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Evidence for receptor-mediated bivalent-cation entry in A10 vascular smooth-muscle cells

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In fura-2-loaded A10 vascular smooth-muscle cells, 1 nM-vasopressin and 200 nM-endothelin evoked a rapid transient rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which was then followed by a maintained elevation of $[Ca^{2+}]_i$. The maintained elevation of $[Ca^{2+}]_i$ was only partially inhibited by 5 μM-nifedipine, but completely abolished in the presence of 1 mM-EGTA. When extracellular Ca^{2+} was replaced with 1 mM-Mn^{2+} (Mn^{2+} quenches fura-2 fluorescence), both endothelin and vasopressin evoked an Mn^{2+} quench of the fluorescence from the intracellularly trapped fura-2, even in the presence of 5 μM-nifedipine. These data suggest that both vasopressin and endothelin promote a bivalent-cation influx and provide further evidence for receptor-mediated Ca^{2+} entry in vascular smooth muscle.

INTRODUCTION

There appear to be three mechanisms by which agonists elevate intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$): the release of stored Ca^{2+} via second messengers [1,2], activation of surface-membrane voltage-operated channels [3] and via the more contentious surface-membrane receptor-operated channels [4–7]. Endothelin and vasopressin have been shown to act on vascular smooth muscle via the release of stored Ca^{2+} [8–10] and through voltage-operated channels [10–12]. Some recent studies suggest that both endothelin [11] and vasopressin [13] can promote a Ca^{2+}-permeable, but non-selective, cation conductance, the nature of which varies slightly between the agonists. Although this conductance may not meet all the requirements of receptor-operated channels (see Rink [6] for discussion), it could be considered a form of receptor-mediated Ca^{2+} entry [7]. Considerable evidence for receptor-mediated Ca^{2+} entry in 'non-excitable' cells has been gained from experiments where Mn^{2+}, added outside the cells, was shown after agonist stimulation to quench the fluorescence of the intracellularly trapped Ca^{2+} indicators quin2 or fura-2 [14–17]. This agonist-evoked quench, which was also shown to be inhibited by Ni^{2+}, was taken to indicate a bivalent-cation influx.

In cultured A10 vascular smooth-muscle cells, vasopressin and endothelin evoke a biphasic elevation of $[Ca^{2+}]_i$, the maintained plateau component of which is partially inhibited by nifedipine [10]. Here we investigate the vasopressin- and endothelin-evoked $[Ca^{2+}]_i$ rises which are only partially sensitive to dihydropyridine inhibition. Using Mn^{2+}-quench experiments, we have identified an agonist-evoked bivalent-cation influx, examined the effects of nifedipine on this influx and provide further evidence for receptor-mediated Ca^{2+} entry in vascular smooth muscle.

MATERIALS AND METHODS

A10-cell culture

A10 cells (CRL 1476) were cultured in Dulbecco's modified Eagle medium containing 10% (v/v) foetal-calf serum (Gibco) at 37 °C in O_2/CO_2 (19:1). The culture was maintained by passaging confluent flasks 1 in 5. For fura-2 loading, a 260 ml flask was subcultured into three Petri dishes (100 mm diameter) containing 19 mm-diameter type-0 glass coverslips and the cells grown to confluence.

Fura-2 loading

The Dulbecco's medium was replaced with a physiological saline [containing, in mM: NaCl, 130; KCl, 2.5; $MgSO_4$, 1; Na_2HPO_4, 1; Tes, 10 (pH 7.4 at 37 °C) and glucose (15) with bovine serum albumin (2 mg/ml; Sigma) and 1 mM-$CaCl_2$ added] in which the cells were incubated for 1 h at 37 °C before loading for a further 1 h with 2 μM-fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes) [18]. After loading, the cells were washed with fresh physiological saline and then kept at 20 °C until required.

Fluorescence measurements

For fluorescence measurements, the coverslips were mounted in the base of a Perspex chamber set in a thermostatically controlled (37 °C) stage on a Nikon Diaphot epifluorescence inverted microscope. The cells were illuminated with u.v. light alternating at two wavelengths, either 340 and 380 nm or 340 and 360 nm [18], using a Photon Technology Incorporated Deltascan dual-excitation fluorimeter, which was also used to collate and process the data. The emission was monitored at 510 nm from a field containing 10–20 cells. The chamber was filled with 1 ml of physiological saline either nominally Ca^{2+}-free or containing 1 mM-$CaCl_2$. In experiments where Mn^{2+} fluxes were studied, 1 mM-$MnCl_2$ was added to the chamber during the experimental recording, as were the following reagents: [Arg^8]vasopressin (Sigma), endothelin (ET2, Peninsula), $NiCl_2$, from stocks in water with 1 in 100 to 1 in 1000 dilutions. Nifedipine (Sigma) was added from a 20 mM stock in dimethyl sulphoxide. Unless indicated otherwise, values are representative of those obtained in at least four similar experiments.

RESULTS

Vasopressin and endothelin-evoked $[Ca^{2+}]_i$ transients

As seen in previous experiments [10], vasopressin (1 nM) and endothelin (200 nM) in the presence of 1 mM extracellular free Ca^{2+} (Ca^{2+}_o) evoked a rapid rise in $[Ca^{2+}]_i$, which reached a peak at about 300–600 nM for endothelin and 600–800 nM for vaso-

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; Ca^{2+}_o, extracellular free Ca^{2+}.

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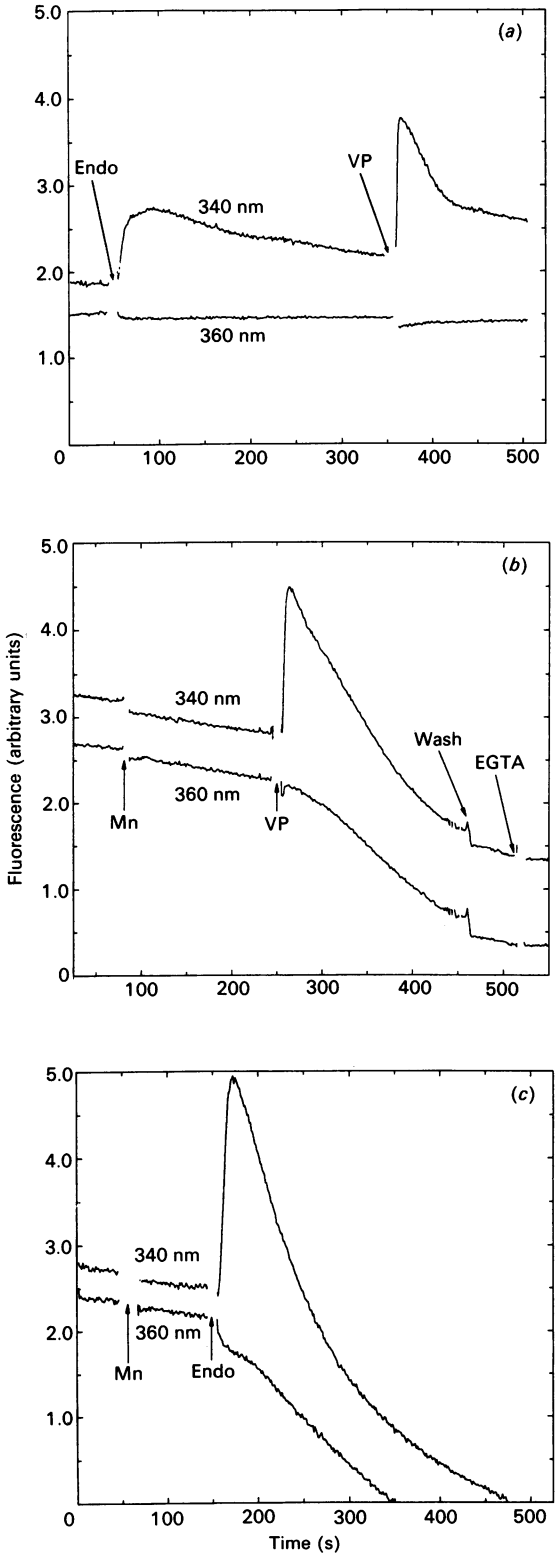

Fig. 1. Effects of agonists on the 340 and 360 nm signals from fura-2-loaded A10 cells in the presence or absence of 1 mM-Mn^{2+}

(*a*) Shows the effect of 200 nM-endothelin (Endo) and 1 nM-vasopressin (VP) on the 340 and 360 nm excitation signals of fura-2 in the presence of 1 mM Ca^{2+}_o. (*b*) Effect of 1 nM-vasopressin on the 340 and 360 nm signals from fura-2 in the presence of 1 mM-Mn^{2+}. 'Mn' indicates the addition of 1 mM-$MnCl_2$ to the nominally Ca^{2+}-free bathing saline. (*c*) Effect of adding 200 nM-endothelin (Endo) on the 340 and 360 nm signals from fura-2 in the presence of 1 mM-Mn^{2+}. 'Mn' indicates the addition of 1 mM-$MnCl_2$ to the nominally Ca^{2+}-free bathing saline.

Table 1. Quantitative analysis of the agonist-evoked quench of the 360 nm signal

Values show the rate of decline in the 360 nm signal of fura-2 evoked by vasopressin and endothelin, in the presence or absence of 5 μM-nifedipine. Fluorescence intensity is expressed as counts (c.p.s.) measured with a photon-counting photomultiplier. Values are means ± S.E.M. calculated over a 100 s interval during the peak linear decrease in the 360 nm signal. Significant (*t* test; $P > 0.05$) differences between various values are shown by superscript letters (^{a,b,c}).

Conditions	Δ Fluorescence at 360 nm (c.p.s./s)	*n*
Vasopressin (1 nM)	1876 ± 256^{ab}	5
Endothelin (200 nM)	2456 ± 250^{ac}	4
Vasopressin (1 nM) + nifedipine (5 μM)	1242 ± 240^{b}	4
Endothelin (200 nM) + nifedipine (5 μM)	1160 ± 220^{c}	4

pressin. With both agonists, after the initial transient, $[Ca^{2+}]_i$ declined to a plateau level above the previous resting value (see, e.g., Fig. 1*a*). In the absence of extracellular Ca^{2+} (1 mM-EGTA added), only the initial transient remained on addition of 200 nM-endothelin or 1 nM-vasopressin. Addition of 5 μM-nifedipine caused a decrease in $[Ca^{2+}]_i$ during this plateau phase, but did not return $[Ca^{2+}]_i$ to its resting value. Such results can be taken to suggest that a Ca^{2+} influx has been stimulated that is insensitive to nifedipine [10].

Mn^{2+}-quench experiments

Fig. 1 shows the 340 and 360 nm signals from the fura-2-loaded A10 cells. On addition of endothelin (200 nM) or vasopressin (1 nM) in the presence of 1 mM-Ca^{2+}_o, there is a transient peak in the 340 nm signal, followed by a sustained elevation of the fluorescence. The 340 nm signal, as expected, is reflecting the changes in $[Ca^{2+}]_i$ seen in the previous study [10] derived from the ratio of the 340 and 380 nm signals. On stimulation with agonists there is no real change in the 360 nm signal, which represents the 'isosbestic point' on the fura-2 excitation spectrum where fluorescence intensity does not change in respect of the extent of Ca^{2+} binding [18]. When the cells are stimulated in the presence of 1 mM-Mn^{2+}_o (1 mM-Mn^{2+}_o replacing the 1 mM-Ca^{2+}_o), the 340 nm signal shows the same initial response, but the fluorescence decays to well below the previous resting value (Figs. 1*b* and 1*c*), suggesting that either $[Ca^{2+}]_i$ has decreased to well below its resting value of ~ 100 nM or that there has been a dramatic decrease in the fluorescence intensity independent of changes in $[Ca^{2+}]_i$. In the presence of 1 mM-Mn^{2+}_o the 360 nm signal shows marked decrease beginning immediately on addition of endothelin or vasopressin.

Effects of nifedipine and Ni^{2+} on the Mn^{2+} influx

In experiments where 5 μM-nifedipine was added before either endothelin or vasopressin, the rate of decrease of the 340 and 360 nm signals appeared slightly lower (Table 1), but was nonetheless still present (Figs. 2*a* and 2*b*). Such concentrations of nifedipine are sufficient to inhibit completely noradrenaline- and KCl-evoked contractions in rat aortic and other vascular smooth muscles [19]. Electrophysiological studies also confirm that, in A10 cells, the L-type Ca^{2+} channel is sensitive to dihydropyridine inhibition [20,21]. Table 1 shows a quantitative comparison of the decrease in 360 nm florescence evoked by vasopressin and endothelin in the presence or absence of nifedipine. With both agonists 5 μM-nifedipine caused a significant diminution in the rate of decline in the 360 nm signal. Also, in cells with no added nifedipine or Ni^{2+}, endothelin appeared to promote a significantly

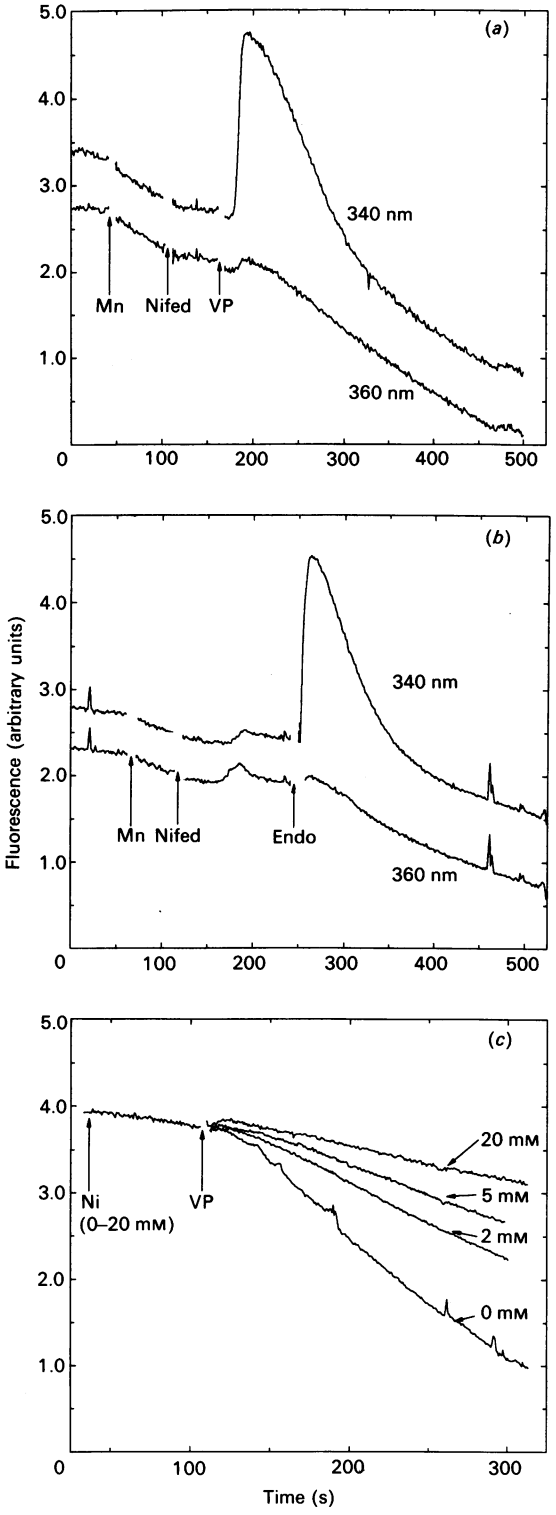

Fig. 2. The effect of nifedipine and Ni^{2+} on the agonist-evoked quench of fura-2-loaded A10 cells

(*a*) Effect of 5 μM-nifedipine (Nifed) on the 1 nM-vasopressin (VP)-evoked changes in the 340 and 360 nm signals from fura-2 in the presence of 1 mM-Mn^{2+}. (*b*) Effect of 5 μM-nifedipine (Nifed) on the 1 nM-vasopressin (VP)-evoked changes in the 340 and 360 nm signals from fura-2 in the presence of 1 mM-Mn^{2+}. (*b*) Effect of 5 μM-nifedipine (Nifed) on the 200 nM-endothelin (Endo)-evoked changes in the 340 and 360 nm signals from fura-2 in the presence of 1 mM-Mn^{2+}. (*c*) Effect of 0, 2, 5 and 20 mM-Ni^{2+} added as indicated (Ni) on the 1 nM-vasopressin (VP)-evoked quench of the 360 nm signal from fura-2 in the presence of 1 mM-Mn^{2+}. Each trace is an average combining results of three separate experiments.

greater decrease in the 360 nm signal than vasopressin. Interestingly, on addition of 5 μM-nifedipine the basal quench of the fluorescence signals seen after the addition of 1 mM-Mn^{2+} was inhibited, implying that nifedipine blocks a basal Mn^{2+} influx. Ni^{2+} (1–20 mM), added before vasopressin and in the presence of 1 mM-Mn^{2+}, inhibited the decrease in the 360 nm signal (Fig. 2*c*).

DISCUSSION

Endothelin and vasopressin both evoke a biphasic elevation of $[Ca^{2+}]_i$. The initial transient, still present when extracellular Ca^{2+} is removed, is apparently due to the release of internal stored Ca^{2+}. The second phase, a maintained plateau of elevated Ca^{2+}, was in part inhibited by nifedipine, suggesting that Ca^{2+} entry through L-type voltage-gated channels contributes in part to this plateau. The agonist-evoked decrease in fura-2 fluorescence seen in the presence of $Mn^{2+}{}_o$ suggests that Mn^{2+} is entering the cells. This conclusion is supported by the fact that Ni^{2+}, which also quenches fura-2 fluorescence, inhibits the this agonist-evoked Mn^{2+} quench. Clearly this can only happen if the A10 cells are intact and the fura-2 is trapped inside the cells. The 340 nm signal in these experiments will depend on the relative concentrations of Mn^{2+} and Ca^{2+}. The increase in the 340 nm signal seen on stimulation remains even in the presence of $Mn^{2+}{}_o$; this most likely occurs because, at such an early stage in stimulation, the release of stored Ca^{2+} will elevate $[Ca^{2+}]_i$, while insufficient Mn^{2+} has entered the cells to quench this rise in signal.

The results demonstrate that, even after the addition of nifedipine, endothelin and vasopressin stimulate a bivalent-cation influx into vascular smooth-muscle cells. It is also apparent that this influx occurs immediately on addition of the agonists and during the transient associated with the release of stored Ca^{2+}. Unfortunately these experiments cannot determine where inside the cell the Mn^{2+}, and consequently, under normal circumstances, the Ca^{2+}, might be entering. The nifedipine-sensitive component of the bivalent-cation influx is most likely due to Mn^{2+} directly entering the cytosol; however, the nifedipine-insensitive component may represent Ca^{2+} entering the cytosol or the Ca^{2+} stores. Interestingly, in the rat parotid, no agonist-evoked Mn^{2+} quench of fura-2 loaded cell can be observed [15], even though recent evidence suggest a Ca^{2+} influx may occur [22].

A non-selective cation conductance has been reported in the related cell line A7r5, observed after stimulation with endothelin or vasopressin [10,12]. These authors suggested that the influx of cations through this channel causes a depolarization, which opens L-type Ca^{2+} channels, and consider that the channel alone may carry sufficient Ca^{2+} to elevate $[Ca^{2+}]_i$. It may be that the results seen here represent Mn^{2+} entering the cytosol through such a channel. Preliminary experiments suggest that vasopressin can stimulate such a conductance in A10 cells (H. F. Brown & A. W. M. Simpson, unpublished work). Clearly patch-clamp studies using Mn^{2+} and Ca^{2+} as conducting cations and Ni^{2+} as an inhibitor are required to determine if Mn^{2+} enters through this Ca^{2+} permeable non-selective cation channel. It is also noteworthy that nifedipine inhibited the basal Mn^{2+} influx, suggesting that even in resting cells Ca^{2+} enters via L-type Ca^{2+} channels, an observation that might be predicted from other systems where L-type channels show some open probability even at holding potentials of about -70 mV [23].

In conclusion, the results presented here show that the smooth-muscle agonists endothelin and vasopresin promote a bivalent-cation influx into A10 cells. The bivalent-cation influx occurs in part via L-type Ca^{2+} channels and in part by a process insensitive to nifedipine. The bivalent-cation influx occurs immediately on

addition of the agonist and during the rise in $[Ca^{2+}]_i$ attributed to the release of stored Ca^{2+} and could be considered as evidence for receptor-mediated Ca^{2+} entry in these cells.

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