Gd^{3+} present throughout to inhibit CCE, arachidonic acid stimulates Ca^{2+} entry via NCCE (ii). (b) Reciprocal regulation of the two Ca^{2+} entry pathways by arachidonic acid.

These findings indicate that the ability of AVP to inhibit the Ca^{2+} (or Mn^{2+}) signals evoked by CCE resulted from an inhibition of the CCE pathway, and not from a decrease in the driving force for Ca^{2+} entry mediated by either inhibition of K^+ channels or activation of non-selective cation channels.

Arachidonic acid, the immediate product of DAG lipase activity, has been reported to inhibit CCE in some cells [22,23]. Because arachidonic acid is lipophilic and would normally be produced at the plasma membrane (from DAG in A7r5 cells) and then affect Ca^{2+} channels in the plasma membrane, it is impossible to present exogenous arachidonic acid in a way that mimics faithfully the arachidonic acid made in response to receptor activation [8]. Therefore we examined the effects of only a single high concentration of arachidonic acid (50 μ M) on the two Ca^{2+} entry pathways, and exploited the distinct pharmacological properties of CCE and NCCE to provide evidence for the selective actions of arachidonic acid. In cells where the intracellular stores had been completely emptied by thapsigargin/ionomycin, and with NCCE blocked by LOE-908 (30 μ M), arachidonic acid inhibited CCE (Figure 6a). Conversely, in thapsigargin/ionomycin-treated cells with the CCE blocked by 1 μ M Gd^{3+} , arachidonic acid rapidly activated Ca^{2+} entry (Figure 6a). We demonstrated previously [5] that arachidonic acid stimulated Sr^{2+} entry, but not Mn^{2+} entry, and that arachidonic acid-evoked Ca^{2+} entry was inhibited by 100 μ M Gd^{3+} . Each of these characteristics is consistent with the selective activation of NCCE by arachidonic acid. We have now extended the characterization by demonstrating that arachidonic acid-evoked Ca^{2+} entry is also selectively inhibited by LOE-908. After exposure to arachidonic acid for 150 s, the increase in $[Ca^{2+}]_i$ was 112 ± 17 nM ($n = 3$) (similar to the response when NCCE was maximally activated by AVP, 90 ± 10 nM; Figure 2b), but in the presence of 30 μ M LOE-908, the increase was only 3 ± 1 nM.

Figure 7 CCE is active only after removal of AVP

Cells were stimulated with either 200 nM (**a**) or 30 nM (**b**) AVP under conditions where both Ca^{2+} entry pathways were active (CCE + NCCE), only NCCE was active (1 μM Gd^{3+} to block CCE), only CCE was active (30 μM LOE-908 to block NCCE) or all Ca^{2+} entry was inhibited (Neither, 100 μM Gd^{3+}). Removal of AVP was accompanied by addition of the peptide $\text{V}_{1\text{A}}$ antagonist (100 nM) to minimize re-association of AVP with its receptor. Results from at least three experiments are summarized in the histograms (means $\pm$ S.E.M.), which show the increases in $[\text{Ca}^{2+}]_c$ recorded under each of the conditions at the times indicated.

We conclude that arachidonic acid, which is released from DAG by DAG lipase, co-ordinates the activities of the two AVP-regulated Ca^{2+} entry pathways by stimulating NCCE and inhibiting CCE (Figure 6b). Thapsigargin, by providing only the stimulatory element of the signals that would pass normally from receptors to the CCE pathway (i.e. store depletion), exaggerates the contribution of CCE to receptor-regulated Ca^{2+} entry.

Rapid switching between Ca^{2+} entry pathways after addition and removal of AVP

To resolve the relative contributions of CCE and NCCE to the Ca^{2+} signals evoked by a physiological stimulus, cells were exposed to a maximal or submaximal concentration of AVP under conditions where both, neither or only one of the two Ca^{2+} entry pathways were selectively blocked (Figure 7). The Ca^{2+} signals recorded in the presence of AVP were similar whether both Ca^{2+} entry pathways were blocked (100 μM Gd^{3+}) or only NCCE was blocked (30 μM LOE-908); in each case the increase in $[\text{Ca}^{2+}]_c$ resulted entirely from release of intracellular Ca^{2+} stores. Furthermore, the more sustained Ca^{2+} signals evoked by AVP were unaffected by blocking of the CCE pathway. Our results therefore demonstrate that, in the presence of either concentration of AVP, the stimulated Ca^{2+} entry is mediated entirely by NCCE (Figure 7).

After rapid removal of AVP, Ca^{2+} entry via NCCE was rapidly switched off and a large Ca^{2+} signal mediated by CCE

was observed, which terminated as the intracellular stores refilled. This CCE signal was most pronounced in cells stimulated with AVP when NCCE was blocked (Figure 7). Under these conditions, there can have been no Ca^{2+} entry via NCCE during the early phase of the response to refill the intracellular stores, but the empty stores would have been unable to evoke CCE until relieved of the inhibition provided by arachidonic acid (Figure 7a). These results therefore establish that inhibition of CCE by AVP is rapidly reversible, which is consistent with the rapid metabolism of arachidonic acid.

The results with a submaximal concentration of AVP are more significant, because they demonstrate that under conditions where the NCCE pathway had been active continuously during the early phase of the response, there was a significant stimulation of Ca^{2+} entry via CCE when AVP was removed (Figure 7b). With sub-maximal stimulation, therefore, all Ca^{2+} entry is via NCCE when AVP is present, but this Ca^{2+} entry is insufficient to refill the intracellular Ca^{2+} stores. After removal of AVP, NCCE rapidly switches off and CCE is rapidly activated and remains active until the stores have refilled.

Conclusions

Cytosolic Ca^{2+} is potentially toxic and with Ca^{2+} entry pathways providing access to a limitless source of Ca^{2+} , it is to be expected that cells tightly regulate each Ca^{2+} entry pathway [3], but it is

Figure 8 Co-ordinate regulation of different Ca^{2+} entry pathways

(a) Conformational interactions between ligand-gated ion channels (i) [24], switching between voltage-gated and voltage-independent pathways after depolarization (ii) [25], and the reciprocal regulation of CCE and NCCE (iii) [26], have each been suggested to co-ordinate Ca^{2+} entry via different pathways. (b) We suggest that reciprocal regulation of CCE and NCCE in A7r5 cells may allow active switching between stimulation (contraction for vascular smooth muscle cells mediated by NCCE) when the receptor is occupied (ii) and recovery (relaxation mediated by CCE) when AVP dissociates from its receptor (iii).

also important that the activities of different Ca^{2+} entry pathways are effectively co-ordinated (Figure 8a).

In myenteric neurones, activation of a subclass of purinoceptor (P2X_2) by ATP is conformationally coupled to inhibition of associated nicotinic acetylcholine receptors, such that the synaptic currents (carried partly by Ca^{2+}) evoked by simultaneous activation of both receptors are less than the sum of the currents evoked by independent activation of each receptor [24] (Figure 8a). In colonic smooth muscle, the Ca^{2+} entry that drives refilling of intracellular Ca^{2+} stores switches from a dependence on CCE to L-type Ca^{2+} channels, as the membrane depolarizes [25] and voltage-gating of the L-type channel compensates for the reduced driving force for Ca^{2+} entry (Figure 8a). In HEK-293 cells, the Ca^{2+} entry evoked by modest stimulation of muscarinic acetylcholine receptors appears to be mediated by an arachidonic acid-activated Ca^{2+} entry channel (I_{ARC}), whereas CCE is almost entirely responsible for the Ca^{2+} entry evoked by more intense stimulation [26] (Figure 8a). The mechanisms controlling this switch from NCCE to CCE are controversial. Inhibition of I_{ARC}

by sustained increases in $[\text{Ca}^{2+}]_i$ [26], or some unidentified consequence of activating CCE [23], have each been proposed to attenuate NCCE as the stimulus intensity increases. The lack of CCE during weak stimulation is likely to result simply from inadequate emptying of intracellular Ca^{2+} stores [26], but active repression of CCE by arachidonic acid has also been suggested [23], although there is no evidence to suggest that activation of muscarinic receptors inhibits CCE in these cells [26]. In summary, co-ordinated regulation of different Ca^{2+} entry pathways is likely to be widespread, but the mechanisms are incompletely understood.

We chose to measure cytosolic Ca^{2+} signals (rather than currents), focused on the effects of a physiological extracellular stimulus (AVP), and used thoroughly characterized inhibitors of the Ca^{2+} entry pathways to define the physiological roles of CCE and NCCE in mediating cytosolic Ca^{2+} signals in A7r5 cells. We conclude that arachidonic acid, released by the sequential activities of phospholipase C and DAG lipase, co-ordinates the contributions of two receptor-regulated Ca^{2+} entry pathways, such that each is activated in a specific temporal sequence. By activating NCCE and simultaneously inhibiting CCE, arachidonic acid ensures that when the extracellular stimulus is present, only the NCCE pathway contributes to Ca^{2+} entry, and CCE contributes only after removal of the stimulus. CCE may therefore, as commonly supposed [3], allow refilling of intracellular stores after removal of a stimulus. More importantly, because different Ca^{2+} entry pathways can selectively regulate different intracellular responses [27,28], reciprocal regulation of the two pathways may allow an extracellular stimulus to abruptly initiate a response (via NCCE) when it binds to its receptor and then reverse it actively (via CCE) when the stimulus dissociates (Figure 8b).

In vascular smooth muscle, Ca^{2+} is the major stimulus for contraction, and cAMP and cGMP cause relaxation [29]. Ca^{2+} entry via the CCE pathway selectively stimulates certain isoforms of adenylate cyclase [30] and constitutive NO synthase [27] (leading to cGMP formation); both enzymes are expressed in vascular smooth muscle [29,31]. We speculate, therefore, that AVP may cause contraction by stimulating Ca^{2+} entry via the NCCE pathway, and removal of AVP may then cause relaxation by activating CCE (Figure 8b).

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REFERENCES

- Berridge, M. J. (1997) Elementary and global aspects of calcium signalling. *J. Physiol.* **499**, 291–306
- Putney, J. W. (1999) TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14669–14671
- Barritt, G. J. (1999) Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular signalling requirements. *Biochem. J.* **337**, 153–169
- Shuttleworth, T. J. and Thompson, J. L. (1999) Discriminating between capacitative and arachidonate-activated Ca^{2+} entry pathways in HEK293 cells. *J. Biol. Chem.* **274**, 31174–31178
- Broad, L. M., Cannon, T. R. and Taylor, C. W. (1999) A non-capacitative pathway activated by arachidonic acid is the major Ca^{2+} entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentrations of vasopressin. *J. Physiol.* **517**, 121–134
- Mignen, O. and Shuttleworth, T. J. (2000) I_{ARC} , a novel arachidonate-regulated, non-capacitative Ca^{2+} entry channel. *J. Biol. Chem.* **275**, 9114–9119
- Munaron, L., Antoniotti, S., Distasi, C. and Lovisolo, D. (1997) Arachidonic acid mediates calcium influx by basic fibroblast growth factor in Balb-c 3T3 fibroblasts. *Cell Calcium* **22**, 179–188
- Luo, D., Broad, L. M., Bird, G. S. J. and Putney, J. W. J. (2001) Signaling pathways underlying muscarinic receptor-induced $[\text{Ca}^{2+}]_i$ oscillations in HEK293 cells. *J. Biol. Chem.* **276**, 5613–5621

- 9 Stevens, T. (2001) Is there a role for store-operated calcium entry in vasoconstriction? *Am. J. Physiol. Lung Cell. Mol. Physiol.* **280**, L866–L869
- 10 Putney, Jr, J. W. (1997) Capacitative Calcium Entry. p. 210, R.G. Landes Company, Austin
- 11 Byron, K. L. and Taylor, C. W. (1995) Vasopressin stimulation of Ca^{2+} mobilization, two bivalent cation entry pathways and Ca^{2+} efflux in A7r5 rat smooth muscle cells. *J. Physiol.* **485**, 455–468
- 12 Thibonnier, M., Bayer, A. L., Simonson, M. S. and Kester, M. (1991) Multiple signaling pathways of V_1 -vascular vasopressin receptors of A_{7r5} cells. *Endocrinology* **129**, 2845–2856
- 13 Migas, I. and Seversen, D. L. (1996) Diacylglycerols derived from membrane phospholipids are metabolized by lipases in A10 smooth muscle cells. *Am. J. Physiol.* **271**, C1194–C1202
- 14 Byron, K. L. and Taylor, C. W. (1993) Spontaneous Ca^{2+} spiking in a vascular smooth muscle cell line is independent of the release of intracellular Ca^{2+} stores. *J. Biol. Chem.* **268**, 6945–6952
- 15 Byron, K. L. (1996) Vasopressin stimulates Ca^{2+} spiking activity in A7r5 vascular smooth muscle cells via activation of phospholipase A_2 . *Circ. Res.* **78**, 813–820
- 16 Morgan, A. J. and Jacob, R. (1994) Ionomycin enhances Ca^{2+} influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. *Biochem. J.* **300**, 665–672
- 17 Manning, M., Lammek, B., Kolodziejczyk, A. M., Seto, J. and Sawyer, W. H. (1981) Synthetic antagonists of *in vivo* antidiuretic and vasopressor responses to arginine-vasopressin. *J. Med. Chem.* **24**, 701–706
- 18 Kawanabe, Y., Okamoto, Y., Hashimoto, N. and Masaki, T. (2001) Characterization of Ca^{2+} channels involved in endothelin-1-induced mitogenic responses in vascular smooth muscle cells. *Eur. J. Pharmacol.* **422**, 15–21
- 19 Shuttleworth, T. J. (1996) Arachidonic acid activates the noncapacitative entry of Ca^{2+} during $[\text{Ca}^{2+}]_i$ oscillations. *J. Biol. Chem.* **271**, 21720–21725
- 20 Broad, L. M., Cannon, T. R., Short, A. D. and Taylor, C. W. (1999) Receptors linked to polyphosphoinositide hydrolysis stimulate Ca^{2+} extrusion by a phospholipase C-independent pathway. *Biochem. J.* **342**, 199–206
- 21 Rogalski, S. L. and Chavkin, C. (2001) Eicosanoids inhibit the G-protein-gated inwardly rectifying potassium channel (Kir3) at the Na^+/PIP_2 gating site. *J. Biol. Chem.* **276**, 14855–14860
- 22 Alonso-Torre, S. R. and García-Sancho, J. (1997) Arachidonic acid inhibits capacitative calcium entry in rat thymocytes and human neutrophils. *Biochim. Biophys. Acta* **1328**, 207–213
- 23 Luo, D., Broad, L. M., Bird, G. S. J. and Putney, J. W. J. (2001) Mutual antagonism of calcium entry by capacitative and arachidonic acid-mediated calcium entry pathways. *J. Biol. Chem.* **276**, 20186–20189
- 24 Khakh, B. S., Zhou, X., Sydes, J., Galligan, J. J. and Lester, H. A. (2000) State-dependent cross-inhibition between transmitter-gated cation channels. *Nature (London)* **406**, 405–410
- 25 McCarron, J. G., Flynn, E. R. M., Bradley, K. N. and Muir, T. C. (2000) Two Ca^{2+} entry pathways mediate InsP_3 -sensitive store refilling in guinea-pig colonic smooth muscle. *J. Physiol.* **525**, 113–124
- 26 Mignen, O., Thompson, J. L. and Shuttleworth, T. J. (2001) Reciprocal regulation of capacitative and arachidonate-regulated noncapacitative Ca^{2+} entry pathways. *J. Biol. Chem.* **276**, 35676–35683
- 27 Lin, S., Fagan, K. A., Li, K.-X., Shaul, P. W., Cooper, D. M. F. and Rodman, D. M. (2000) Sustained endothelial nitric-oxide synthase activation requires capacitative Ca^{2+} entry. *J. Biol. Chem.* **275**, 17979–17985
- 28 Fagan, K. A., Mons, N. and Cooper, D. M. F. (1998) Dependence of the Ca^{2+} -inhibitable adenylyl cyclase of C6-2B glioma cells on capacitative Ca^{2+} entry. *J. Biol. Chem.* **273**, 9297–9305
- 29 Zhang, J., Sato, M., Duzic, E., Kubalak, S. W., Lanier, S. M. and Webb, J. G. (1997) Adenylyl cyclase isoforms and vasopressin enhancement of agonist-stimulated cAMP in vascular smooth muscles. *Am. J. Physiol.* **273**, H971–H980
- 30 Fagan, K. A., Graf, R. A., Tolman, S., Schaak, J. and Cooper, D. M. F. (2000) Regulation of a Ca^{2+} -sensitive adenylyl cyclase in an excitable cell. Role of voltage-gated versus capacitative Ca^{2+} entry. *J. Biol. Chem.* **275**, 40187–40194
- 31 Loesch, A. and Burnstock, G. (1995) Ultrastructural localization of nitric oxide synthase and endothelin in coronary and pulmonary arteries. *Cell Tissue Res.* **279**, 475–483

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