Nitric oxide co-ordinates the activities of the capacitative and non-capacitative Ca^{2+}-entry pathways regulated by vasopressin

Zahid MONEER, Jeanette L. DYER and Colin W. TAYLOR[1]

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, U.K.

In A7r5 vascular smooth muscle cells vasopressin, via arachidonic acid, regulates two Ca^{2+}-entry pathways. Capacitative Ca^{2+} entry (CCE), activated by empty Ca^{2+} stores, is inhibited by arachidonic acid, and non-capacitative Ca^{2+} entry (NCCE) is stimulated by it. This reciprocal regulation ensures that all Ca^{2+} entry is via NCCE in the presence of vasopressin, while CCE mediates a transient Ca^{2+} entry only after removal of vasopressin. We demonstrate that type III NO synthase (NOS III) is expressed in A7r5 cells and that NO inhibits CCE. Inhibition of CCE by vasopressin requires NOS III and the requirement lies downstream of arachidonic acid. Activation of soluble guanylate cyclase by NO and subsequent activation of protein kinase G are required for inhibition of CCE. Stimulation of NCCE by vasopressin also requires NOS III, but the stimulation is neither mimicked by cGMP nor blocked by inhibitors of soluble guanylate cyclase or protein kinase G. We conclude that arachidonic acid formed in response to vasopressin stimulates NOS III. NO then directly stimulates Ca^{2+} entry through NCCE and, via protein kinase G, it inhibits CCE. The additional amplification provided by the involvement of guanylate cyclase and protein kinase G ensures that CCE will always be inhibited when vasopressin activates NCCE.

Key words: A7r5 cell, arachidonic acid, cyclic GMP (cGMP), nitric oxide synthase, vascular smooth muscle.

INTRODUCTION

Receptors that stimulate formation of inositol 1,4,5-trisphosphate (IP_3) typically stimulate both release of Ca^{2+} from intracellular stores and Ca^{2+} entry across the plasma membrane [1]. The latter is often thought to be mediated by the capacitative Ca^{2+} entry (CCE) pathway, which is activated by an unknown signal generated in response to depletion of intracellular Ca^{2+} stores [2]. However, accumulating evidence suggests that non-capacitative Ca^{2+} entry (NCCE) pathways may contribute significantly to the Ca^{2+} entry evoked by receptors that stimulate phosphoinositide hydrolysis [3–5].

In A7r5 vascular smooth muscle cells, at least two Ca^{2+}-entry pathways contribute to the Ca^{2+} entry evoked by vasopressin, a hormone that stimulates phosphoinositide hydrolysis. The pathways are distinguishable by their different ion-permeation properties and sensitivities to blockers [4,6] (Figure 1A). CCE is activated when the intracellular stores are emptied by IP_3, and NCCE is activated by arachidonic acid released by diacylglycerol (DAG) lipase from the DAG produced with IP_3. Arachidonic acid stimulates NCCE in other cells too [5]. We recently demonstrated that the two Ca^{2+}-entry pathways in A7r5 cells do not behave independently. The arachidonic acid that stimulates NCCE simultaneously inhibits CCE, such that all Ca^{2+} entry occurs via NCCE when vasopressin is present, to be followed by a brief burst of Ca^{2+} entry via CCE when vasopressin is removed [6]. Because different Ca^{2+}-entry pathways are known to selectively regulate different cellular responses [7], we suggest that such reciprocal regulation of Ca^{2+}-entry pathways, which also occurs in other cells [8,9], may allow receptor activation to regulate one cellular response (via the Ca^{2+} signal generated by NCCE) and then, when the stimulus is removed, a counteracting cellular response (via the Ca^{2+} signal generated by CCE) [6].

cGMP and NO have been implicated in the regulation of Ca^{2+} entry, but the evidence is conflicting. cGMP has been reported to mediate [10] or enhance [11] agonist-evoked Ca^{2+} entry, with empty stores perhaps directly stimulating NO synthase (NOS) activity and thereby activation of guanylate cyclase and stimulation of CCE [12]. Others either failed to detect an effect of cGMP on CCE [13] or found it to inhibit agonist-evoked Ca^{2+} entry [14]. There is also evidence to suggest a direct effect of NO on Ca^{2+}-entry pathways [15]. Much of this evidence predates the realization that agonists regulate multiple Ca^{2+}-entry pathways [6,16–18]. We therefore considered the possibility that the conflicting results might reflect opposing effects of NO/cGMP signalling pathways on different receptor-regulated Ca^{2+}-entry pathways. This, together with recent evidence showing functional expression of NOS in vascular smooth muscle [19], prompted us to examine the role of NO and cGMP in mediating the reciprocal regulation of CCE and NCCE by vasopressin in A7r5 vascular smooth muscle cells. Our results demonstrate an essential role for NOS III in co-ordinating the activities of the two Ca^{2+}-entry pathways in A7r5 cells.

EXPERIMENTAL

Fura 2 measurements of Ca^{2+} entry

A7r5 cells were cultured on glass coverslips, loaded with fura 2 and the cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured using the methods reported previously [6]. In brief, fura-2-loaded cells on

Abbreviations used: 8-Br-cGMP, 8-bromo-cGMP; $[Ca^{2+}]_i$, cytosolic free $[Ca^{2+}]$; CCE, capacitative Ca^{2+} entry; DAG, diacylglycerol; ETYA, 5,8,11,14-eicosatetraynoic acid; IBMX, isobutylmethylxanthine; IP_3, inositol 1,4,5-trisphosphate; NCCE, non-capacitative Ca^{2+} entry; NOC-18, 2,2′-(hydroxynitrosohydrazino)bis-ethanamine; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PKG, protein kinase G; RHC-80267, 1,6-bis(cyclohexyloximinocarbonylamino)hexane; U73122, 1-[6((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.

[1] To whom correspondence should be addressed (e-mail cwt1000@cam.ac.uk).

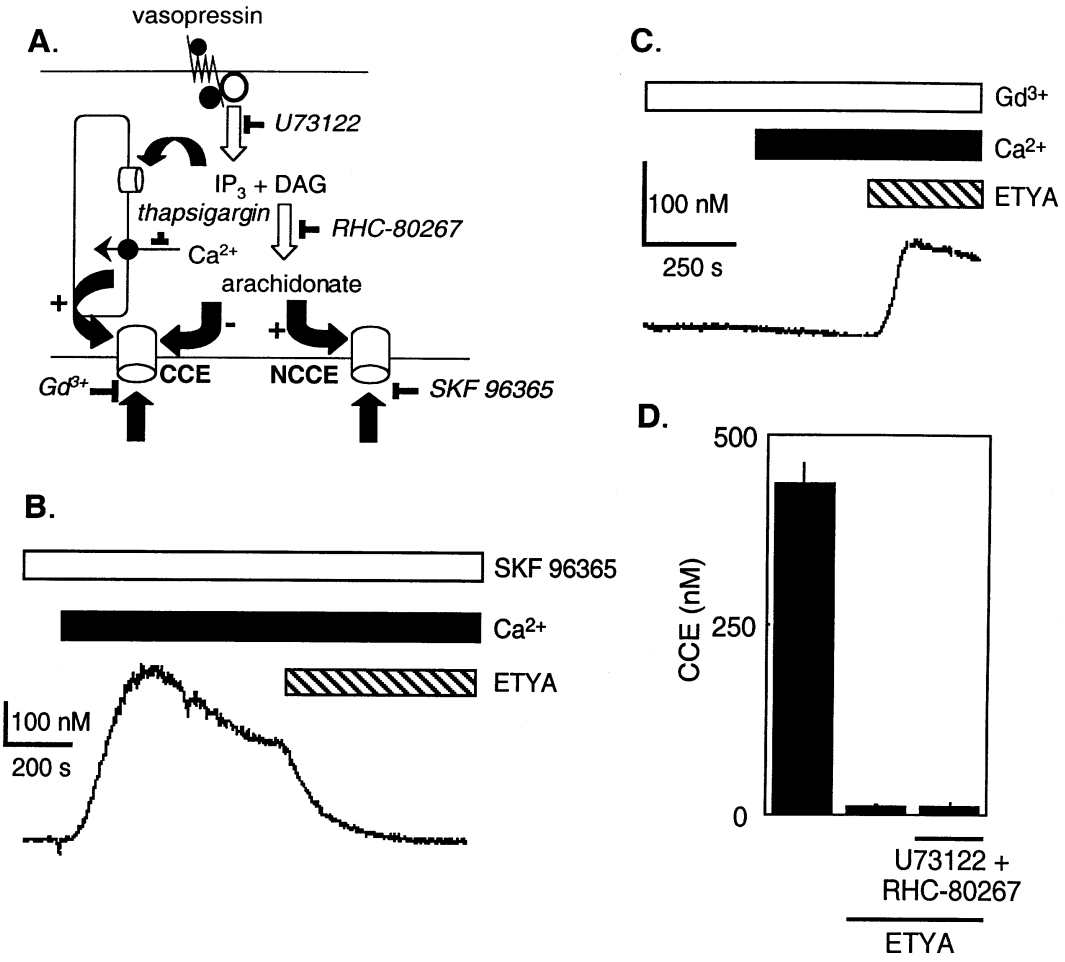


Figure 1 Reciprocal regulation of CCE and NCCE by vasopressin

(**A**) The IP_3 formed in response to vasopressin stimulates release of Ca^{2+} from intracellular stores and so activation of CCE, while diacylglycerol (DAG) is metabolized to arachidonic acid and that both activates NCCE and inhibits CCE. Selective inhibitors of phospholipase C (U73122), DAG lipase (RHC-80267), the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (thapsigargin) and of the two Ca^{2+}-entry pathways (1 μM Gd^{3+} and SKF 96365) are shown by hammerheads. (**B**) Restoration of extracellular Ca^{2+} to cells pre-treated with thapsigargin/ionomycin to empty the intracellular Ca^{2+} stores evoked a large CCE signal that was inhibited by ETYA (50 μM). SKF 96365 (100 nM) was present throughout to inhibit NCCE. (**C**) In the presence of 1 μM Gd^{3+} to inhibit CCE, addition of ETYA (50 μM) to cells with empty Ca^{2+} stores stimulated Ca^{2+} entry via the NCCE pathway. (**D**) ETYA (50 μM) completely inhibited the CCE Ca^{2+} signal evoked by restoration of extracellular Ca^{2+} to cells with empty Ca^{2+} stores, even in the presence of U73122 (30 μM) and RHC-80267 (50 μM). SKF 96365 (100 nM) was present to inhibit NCCE.

glass coverslips were mounted in the lightpath of a fluorescence spectrometer (Hitachi F4500) and alternately excited with light of wavelengths 340 and 380 nm, while collecting emitted light (510 nm). After correction for autofluorescence (following addition of 1 μM ionomycin and 1 mM $MnCl_2$), fluorescence ratios were calibrated to $[Ca^{2+}]_i$ by means of a look-up table created from Ca^{2+} standard solutions (Molecular Probes, Leiden, The Netherlands). Cells were continuously perfused (17 ml/min) at 20 °C with Hepes-buffered saline (HBS; NaCl, 135 mM; KCl, 5.9 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 1.5 mM; Hepes, 11.6 mM; glucose, 11.5 mM; pH 7.3). Where appropriate intracellular Ca^{2+} stores were emptied by pre-incubating cells for 10 min with thapsigargin (1 μM) and ionomycin (1 μM) in Ca^{2+}-free HBS. As shown previously, this method completely empties the intracellular Ca^{2+} stores and the only Ca^{2+} entry it activates is via CCE [6]. All media included verapamil (10 μM) to inhibit L-type Ca^{2+} channels. The half-time for exchange of media within the cuvette was 9.6 ± 0.3 s. All traces are typical of at least three experiments and results are shown as means $\pm$ S.E.M. of at least three independent experiments.

Antibody methods

Cells were detached from culture dishes and suspended in lysis buffer (Tris/HCl, 10 mM; EDTA, 1 mM; aprotinin, 150 nM; pepstatin, 1 μM; soya bean trypsin inhibitor, 20 μg/ml; benzamidine, 1 mM; captopril, 100 μM; leupeptin, 1 μM; bestatin, 1 μM; PMSF, 250 μM; pH 8). After lysis by freeze-thawing, the cells were centrifuged (20000 ***g***, 30 min) and the supernatant and pellets were rapidly frozen. Standard methods were used for SDS/PAGE and immunoblotting [20] using mouse antisera (1:2500) selective for each NOS isoform (Transduction Laboratories, Oxford, U.K.) and a secondary goat anti-mouse IgG (1:1000) coupled to horseradish peroxidase (Sigma, Poole, Dorset, U.K.). The blots were then visualized using enhanced chemiluminescence reagents [20].

cGMP assay

Confluent cultures of A7r5 cells in 35 mm dishes were incubated with isobutylmethylxanthine (IBMX; 100 μM, 5 min) in HBS and then with appropriate additions for a further 5 min in the

continued presence of IBMX. Incubations were terminated by aspiration of the medium and addition of ice-cold ethanol (65%, 0.5 ml). After 5 min, the medium was removed, the dishes were washed (0.5 ml of 65% ethanol) and the pooled extracts were centrifuged (20000 ***g***, 5 min). The supernatants were then dried under vacuum. The cGMP contents of a series of dilutions of the samples were then determined using a cGMP enzyme immunoassay (Biotrak; Amersham Biosciences, Little Chalfont, Bucks., U.K.) according to the manufacturer's instructions.

Materials

A7r5 cells were originally from the American Type Culture Collection (Rockville, MD, U.S.A.). A 2 mM stock solution of NO was prepared by gassing deionized water first with nitrogen for 15 min and then with NO (Messer, Frankfurt, Germany) for 10 min [21]. This saturated solution of NO was then diluted directly into the physiological saline within the cuvette to give a final concentration of 1 μM. Arachidonic acid, ETYA (5,8,11,14-eicosatetraynoic acid), NOC-18 [2,2′-(hydroxynitrosohydrazino)-bis-ethanamine], ODQ (1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), RHC-80267 [1,6-bis(cyclohexyloximinocarbonylamino)-hexane], U73122 {1-[6((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione} and 8-bromo-cGMP (8-Br-cGMP) were from Calbiochem (Nottingham, U.K.). L-Arginine, L-NAME (N^G-nitro-L-arginine) and verapamil were from Sigma. KT 5283 and SKF 96365 were from Tocris (Avonmouth, U.K.). The targets of the drugs are shown in Figures 1(A), 3(C) and 4(B). Other suppliers were reported previously [6].

RESULTS

Reciprocal regulation of CCE and NCCE by arachidonic acid

We reported previously that Gd^{3+} (1 μM) and LOE-908 (30 μM) selectively inhibit CCE and NCCE respectively, without affecting the other pathway [6]. Here we use SKF 96365, which is more readily available than LOE-908, to selectively inhibit NCCE. SKF 96365 (100 nM) reduced vasopressin-evoked NCCE by $93 \pm 3\%$ ($n = 3$) without affecting CCE ($3 \pm 9\%$ inhibition). Throughout this study, we used 1 μM Gd^{3+} to selectively block CCE and 100 nM SKF 96365 to selectively block NCCE (Figure 1A).

Restoration of extracellular Ca^{2+} to A7r5 cells in which the intracellular Ca^{2+} stores had been completely emptied by thapsigargin and ionomycin evoked a large increase in the $[Ca^{2+}]_i$ as Ca^{2+} entered the cells via the CCE pathway (Figure 1B). In the presence of SKF 96365 to block NCCE, ETYA, a stable analogue of arachidonic acid, completely inhibited CCE (Figure 1B). With CCE blocked by Gd^{3+}, ETYA stimulated Ca^{2+} entry via the NCCE pathway (Figure 1C). Inhibition of either phospholipase C (U73122, 30 μM) or DAG lipase (RHC-80267, 50 μM) abolished the ability of vasopressin to inhibit CCE and activate NCCE [4,6] but, as expected, neither inhibitor prevented ETYA from inhibiting CCE (Figure 1D). These results confirm that arachidonic acid mediates the reciprocal regulation of the two Ca^{2+}-entry pathways by vasopressin [6] and that the metabolism of arachidonic acid is not required. We [6] and others [5,8] had therefore assumed that arachidonic acid directly stimulated NCCE and directly inhibited CCE. Here we examine more closely the mechanisms underlying reciprocal regulation of the two pathways by vasopressin and arachidonic acid.

Inhibition of CCE by vasopressin is mediated by NO

Addition of either the NO donor, NOC-18 (half-maximal inhibitory concentration, $IC_{50} = 62 \pm 8$ μM), or NO (1 μM) itself completely and reversibly abolished CCE (Figures 2A and 2B). By contrast in control experiments, restoration of extracellular Ca^{2+} for a second time evoked an increase in $[Ca^{2+}]_i$ that was $91 \pm 7\%$ ($n = 5$) of the initial CCE response. Using isoform-selective antibodies to NOS, we detected NOS III (also known as endothelial NOS or eNOS) in A7r5 cells, but not NOS I or NOS II. The NOS III was located exclusively in the membrane fraction (Figure 2C), in keeping with results from many other

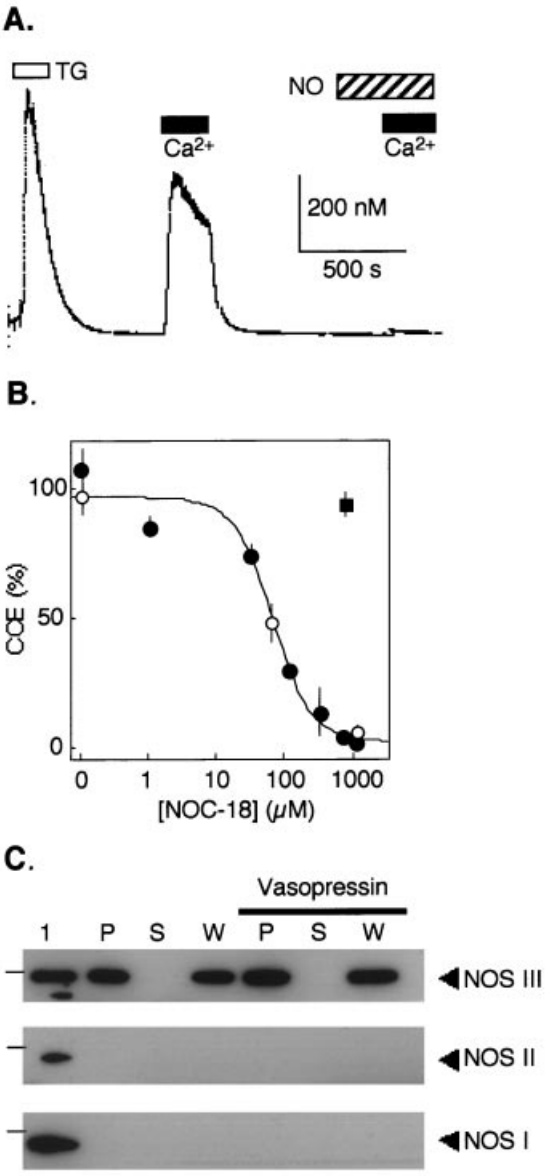


Figure 2 Inhibition of CCE by NO

(**A**) Restoration of extracellular Ca^{2+} to cells treated with thapsigargin/ionomycin (TG) evoked a large CCE signal that was inhibited by NO (1 μM). (**B**) NOC-18 caused a concentration-dependent inhibition of CCE (●), which fully reversed after its removal (■). Inhibition of CCE by NOC-18 was unaffected by a combination of U 73122 (30 μM) and RHC-80267 (50 μM; ○). SKF 96365 (100 nM) was present throughout (**A**) and (**B**) to block NCCE. (**C**) Lanes were loaded with 10 μg of protein from lysed A7r5 cells (W) or from the supernatant (S) or pellet fractions (P), each prepared from unstimulated cells or from cells stimulated with vasopressin (100 nM, 5 min). Lane 1 in each of the three blots shows lysates from cells expressing predominantly one isoform of NOS: I (rat pituitary), II (stimulated mouse macrophages) and III (human endothelial cells). Lines (left) show positions of the 150 kDa marker.

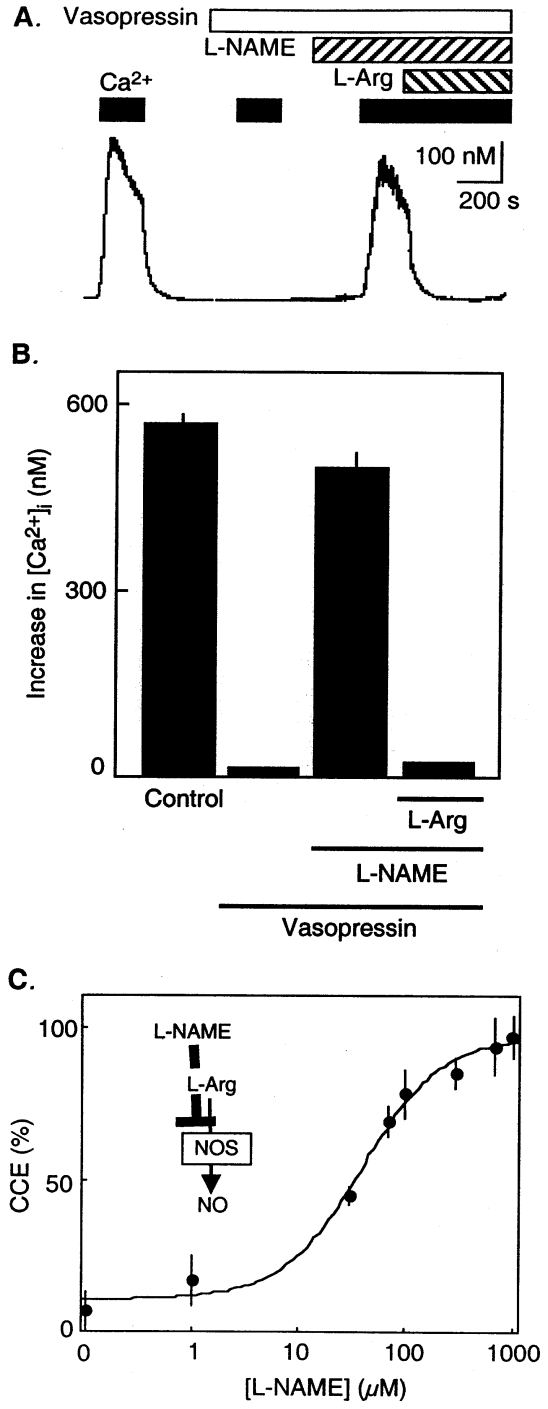


Figure 3 NO mediates inhibition of CCE by vasopressin

(**A**) Vasopressin completely inhibited the CCE evoked by restoration of extracellular Ca^{2+} to cells with empty Ca^{2+} stores, and the inhibition was reversed by L-NAME (700 μM). Addition of L-arginine (2.1 mM) then reversed the effect of L-NAME. (**B**) Summarizes results from four similar experiments. (**C**) The concentration-dependent reversal by L-NAME of the inhibition of CCE by vasopressin (50 nM). SKF 96365 (100 nM) was present throughout (**A**)–(**C**) to block NCCE.

cell types [22]. Vasopressin had no obvious effect on the distribution of NOS III (Figure 2C).

Our earlier work established that vasopressin inhibited CCE and that the inhibition was not a consequence of either membrane depolarization (it persisted in medium containing 145 mM KCl) or the increased activity of Ca^{2+} pumps (vasopressin inhibited unidirectional Ba^{2+} and Mn^{2+} entry, neither of which are transported by Ca^{2+} pumps) [6]. We therefore assessed whether inhibition of CCE by vasopressin was mediated by NO. Because vasopressin also activates NCCE, its effect on CCE was examined in cells with completely empty Ca^{2+} stores (to maximally activate CCE) and with the NCCE pathway blocked by SKF 96365. Under these conditions, vasopressin (100 nM) completely inhibited CCE (Figure 3A), but in the presence of L-NAME, which inhibits NOS by competing with the natural substrate L-arginine, there was a concentration-dependent ($IC_{50} = 39 \pm 5$ μM) reversal of the inhibition by vasopressin (Figures 3B and 3C). The effect of L-NAME was itself reversed by addition of L-arginine (Figures 3A and 3B). Another structurally unrelated inhibitor of NOS, 7-nitroindazole (10 μM), also blocked the ability of vasopressin to inhibit CCE (results not shown). These results establish that inhibition of CCE by vasopressin requires activation of NOS. Inhibitors of phospholipase C (U73122, 30 μM) or DAG lipase (RHC-80267, 50 μM) completely blocked the inhibition of CCE by vasopressin [6] without affecting the ability of either ETYA (50 μM, Figure 1D) or NOC-18 (Figure 2B, ○) to inhibit CCE. NOS must therefore lie downstream of arachidonic acid in the sequence linking the vasopressin receptor to inhibition of CCE.

Vasopressin inhibits CCE via protein kinase G (PKG)

A major intracellular target of NO is soluble guanylate cyclase. We therefore examined whether cGMP and PKG were involved in the inhibition of CCE by NO. The membrane-permeant analogue of cGMP, 8-Br-cGMP, caused a complete and concentration-dependent ($IC_{50} = 120 \pm 17$ μM) inhibition of CCE (Figures 4A and 4B). The effect of 8-Br-cGMP was blocked by a selective inhibitor of PKG (KT 5823, Figure 5). Activation of PKG therefore mimics the inhibitory effect of vasopressin on CCE.

Selective inhibitors of either NO-sensitive guanylate cyclase (ODQ; $IC_{50} = 44 \pm 8$ nM) [23] or PKG (KT 5823; $IC_{50} = 86 \pm 10$ nM) caused complete and concentration-dependent reversal of the inhibition of CCE by the NO donor NOC-18, NO itself or vasopressin (Figures 4C–4F). As expected, inhibition of NOS by L-NAME had no effect on the ability of 8-Br-cGMP (1 mM) to inhibit CCE (Figure 5), confirming that NOS is upstream of cGMP in the pathway linking vasopressin to inhibition of CCE. Vasopressin therefore inhibits CCE via NO, stimulation of cGMP formation and subsequent activation of PKG.

Vasopressin and ETYA stimulate cGMP formation

Because our pharmacological analyses clearly implicate NO and cGMP in mediating the inhibitory effect of vasopressin on CCE (Figures 2–5), we measured the effects of vasopressin and the stable arachidonic acid analogue, ETYA, on cGMP formation. In the presence of IBMX to inhibit cyclic nucleotide phosphodiesterases (see the Experimental section), the cGMP content of A7r5 cells increased significantly (Student's t test, $P < 0.01$) from 180 ± 24 fmol/35 mm dish ($n = 5$) to 1528 ± 179 fmol/dish during a 5 min incubation with 100 nM vasopressin, and to 566 ± 94 fmol/dish during a 5 min incubation with 50 μM ETYA.

Activation of NCCE by vasopressin requires NO but not cGMP

Arachidonic acid, released from the DAG produced when vasopressin activates its receptor and stimulates phospholipase C, both inhibits CCE and stimulates NCCE [6] (Figure 1A). We

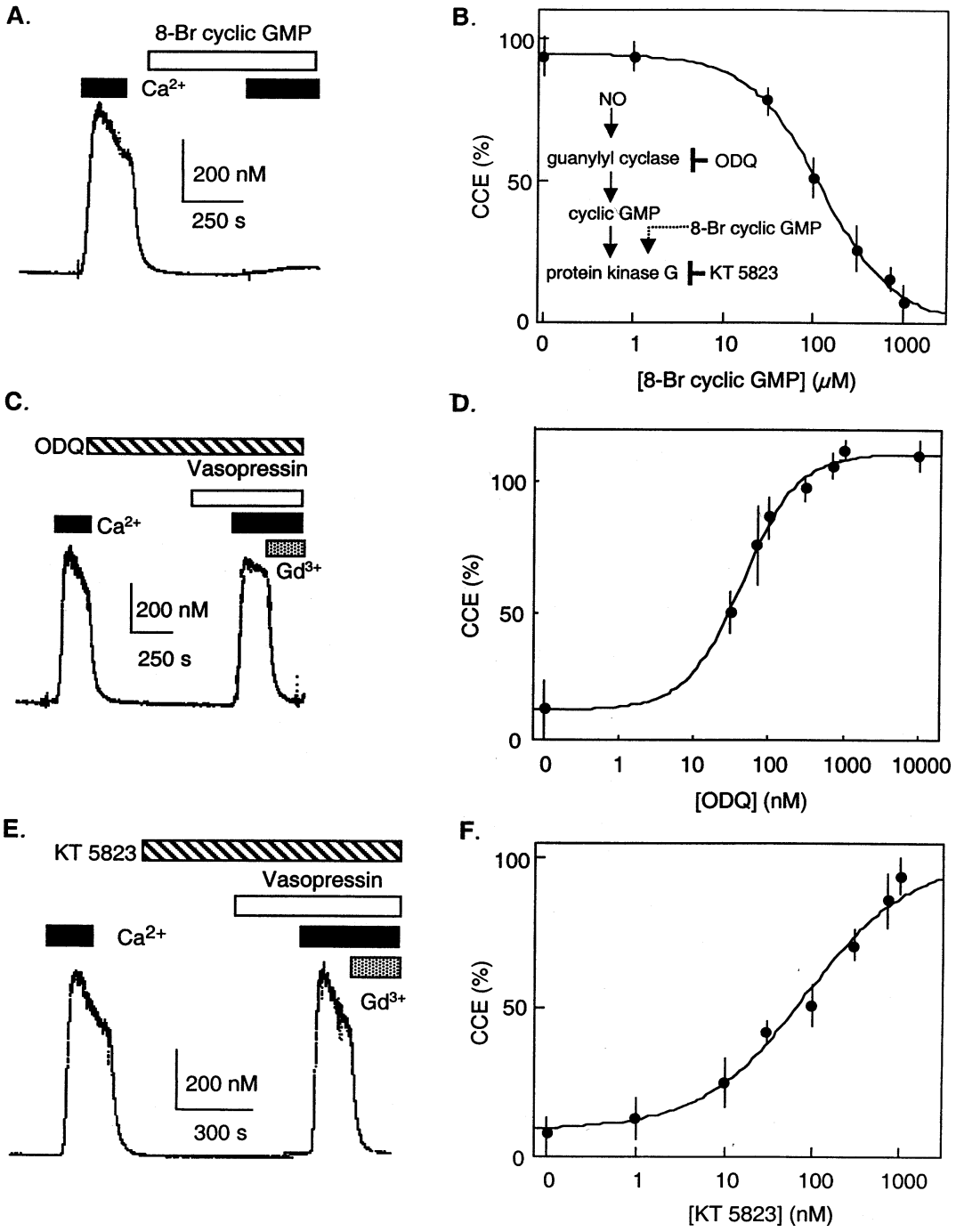


Figure 4 Inhibition of CCE by vasopressin requires PKG

(**A** and **B**) 8-Br-cGMP (1 mM) caused a complete (**A**) and concentration-dependent (**B**) inhibition of CCE. The inset to (**B**) shows the signalling sequence from NO to PKG and the inhibitors (hammerheads) used. (**C**, **D**) ODQ (10 μM) caused a complete (**C**) and concentration-dependent (**D**) reversal of the inhibition of CCE by vasopressin (50 nM). Gd^{3+} (1 μM) rapidly reversed the Ca^{2+} signal confirming that it was mediated by CCE. (**E**, **F**) KT 5823 (1 μM) caused a complete (**E**) and concentration-dependent (**F**) reversal of the inhibition of CCE by vasopressin (50 nM). SKF 96365 (100 nM) was present throughout all experiments to block NCCE.

therefore sought to establish whether NO and cGMP were also required for vasopressin to stimulate NCCE. In the presence of 1 μM Gd^{3+} to inhibit CCE, addition of vasopressin (Figure 6A) or ETYA (Figure 1C) to cells with empty intracellular Ca^{2+} stores stimulated Ca^{2+} entry. The effect of vasopressin or ETYA was mimicked by NOC-18 or NO (Figures 6B and 6C) and abolished by L-NAME (Figures 6A, 6D and 6E). The effect of L-NAME was itself reversed by addition of L-arginine (Figure 6E). Stimulation of NCCE by vasopressin is therefore mediated by NO and the requirement for NO lies downstream of arachidonic acid.

Although PKG mediates the inhibitory effect of vasopressin on CCE (Figures 4 and 5), it is not required for activation of NCCE. Addition of 8-Br-cGMP, at a concentration (1 mM)

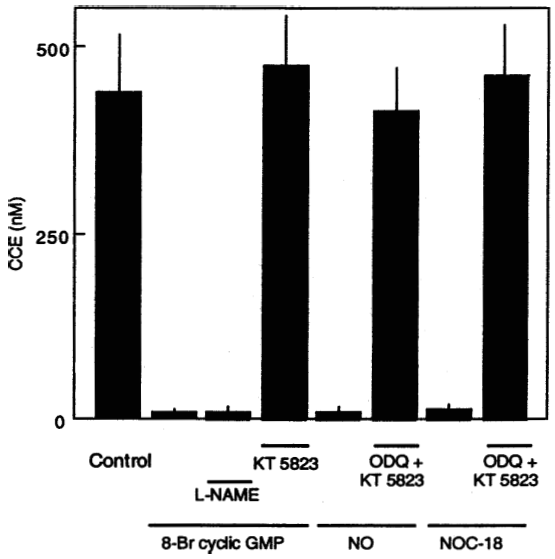


Figure 5 Inhibition of CCE by NO requires PKG

In experiments similar to those shown in Figure 4, intracellular stores were emptied using ionomycin and thapsigargin and the amplitude of the CCE signal was then measured after restoration of extracellular Ca^{2+}. Control responses and the responses observed in the presence of 8-Br-cGMP (1 mM), NO (1 μM) or NOC-18 (1 mM) are shown as the peak change in $[Ca^{2+}]_i$ after restoration of extracellular Ca^{2+} (mean $\pm$ S.E.M. from three or more experiments). 8-Br-cGMP and NOC-18, and where indicated ODQ (10 μM), L-NAME (1 mM) and KT 5823 (1 μM) were present for 5 min before and then during Ca^{2+} restoration. NO was added with the extracellular Ca^{2+}. SKF 96365 (100 nM) was present throughout to inhibit NCCE.

greater than that required to inhibit CCE (Figure 4B), failed to activate NCCE (Figure 7). Furthermore, concentrations of ODQ (10 μM, to inhibit soluble guanylate cyclase) or KT 5823 (10 μM, to inhibit PKG) in excess of those that completely blocked inhibition of CCE by vasopressin (Figures 4D and 4F) had no effect on activation of NCCE by vasopressin (Figures 7B and 7C). We conclude that neither cGMP nor PKG is required for activation of NCCE (Figure 7D).

NO determines which pathway mediates vasopressin-evoked Ca^{2+} entry

Vasopressin empties intracellular Ca^{2+} stores and might thereby be expected to activate CCE, but NO produced in response to vasopressin also causes inhibition of CCE (Figures 2–5) and activation of NCCE (Figures 6 and 7) such that in the presence of vasopressin all Ca^{2+} entry occurs via the NCCE pathway [6]. A clear prediction of this scheme is that with NOS inhibited, vasopressin should stimulate a large Ca^{2+}-entry signal mediated entirely by the CCE pathway. The results shown in Figure 8 confirm this prediction. The Ca^{2+} entry evoked by vasopressin (100 nM) was insensitive to Gd^{3+} (1 μM) but completely blocked by SKF 96365 (100 nM; Figure 8A), confirming that the NCCE pathway was entirely responsible. However, in the presence of L-NAME to inhibit NOS, vasopressin evoked a much larger Ca^{2+}-entry signal that was insensitive to SKF 96365 but completely blocked by Gd^{3+} (1 μM; Figure 8B). As expected, L-arginine reversed the effect of L-NAME, such that all vasopressin-evoked Ca^{2+} entry was again mediated by the SKF 96365-sensitive NCCE pathway (Figure 8C). These results establish that when NOS is inhibited all vasopressin-evoked Ca^{2+} entry occurs via the CCE pathway.

DISCUSSION

Co-ordinated regulation of CCE and NCCE

Many Ca^{2+}-regulating proteins are influenced by the NO/cGMP signalling pathway [24]. These include proteins responsible for synthesis of Ca^{2+}-mobilizing messengers, IP_3 and ryanodine receptors, the Na^+/Ca^{2+} exchanger, Ca^{2+} pumps in both the endoplasmic reticulum and plasma membrane, and a variety of plasma-membrane Ca^{2+} channels [24,25]. Most are regulated by PKG-mediated phosphorylation, but for some nitrosylation of reactive thiol groups allows direct and reversible regulation by NO [26,27]. Our results suggest another role for NO, namely the co-ordination of two Ca^{2+}-entry pathways by mechanisms that involve both a direct action of NO and regulation via PKG.

In several cell types NCCE is more important than CCE in mediating the Ca^{2+} entry evoked by receptor activation [4,28,29] and arachidonic acid is emerging as an important co-ordinating signal that both stimulates NCCE [4,9] and inhibits CCE [6,8,9]. Our results establish the presence of NOS III in A7r5 cells (Figure 2C) and they provide the first evidence that NOS III is an obligatory link between receptor activation and reciprocal regulation of CCE and NCCE. Furthermore, by identifying the signalling cascades that regulate the two Ca^{2+}-entry pathways, we suggest a molecular mechanism to account for the sequential activation of first NCCE and then CCE during addition and then removal of vasopressin (Scheme 1).

Dual regulation of CCE

In A7r5 cells most Ca^{2+} entry evoked by receptor activation enters via NCCE [4], yet the Ca^{2+} signal recorded after activation of CCE by thapsigargin is about five times greater than that evoked by maximal activation of NCCE (Figures 1B and 1C). The paradox is resolved by the demonstration that a physiological stimulus (vasopressin) stimulates formation of both IP_3 and DAG, which deliver opposing signals to the CCE pathway. IP_3 empties the intracellular stores and so activates CCE, while arachidonic acid released from DAG initiates a cascade that inhibits CCE (Figure 1A and Scheme 1). Thapsigargin applies only the accelerator, while receptor activation applies the accelerator and the brakes.

Both arachidonic acid [6,8,9] and NO [25] have been shown to inhibit CCE. Inhibition of CCE by vasopressin in A7r5 cells does not result from changes in membrane potential [6], nor from stimulated Ca^{2+} uptake into intracellular stores [25,30] because in all our experiments the Ca^{2+}-ATPase of the endoplasmic reticulum was inhibited by thapsigargin. NO can also inhibit CCE by uncoupling mitochondria so that they are unable to prevent inhibitory Ca^{2+} feedback on to the CCE channel [31]. This cannot be the explanation for our results because vasopressin inhibits Mn^{2+} and Ba^{2+} entry via CCE (neither provides negative feedback) [6] and inhibition of CCE requires activation of PKG (Figures 4 and 5), whereas NO directly inhibits mitochondria [31]. Inhibition of CCE by vasopressin is likely, therefore, to reflect a direct effect of the signalling pathways on the CCE channel.

Recent results from endothelial cells [25] and vascular smooth muscle [30] have suggested that NOS mediates negative feedback to CCE, such that Ca^{2+} entering by CCE binds to calmodulin, activates NOS, and NO then inhibits CCE, either directly [31] or via PKG [25]. Our results suggest that vasopressin, even in the absence of Ca^{2+} entry [6], stimulates NOS III, and that the subsequent activation of PKG inhibits CCE (Scheme 1). The involvement of NOS III is interesting because although it is

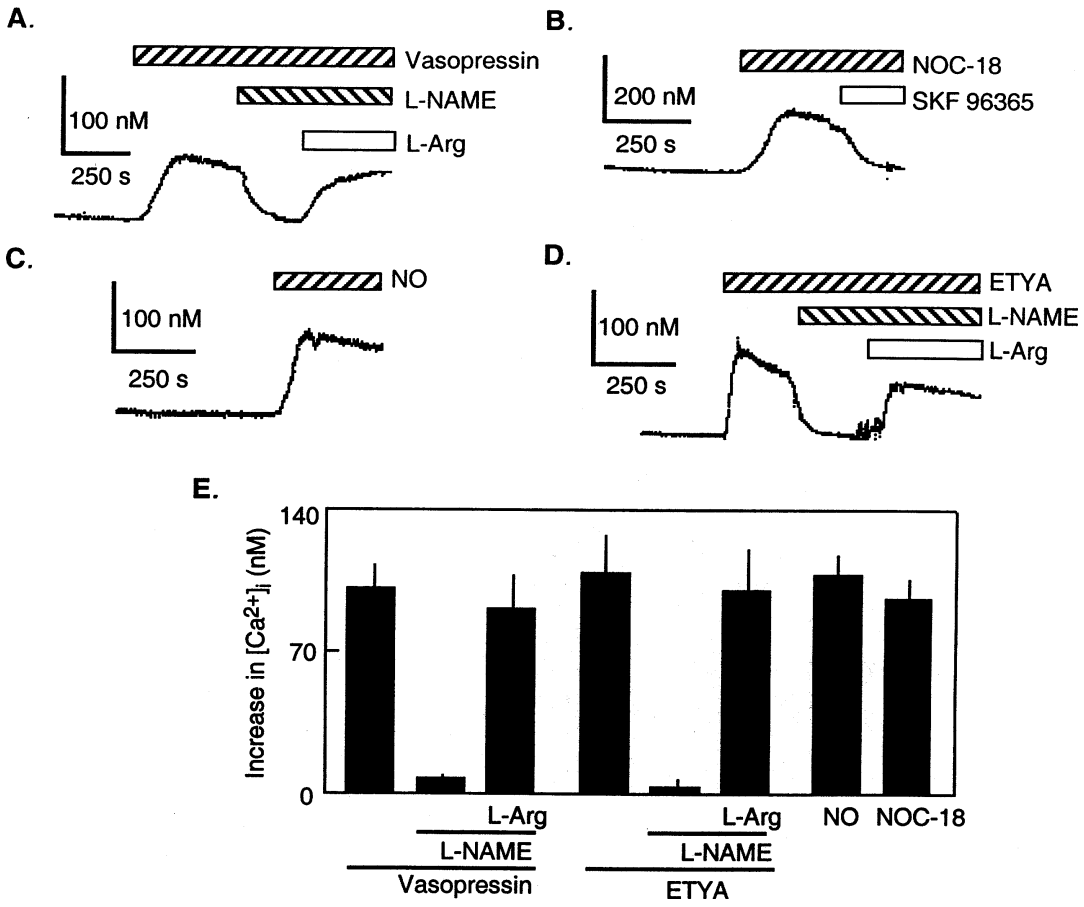


Figure 6 Activation of NCCE is mediated by NO

(**A**) Addition of vasopressin (50 nM) to cells with empty intracellular Ca^{2+} stores stimulated NCCE, which was inhibited by L-NAME (700 μM) and then reversed by L-arginine (2.1 mM). (**B**, **C**) NOC-18 (1 mM) or NO (1 μM) also stimulate NCCE. SKF 96364 (100 nM) reversed the Ca^{2+} signal confirming that it was mediated by NCCE. (**D**) L-NAME (700 μM) prevented the activation of NCCE by ETYA (50 μM), and the effect was reversed by L-arginine (2.1 mM). (**E**) Summarizes results from at least three similar experiments showing that NO and NOC-18 mimic the stimulation of NCCE by vasopressin and ETYA. The effects of the latter are inhibited by L-NAME (700 μM), and show that inhibition is reversed by L-arginine (2.1 mM). In all traces 1 μM Gd^{3+} was present to inhibit CCE.

stimulated by Ca^{2+}/calmodulin, in common with the other NOS isoforms, it is also stimulated after phosphorylation by a variety of protein kinases, including protein kinase B [22]. In A7r5 cells, arachidonic acid is upstream of NOS III in the signalling sequence and its metabolism is not required for NOS activation [4] (Figures 1B–1D), but we have yet to resolve how arachidonic acid stimulates NOS III. Vasopressin has no obvious effect on the distribution of NOS III (Figure 2C) and we are unaware of evidence that NOS III is directly stimulated by arachidonic acid. Wortmannin (100 nM, 15 min), which inhibits phosphoinositide 3-kinase and so formation of the 3-phosphorylated phosphoinositides that activate protein kinase B, had no effect on inhibition of CCE by vasopressin (results not shown). Identifying the link between arachidonic acid and stimulation of NOS III is an issue for future work.

Activation of NCCE by NO

Our assumption that arachidonic acid directly regulates NCCE was too simple [6]: arachidonic acid stimulates NCCE only via its ability to stimulate NOS III (Figure 6). It remains to be shown whether the other cells in which arachidonic acid stimulates NCCE [5] are likewise dependent on activation of NOS. Inhibition of CCE and stimulation of NCCE by vasopressin share an identical signalling pathway as far as NO, but thereafter there are significant differences. Activation of the cGMP cascade is required for inhibition of CCE (Figures 4 and 5), but this pathway is not required for stimulation of NCCE (Figure 7). Instead, NO itself stimulates NCCE. The simplest mechanism would be to suggest that S-nitrosylation of critical thiol groups on the NCCE channel causes its opening. A similar mechanism has been proposed for modulation of ryanodine receptors [32] and other channels by NO (see references in [27]). However, until we have identified the channel that mediates NCCE, presumably a member of the trp family [33], and possibly trp6 [34,35], we cannot establish whether the effect of NO is on the channel itself or another intermediary protein. Earlier work claimed that NO stimulated CCE in DDT1-MF-2 cells by causing S-nitrosylation of the channel [15,36], but the properties of this NO-stimulated Ca^{2+} entry seem more consistent with it being mediated by NCCE. Activation of NCCE by S-nitrosylation may therefore be a characteristic of other cells too.

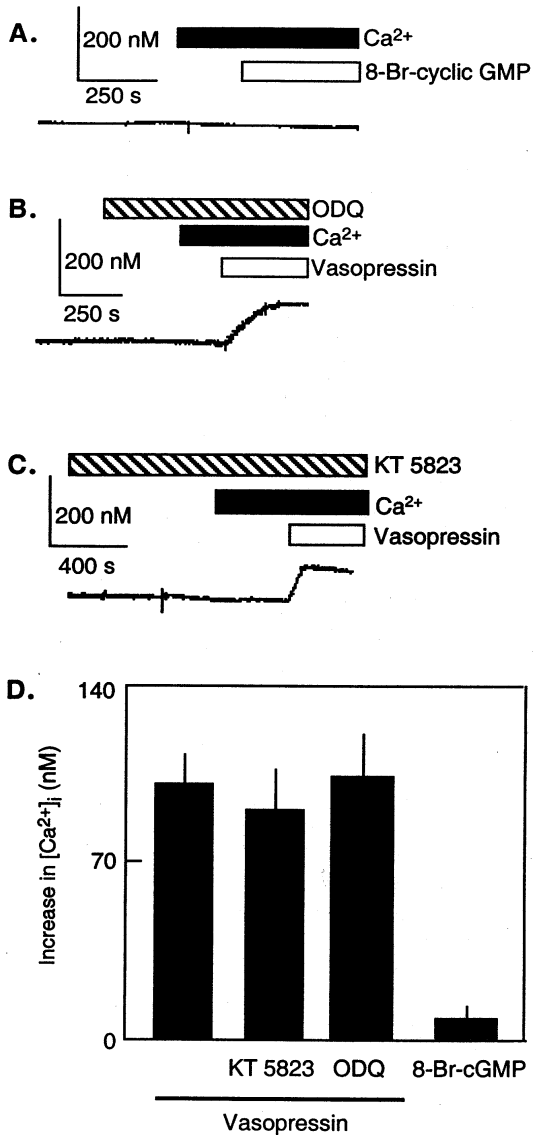


Figure 7 Activation of NCCE is not mediated by PKG

(**A**) 8-Br-cGMP (1 mM) did not stimulate Ca^{2+} entry via NCCE. (**B**, **C**) Neither ODQ (10 μM) nor KT 5823 (1 μM) affected the ability of vasopressin to stimulate Ca^{2+} entry via NCCE. (**D**) Summarizes results from three similar experiments. Experiments were performed on cells with empty intracellular Ca^{2+} stores and with 1 μM Gd^{3+} present to block CCE.

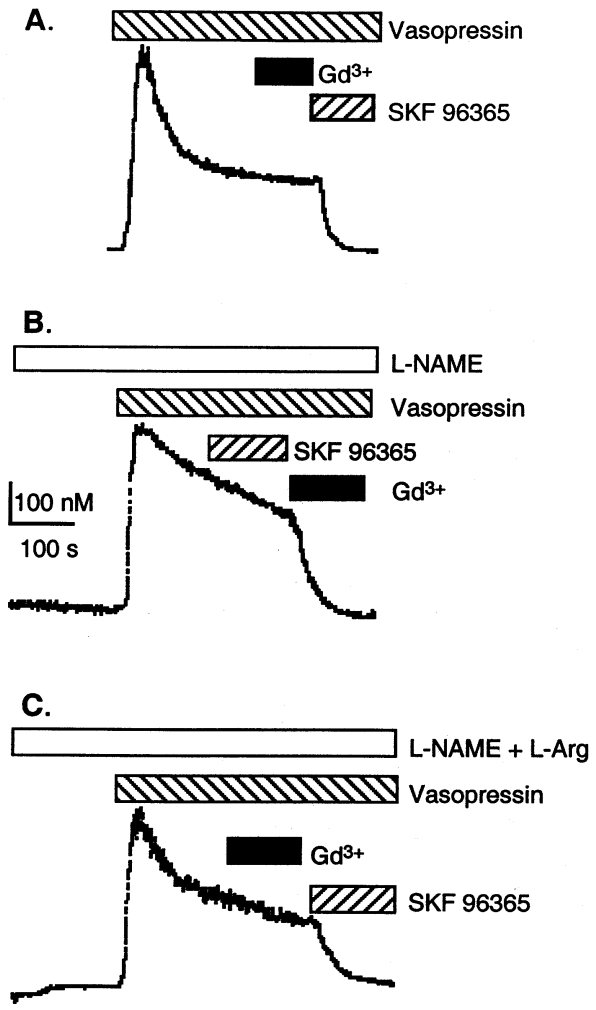


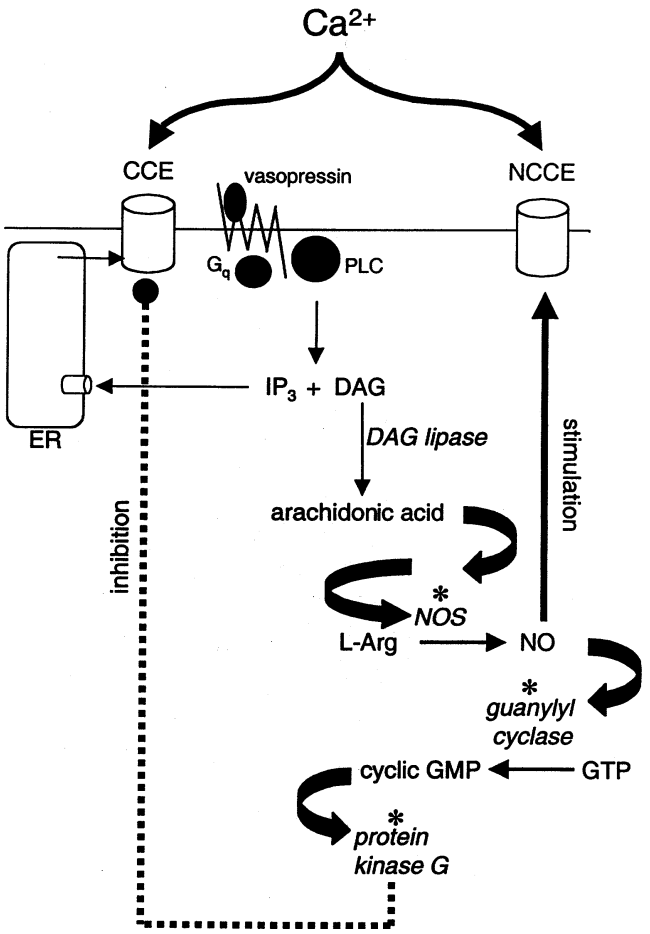
Figure 8 NO determines which pathway mediates vasopressin-evoked Ca^{2+} entry

Cells were stimulated with vasopressin (100 nM) in normal HBS alone (**A**) or in the presence of L-NAME (700 μM, **B**), or L-NAME and L-arginine (2.1 mM, **C**). After the initial Ca^{2+} mobilization phase of the response to vasopressin, the effects of Gd^{3+} (1 μM) and SKF 96365 (100 nM) on the Ca^{2+}-entry phase were assessed. The scale bar applies to all three traces.

Conclusions: regulation of Ca^{2+} entry by both limbs of the phosphoinositide pathway

IP_3 is widely assumed to be the intracellular messenger with prime responsibility for regulating both phases of the Ca^{2+} signal evoked by receptors that stimulate phospholipase C: its receptor in the endoplasmic reticulum mediates Ca^{2+} release from the intracellular stores and the empty stores then trigger the second phase of the response by activating CCE [2]. We propose that in A7r5 cells, and perhaps in other cells too, both limbs of the phosphoinositide pathway (IP_3 and DAG) work together to co-ordinate Ca^{2+} entry, with DAG initiating a cascade that both stimulates NCCE and inhibits CCE. Because the two initiating signals are produced together, there is an inextricable reciprocal regulation of the two pathways, but the properties of the signalling cascades that link vasopressin to CCE and NCCE are also crucial. Each pathway begins with a messenger (IP_3 or DAG) produced in identical amounts when vasopressin activates phospholipase C, although each is, of course, then rapidly metabolized by different pathways. There is no further amplification between IP_3 and release of Ca^{2+} from intracellular stores, whereas the link between DAG and inhibition of CCE has many steps, with several (asterisks in Scheme 1) providing amplification. Because amplifying cascades increase the sensitivity of the final response to the initial stimulus, we suggest that the complex sequence linking DAG to inhibition of CCE may ensure that powerful inhibition occurs before CCE can be activated by the simpler sequence that leads to store depletion. Because arachidonic acid [8,9,37] and NO via PKG can inhibit CCE [25] in other cells too, we speculate that a mismatch in amplification between the stimulatory and inhibitory limbs of the pathways that reciprocally regulate CCE may be a common feature that ensures complete inhibition of CCE during receptor activation.

The stimulus for NCCE (NO) leaves the signalling cascade earlier than the signal that inhibits CCE (PKG) and before the



Scheme 1 Co-ordination of Ca^{2+}-entry pathways by NO

Binding of vasopressin to its receptor activates G_q and thereby stimulates phospholipase C (PLC), leading to formation of IP_3 and DAG. IP_3 stimulates release of Ca^{2+} from intracellular stores and the empty stores then provide a signal that activates CCE. Release of arachidonic acid from DAG by DAG lipase initiates the signalling cascade that leads to both inhibition of CCE and activation of NCCE. Arachidonic acid (by mechanisms that remain unknown) stimulates NOS III and the NO produced stimulates soluble guanylate cyclase leading to cGMP formation and activation of PKG. Our results suggest that NO directly activates NCCE, whereas activation of PKG is required for vasopressin to inhibit CCE. We suggest that the steps at which amplification occurs in the latter stages of the signalling pathway (*) are crucial in ensuring that inhibition of CCE over-rides its activation by vasopressin and in ensuring that CCE is inhibited whenever NCCE is stimulated. See text for further details. ER, endoplasmic reticulum.

final amplifying steps of the cascade (Scheme 1). The additional amplification steps in the inhibitory sequence may ensure that vasopressin profoundly inhibits CCE before it stimulates NCCE. This pattern of regulation may thereby provide a robust defence against the cell being swamped by excessive Ca^{2+}: NCCE is active only after inhibition of CCE, and CCE can become active only when the signal that activates NCCE is no longer produced. These properties allow the two Ca^{2+}-entry pathways, each perhaps regulating different intracellular processes [6], to operate in a strict temporal sequence. Vasopressin first activates NCCE and only when the agonist is removed is there a transient phase of Ca^{2+} entry via the CCE pathway.

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