Evidence that Ca^{2+} -release-activated Ca^{2+} channels in rat hepatocytes are required for the maintenance of hormone-induced Ca^{2+} oscillations

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Store-operated Ca^{2+} channels in liver cells have been shown previously to exhibit a high selectivity for Ca^{2+} and to have properties indistinguishable from those of Ca^{2+} -release-activated Ca^{2+} (CRAC) channels in mast cells and lymphocytes [Rychkov, Brereton, Harland and Barritt (2001) *Hepatology* **33**, 938–947]. The role of CRAC channels in the maintenance of hormone-induced oscillations in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in isolated rat hepatocytes was investigated using several inhibitors of CRAC channels. 2-Aminoethyl diphenylborate (2-APB; 75 μM), Gd^{3+} (1 μM) and 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SK&F 96365; 50 μM) each inhibited vasopressin- and adrenaline (epinephrine)-induced Ca^{2+} oscillations (measured using fura-2). The characteristics of this inhibition were similar to those of inhibition caused by decreasing the extracellular Ca^{2+} concentration to zero by addition of EGTA. The effect of 2-APB was reversible. In contrast, LOE-908 $\{(R,S)-(3,4\text{-dihydro-6,7-dimethoxy-isochinolin-1-yl})-2\text{-phenyl-}N,N\text{-di}[2-(2,3,4\text{-trimethoxyphenyl})\text{ethyl}]\text{acetamide mesylate}\}$ (30 μM),

used commonly to block Ca^{2+} inflow through intracellular-messenger-activated, non-selective cation channels, did not inhibit the Ca^{2+} oscillations. In the absence of added extracellular Ca^{2+} , 2-APB, Gd^{3+} and SK&F 96365 did not alter the kinetics of the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by a concentration of adrenaline or vasopressin that induces continuous Ca^{2+} oscillations at the physiological extracellular Ca^{2+} concentration. Ca^{2+} inflow through non-selective cation channels activated by maitotoxin could not restore Ca^{2+} oscillations in cells treated with 2-APB to block Ca^{2+} inflow through CRAC channels. Evidence for the specificity of the pharmacological agents for inhibition of CRAC channels under the conditions of the present experiments with hepatocytes is discussed. It is concluded that Ca^{2+} inflow through CRAC channels is required for the maintenance of hormone-induced Ca^{2+} oscillations in isolated hepatocytes.

Key words: 2-aminoethyl diphenylborate, Ca^{2+} inflow, epinephrine, Gd^{3+} , LOE-908, vasopressin.

INTRODUCTION

In hepatocytes, increases in the concentration of free Ca^{2+} both in the cytoplasmic space ($[\text{Ca}^{2+}]_{\text{cyt}}$) and in the mitochondrial matrix mediate the hormonal regulation of many processes, including the transport and secretion of bile acids, the metabolism of glycogen, glucose, lipids, amino acids and drugs, and the synthesis and secretion of proteins [1–3]. An important form of the hormone-induced cytoplasmic Ca^{2+} signal in hepatocytes is composed of oscillations or waves of increased $[\text{Ca}^{2+}]_{\text{cyt}}$ (Ca^{2+} oscillations), which travel within [4,5] and between [6–8] hepatocytes.

Because some intracellular Ca^{2+} is lost during each Ca^{2+} oscillation [9], the maintenance of hormone-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ requires Ca^{2+} inflow across the plasma membrane through store-operated Ca^{2+} channels (SOCs) and intracellular-messenger-activated, non-selective cation channels (NSCCs) [10]. In liver cells and in other non-excitable animal cells, there are several types of SOCs, which can be distinguished by their different selectivities for Ca^{2+} compared with univalent cations. The Ca^{2+} -release-activated Ca^{2+} (CRAC) channels identified in mast cells and lymphocytes are characterized by a very high selectivity for Ca^{2+} compared with Na^{+} [11,12]. Although it is

clear that SOCs are responsible for refilling the endoplasmic reticulum (ER) Ca^{2+} stores after a large decrease in ER Ca^{2+} , there is no direct evidence that SOCs are required for the maintenance of Ca^{2+} oscillations in hepatocytes or in many other non-excitable cells. Moreover, studies with smooth-muscle cells [13], avian nasal-gland cells [14] and HEK cells transfected with muscarinic acetylcholine receptors [15,16] have shown that arachidonic-acid-activated Ca^{2+} channels, rather than SOCs, fulfil this role in these cell types.

Patch-clamp recording studies with the H4-IIIE rat-liver hepatoma cell line and with rat hepatocytes have shown that properties of SOCs (designated hepatocyte CRAC channels) in these cells are indistinguishable from those of CRAC channels in lymphocytes and mast cells [17]. Although several types of NSCC have been identified in hepatocytes and in liver cell lines [18–21], most have not been well characterized, and their functions are not well defined. One of these types of liver-cell NSCC is activated by maitotoxin, a polycyclic ether derived from a marine dinoflagellate [19,22,23]. Maitotoxin also activates NSCCs in a number of other cell types [24,25]. Although the liver cell NSCCs activated by maitotoxin admit some Ca^{2+} , they principally facilitate Na^{+} inflow. The activation pathway does not appear to involve the release of Ca^{2+} from the ER [19].

Abbreviations used: 2-APB, 2-aminoethyl diphenylborate; $\text{Ca}^{2+}_{\text{cyt}}$, cytoplasmic free Ca^{2+} ; $\text{Ca}^{2+}_{\text{o}}$, extracellular free Ca^{2+} ; CRAC channel, Ca^{2+} -release-activated Ca^{2+} channel; ER, endoplasmic reticulum; fura-2/AM, fura-2 acetoxymethyl ester; LOE-908, $(R,S)-(3,4\text{-dihydro-6,7-dimethoxy-isochinolin-1-yl})-2\text{-phenyl-}N,N\text{-di}[2-(2,3,4\text{-trimethoxyphenyl})\text{ethyl}]\text{acetamide mesylate}$; NSCC, non-selective cation channel; SK&F 96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride; SOC, store-operated Ca^{2+} channel.

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The aim of the present study was to elucidate the roles of CRAC channels in the maintenance of hormone-induced Ca^{2+} oscillations in hepatocytes. The experimental approach used has employed three inhibitors of CRAC channels: 2-aminoethyl diphenylborate (2-APB) [23,26–31], Gd^{3+} [17,32] and SK&F 96365 (1- $\{\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl]-1H-imidazole hydrochloride) [33], and LOE-908 $\{(R,S)-(3,4\text{-dihydro-6,7-dimethoxy-isochinolin-1-yl})-2\text{-phenyl-}N,N\text{-di}[2-(2,3,4\text{-trimethoxyphenyl})\text{ethyl}]\text{acetamide mesylate}\}$, an inhibitor of Ca^{2+} entry through NSCCs [34–36]. 2-APB was originally identified as an inhibitor of Ca^{2+} release through $\text{Ins}(1,4,5)\text{P}_3$ receptors [37]. Subsequently, it was shown to inhibit $\text{Ins}(1,4,5)\text{P}_3$ receptor function in several cell types [26,38–42], with the degree of inhibition depending on the concentration of $\text{Ins}(1,4,5)\text{P}_3$ [43]. However, in many cell types, 2-APB inhibits Ca^{2+} inflow across the plasma membrane through SOC channels [23,27,28,44–47], often with little effect on Ca^{2+} release from the ER through $\text{Ins}(1,4,5)\text{P}_3$ receptors [23,27–29,45–48]. Although there are reports that 2-APB affects some other types of Ca^{2+} channels and transporters [30,31,42,45,49,50], it has been used effectively to elucidate the role of Ca^{2+} inflow through SOC channels [23,26–31]. In liver cells, 2-APB inhibits Ca^{2+} inflow through CRAC channels (measured using patch-clamp recording and fura-2) with no observable effect on either Ca^{2+} release from $\text{Ins}(1,4,5)\text{P}_3$ receptors [23] or maitotoxin-initiated Ca^{2+} inflow [23]. At low concentrations, Gd^{3+} is a potent and selective inhibitor of hepatocyte CRAC channels [17,32]. The results reported in the present study provide evidence that, under the conditions used here, 2-APB and Gd^{3+} are relatively specific inhibitors of CRAC channels in hepatocytes, and that these channels are required for the maintenance of hormone-induced Ca^{2+} oscillations.

MATERIALS AND METHODS

Materials

Collagenase Type 4 was obtained from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.); maitotoxin, $[\text{Arg}^8]\text{-vasopressin}$ and 2-APB were from Sigma–Aldrich (Castle Hill, New South Wales, Australia), SK&F 96365 was from Sapphire Bioscience (Alexandria, New South Wales, Australia), adrenaline (epinephrine) was from Boehringer Mannheim (Castle Hill, New South Wales, Australia) and fura-2 [used in these experiments as fura-2 acetoxymethyl ester (fura-2/AM)] was from Molecular Probes (Eugene, OR, U.S.A.). LOE-908 was generously given by Boehringer Ingelheim (Biberach an der Riss, Germany). Other reagents were obtained from the sources described previously [18,23].

Isolation and incubation of hepatocytes and measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$

Hepatocytes were isolated from fed male Hooded Wistar rats (approx. 250 g) and cultured on collagen-coated glass coverslips (plating cell density approx. 3.3×10^4 cells/ml) in Dulbecco's modified Eagle's medium containing 2% (w/v) BSA, 1 mM pyruvate and 44 mM NaHCO_3 (DMEM solution), as described previously [23]. For the measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$, cells were loaded with the high-affinity Ca^{2+} indicator fura-2 by incubating coverslips in DMEM solution that contained 5 μM fura-2/AM and 0.02% (w/v) pluronic F-127 for approx. 1 h at 37 °C [51]. The coverslips were washed with Ca^{2+} -free Hanks medium, placed in a thermostat-controlled chamber (34 °C), and fura-2

fluorescence was then measured using a Nikon Diaphot inverted fluorescence microscope, Sutter filter wheel, Photonics intensified CCD (charge-coupled-device) camera (ISIS-3/S20) and Axon Imaging Workbench software (V. 2.2), as described previously [23,51]. At the commencement of fluorescence measurements, cells were in Ca^{2+} -containing Hanks medium, unless otherwise stated. For experiments involving the addition of GdCl_3 , the Hanks medium was sulphate- and phosphate-free. Changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ reflected by the changes in the fluorescence of fura-2 are reported as changes in the fluorescence ratio measured at 340 nm/380 nm.

RESULTS

Effects of 2-APB, Gd^{3+} , SK&F 96365 and LOE-908 on vasopressin-induced Ca^{2+} oscillations

The sensitivity of different preparations of isolated hepatocytes to low concentrations of vasopressin varied slightly (cf. [4]). Hence, in order to obtain similar oscillatory responses, a range of vasopressin concentrations (0.02–0.1 nM) was used with the different cell preparations. In the presence of an extracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) of 1.5 mM, vasopressin induced oscillations in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Figure 1A), as shown previously [4]. (For most treatments, individual hepatocytes exhibited a range of responses. Each Figure is an example of a representative trace.) In the absence of added Ca^{2+}_o , the number of vasopressin-induced Ca^{2+} oscillations was substantially reduced (Figure 1B), as shown previously [4]. The reduction of $[\text{Ca}^{2+}]_o$ to close to zero by the addition of EGTA during vasopressin-induced Ca^{2+} oscillations caused an immediate inhibition of the Ca^{2+} oscillations (Figure 1C). The addition of 2-APB, an inhibitor of CRAC channels [23,27,28,30,45,46,48], before vasopressin caused a substantial inhibition of the Ca^{2+} oscillations (Figure 1D). When 2-APB was added after Ca^{2+} oscillations had been initiated by vasopressin, the oscillations ceased within one oscillation (Figure 1E). The subsequent addition of a 300-fold-higher concentration of vasopressin (in the presence of 2-APB) induced a single spike of increased $[\text{Ca}^{2+}]_{\text{cyt}}$ (Figure 1E). In cells which had been exposed to vasopressin (to induce Ca^{2+} oscillations) and 2-APB, which were then washed to remove these agents, it was possible to reinitiate some Ca^{2+} oscillations by the reintroduction of vasopressin (Figure 1F). [In 61 cells in which vasopressin (0.04 nM) was added after pre-treatment of the cells with vasopressin (0.04 nM) and 2-APB, and the subsequent washout of these agents, 27 cells resumed Ca^{2+} oscillations which were apparent for several cycles.]

In order to verify that 2-APB does not inhibit the ability of low (physiological) concentrations of vasopressin to induce the release of Ca^{2+} from intracellular stores, cells were incubated in the absence of added Ca^{2+}_o . The addition of 75 μM 2-APB before vasopressin (0.1 nM) did not prevent the induction by vasopressin of a Ca^{2+} -release peak (Figure 2A) of similar shape to that seen in the absence of 2-APB (Figure 2B). Typically, between one and three such Ca^{2+} -release peaks were observed, regardless of whether or not 2-APB was present (results not shown). The characteristics of the spikes of increased $[\text{Ca}^{2+}]_{\text{cyt}}$ observed in the presence of 2-APB and absence of added Ca^{2+}_o (Figure 2A) were similar to those of the $[\text{Ca}^{2+}]_{\text{cyt}}$ spikes seen in the presence of added Ca^{2+}_o and 2-APB (Figure 1D). These results, together with those shown in Figure 1(E), where a high concentration of vasopressin was added in the presence of 2-APB, confirm those obtained previously [23], which indicates that, in hepatocytes, 2-APB does not inhibit vasopressin-induced release of Ca^{2+} from the ER.

Figure 1 Oscillations in $[Ca^{2+}]_{cyt}$ triggered by a low concentration of vasopressin are dependent on Ca^{2+} stores, are reversibly inhibited by 2-APB and inhibited by Gd^{3+}

Hepatocytes were loaded with fura-2/AM and changes in fura-2 fluorescence measured as described in the Materials and methods section. Ca^{2+} oscillations were initiated by the addition of 0.02 (A, C), 0.03 (G, H), 0.04 (B, D, F) or 0.1 (E) nM vasopressin in the absence (B) or presence (A, C–H) of 1.5 mM $CaCl_2$ (shown by the horizontal bars). The times for the addition of Ca^{2+} (1.5 mM), vasopressin, EGTA (4 mM), 2-APB (75 μM) and Gd^{3+} (1 μM) are indicated by the horizontal bars. The addition of 0.1% (v/v) DMSO (the vehicle in which 2-APB was dissolved) did not affect the oscillations (A). In (E), a second addition of vasopressin after 16 min increased the concentration of vasopressin to 30 nM. In (F), the cells were perfused with fresh medium between 20.5 and 23 min to remove 2-APB and vasopressin (indicated by the arrow) before the fresh addition of vasopressin (0.04 nM). Each trace shown was obtained from a single cell, and is representative of those obtained for 78 (A), 164 (B), 31 (C), 91 (D), 88 (E), 42 (F), 57 (G) and 98 (H) cells on 2–9 coverslips from 1–7 separate experiments. F340/F380, the ratio of fluorescence measured at 340 nm compared with 380 nm.

The ability of Gd^{3+} to inhibit CRAC channels in hepatocytes [17,32] was confirmed by measuring rates of vasopressin-induced Ca^{2+} inflow in the presence of a range of concentrations of Gd^{3+} (Figure 3). Gd^{3+} caused almost total inhibition of vasopressin-

Figure 2 2-APB does not affect the profile of the change in $[Ca^{2+}]_{cyt}$ induced by a low concentration of vasopressin in the absence of added extracellular Ca^{2+}

Hepatocytes were loaded with fura-2/AM and changes in fura-2 fluorescence were measured as described in the Materials and methods section. An increase in $[Ca^{2+}]_{cyt}$ was initiated by the addition of vasopressin (0.1 nM) in the absence of added Ca^{2+} , and in the presence (A) or absence (B) of 75 μM 2-APB. The times for the addition of vasopressin, 2-APB, or vehicle (0.1% (v/v) DMSO) are indicated by the horizontal bars. The traces shown are representative of those obtained for 88 (A) or 164 (B) cells on 6–9 coverslips from five separate experiments. F340/F380, the ratio of fluorescence measured at 340 nm compared with 380 nm.

Figure 3 Dose-response curve for Gd^{3+} inhibition of vasopressin-stimulated Ca^{2+} inflow

Hepatocytes were loaded with fura-2/AM and changes in fura-2 fluorescence were measured as described in the Materials and methods section. Cells were initially incubated in Ca^{2+} -free medium and vasopressin (40 nM) was added to deplete Ca^{2+} stores. Sequential additions of $GdCl_3$ (0–1 μM) and $CaCl_2$ (final concentration 1.5 mM) followed. The rate of Ca^{2+} inflow (expressed as the rate of increase in fluorescence ratio) was calculated from the initial slope of the increase in ratio following Ca^{2+} addition. The data are mean values $\pm$ S.E.M. for at least 22 cells for each concentration of Gd^{3+} from one of two similar experiments. The curve was fitted by means of a non-linear regression function in the GraphPad Prism software.

Figure 4 SK&F 96365 inhibits Ca^{2+} oscillations induced by low concentrations of both vasopressin and adrenaline (epinephrine)

Hepatocytes were loaded with fura-2/AM and changes in fura-2 fluorescence were measured as described in the Materials and methods section. Ca^{2+} oscillations were initiated by the addition of 0.04 nM vasopressin (A) or 0.5 μM adrenaline (B) in the presence of 1.5 mM CaCl_2 . The times for the addition of Ca^{2+} (1.5 mM), vasopressin (0.04 nM), adrenaline (0.5 μM) and SK&F 96365 (50 μM) are indicated by the horizontal bars. The addition of 0.1% (v/v) DMSO (the vehicle for SK&F 96365) did not affect the oscillations induced by either vasopressin (Figure 1A) or adrenaline (Figure 5A). Each trace was obtained from a single cell and is representative of those obtained for 66 (A) and 13 (B) cells on 1–4 coverslips from 1–3 separate experiments. F340/F380, the ratio of fluorescence measured at 340 nm compared with 380 nm.

induced Ca^{2+} inflow, with an IC_{50} of approx. 0.07 μM (Figure 3). The residual Ca^{2+} inflow rate of 0.031 ± 0.004 [41] ratio units/min measured in the presence of 1 μM Gd^{3+} was comparable with the basal Ca^{2+} inflow rate of 0.039 ± 0.008 [37] ratio units/min measured in the absence of vasopressin or Gd^{3+} . The addition of Gd^{3+} (1 μM) before vasopressin substantially decreased the frequency of vasopressin-induced Ca^{2+} oscillations compared with those in control cells, and led to cessation of oscillations (compare Figures 1G and Figure 1A). Likewise, when 1 μM Gd^{3+} was added to cells exhibiting vasopressin-induced Ca^{2+} oscillations, a substantial decrease in the frequency of the oscillations was observed (compare Figures 1H and 1A). In the absence of added Ca^{2+} , the prior addition of 1 μM Gd^{3+} did not affect the ability of vasopressin (0.04 nM) to induce Ca^{2+} release from the ER (results not shown).

SK&F 96365 is an inhibitor of Ca^{2+} inflow through SOCs, as well as through voltage-operated Ca^{2+} channels [33]. Since hepatocytes do not contain voltage-operated Ca^{2+} channels [18], any effects of SK&F 96365 will be likely to reflect actions on SOCs. Ca^{2+} oscillations induced by vasopressin were inhibited by 50 μM SK&F 96365 (Figure 4A), but were not affected by vehicle [0.1% (v/v) DMSO] (Figure 1A, and results not shown). In the absence of added Ca^{2+} , Ca^{2+} -release peaks induced by 0.01 nM

Figure 5 Oscillations in $[\text{Ca}^{2+}]_{\text{cyt}}$ triggered by a low concentration of adrenaline (epinephrine) are dependent on extracellular Ca^{2+} and are inhibited by 2-APB

Hepatocytes were loaded with fura-2/AM and changes in fura-2 fluorescence were measured as described in the Materials and methods section. Ca^{2+} oscillations were initiated by the addition of adrenaline (5 μM) in the presence (A–C) or absence (D, E) of 1.5 mM CaCl_2 . The times for the addition of Ca^{2+} (1.5 mM), adrenaline (5 μM), 2-APB (75 μM), DMSO (0.1%, v/v), EGTA (2 mM) and vasopressin (40 nM) are indicated by the horizontal bars. In (B), the second addition of Ca^{2+} increased the total concentration of added Ca^{2+} to 2.5 mM. In (D) and (E), an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was initiated by the addition of adrenaline (5 μM) in the absence of added Ca^{2+} , and in the presence (D) or absence (E) of 75 μM 2-APB. Similar profiles of the change in $[\text{Ca}^{2+}]_{\text{cyt}}$ were obtained with these conditions. Each trace shown was obtained from a single cell and is representative of those obtained for 31 (A), 7 (B), 32 (C), 48 (D) or 74 (E) cells on 1–3 coverslips from two separate experiments. F340/F380, the ratio of fluorescence measured at 340 nm compared with 380 nm.

vasopressin were similar in the absence or presence of 50 μM SK&F 96365 (results not shown).

LOE-908 (10–30 μM) has been used to inhibit Ca^{2+} inflow through NSCCs [34–36]. When added to cells exhibiting vasopressin-induced Ca^{2+} oscillations, LOE-908 (30 μM) did not bring about any noticeable change to the oscillations (results not shown).

Effects of 2-APB, Gd^{3+} , SK&F 96365 and LOE-908 on adrenaline-induced Ca^{2+} oscillations

As noted for vasopressin, different cell preparations displayed different sensitivities to adrenaline [4]. In order to initiate Ca^{2+}

Figure 6 Ca²⁺ oscillations initiated by a low concentration of adrenaline (epinephrine) are inhibited by 1 μ M Gd³⁺

Hepatocytes were loaded with fura-2/AM and changes in fura-2 fluorescence were measured as described in the Materials and methods section. Ca²⁺ oscillations were initiated by the addition of adrenaline (final concentration 0.1 μ M) in the presence (A–C) or absence (D) of 1.5 mM CaCl₂. The times for the addition of Ca²⁺ (1.5 mM), adrenaline (0.1 μ M), Gd³⁺ (1 μ M) and vehicle (incubation medium) are indicated by the horizontal bars. The addition of an aliquot of incubation medium (vehicle for GdCl₃) did not affect oscillations (A). Each trace was obtained from a single cell and is representative of those obtained for 75 (A), 72 (B), 77 (C) or 115 (D) cells on 2–4 coverslips from one or two separate experiments. F340/F380, the ratio of fluorescence measured at 340 nm compared with 380 nm.

oscillations rather than sustained Ca²⁺ inflow, a range of adrenaline concentrations (0.1–5 μ M) was used with different cell preparations. As shown previously for other α -adrenergic agonists [4,22], in the presence of 1.5 mM Ca²⁺_o, adrenaline induced Ca²⁺ oscillations (Figure 5A), which were inhibited by the addition of EGTA (Figure 5B). The subsequent addition of CaCl₂ to restore [Ca²⁺]_o to approx. 0.5 mM restored Ca²⁺ oscillations (Figure 5B). Of 12 cells (from a population of 68 cells) that showed oscillations before EGTA addition, seven cells exhibited between two and seven further oscillations when Ca²⁺_o was added after EGTA (Figure 5B shows one such example).

The addition of 2-APB to cells exhibiting adrenaline-induced Ca²⁺ oscillations inhibited these oscillations (compare Figures 5C and 5A). The inhibition of adrenaline-induced Ca²⁺ oscillations by 2-APB was reversed by washing the cells to remove adrenaline and 2-APB, and making a fresh addition of adrenaline (results not shown). The addition of a high concentration of vasopressin (40 nM, in the presence of 2-APB) to cells treated previously with adrenaline (to induce Ca²⁺ oscillations) and with 2-APB induced a single spike of increased [Ca²⁺]_{cyt} (Figure 5C), confirming further that 2-APB does not inhibit vasopressin-induced Ca²⁺ release from the ER. For cells incubated in the absence of added Ca²⁺_o, the addition of 2-APB (75 μ M) before adrenaline did not cause a detectable change in the shape, frequency or amplitude of adrenaline-induced Ca²⁺ oscillations. The results obtained for a cell exhibiting a single adrenaline-induced Ca²⁺ oscillation are shown in Figure 5(D) (cf. results

Figure 7 Ca²⁺ inflow stimulated by maitotoxin cannot restore [Ca²⁺]_{cyt} oscillations previously inhibited by 2-APB

Hepatocytes were loaded with fura-2-AM and fura-2 fluorescence measured as described in the Materials and methods section. Ca²⁺ oscillations were initiated by the addition of 0.1 μ M adrenaline (epinephrine) in the presence of 1.5 mM CaCl₂. The times for the addition of Ca²⁺ (1.5 mM), adrenaline, 2-APB (75 μ M), DMSO (0.1% (v/v)), and maitotoxin (40 pM) are indicated by the horizontal bars. Each trace shown was obtained from a single cell and is representative of the traces obtained for 19 (A) or 24 (B) cells from one experiment. F340/F380, the ratio of fluorescence measured at 340 nm compared with 380 nm.

obtained in the absence of 2-APB; Figure 5E). When Gd³⁺ (1 μ M) was added before adrenaline, Ca²⁺ oscillations were substantially reduced (compare Figures 6B and 6A), whereas the addition of Gd³⁺ to cells exhibiting adrenaline-induced Ca²⁺ oscillations inhibited the oscillations (Figure 6C). The diminished response to adrenaline in the presence of Gd³⁺ and added Ca²⁺_o (Figure 6B) was similar to that seen when adrenaline was added in the absence of added Ca²⁺_o or Gd³⁺ (Figure 6D).

Ca²⁺ oscillations initiated by adrenaline were blocked by 50 μ M SK&F 96365 (Figure 4B). Vehicle for SK&F 96365 [0.1% (v/v) DMSO] did not affect the Ca²⁺ oscillations (Figure 5A). LOE-908 (30 μ M) did not cause any detectable change in Ca²⁺ oscillations induced by adrenaline (results not shown).

Effect of maitotoxin on Ca²⁺ oscillations in the presence of 2-APB

The ability of maitotoxin-activated NSCCs [19,22,23] to reinstate Ca²⁺ oscillations in cells treated with adrenaline [to supply Ins(1,4,5)P₃] and 2-APB (to inhibit CRAC channels) was investigated. Several concentrations of maitotoxin were tested. However, it was not possible to find conditions under which the addition of maitotoxin restored Ca²⁺ oscillations. A typical result is shown in Figure 7(A). (In 19 cells to which 40 pM maitotoxin was added after 0.1 μ M adrenaline and 2-APB, no cells treated with maitotoxin exhibited oscillations.) The addition of maitotoxin to cells in which oscillations had been induced by adrenaline

in the absence of 2-APB caused the oscillations to cease after about four further oscillations (Figure 7B) (cf. [22]). Subsequently, a sustained increase in $[Ca^{2+}]_{\text{cyt}}$ was observed. Results similar to those shown in Figures 7(A) and 7(B) were obtained when vasopressin was used in place of adrenaline (results not shown).

DISCUSSION

The present results show that 2-APB, Gd^{3+} and SK&F 96365 inhibit hormone-induced Ca^{2+} oscillations in freshly isolated rat hepatocytes at concentrations that selectively inhibit CRAC channels ([17,23,33] and the present study). The effects of each agent on hormone-induced Ca^{2+} oscillations are comparable with those caused by decreasing the $[Ca^{2+}]_o$ to almost zero. LOE-908, an inhibitor of NSCCs which has been used to discriminate between SOC and NSCCs [34–36,52], did not inhibit hormone-induced Ca^{2+} oscillations. The absence of inhibition by LOE-908 indicates that NSCCs are not involved in the maintenance of hormone-induced Ca^{2+} oscillations in rat hepatocytes.

It has clearly been shown that 2-APB inhibits CRAC channels in cells in which these have been identified [23,27,28,30,45,46,48]. In some cell types, 2-APB inhibits Ca^{2+} outflow through $Ins(1,4,5)P_3$ receptors [26,37–42,44,48,53]. In addition, recent studies have shown that 2-APB can inhibit some other Ca^{2+} channels and transporters [30,42,45,49,50]. However, there is no evidence that 2-APB inhibits $Ins(1,4,5)P_3$ receptors [23] or other Ca^{2+} channels and transporters in liver cells ([23] and the present study). The observation that vasopressin can reinitiate Ca^{2+} oscillations when 2-APB has been removed indicates that 2-APB does not induce irreversible changes in Ca^{2+} channels and transporters in hepatocytes.

In the absence of added Ca^{2+}_o , 2-APB did not inhibit the transient increase in $[Ca^{2+}]_{\text{cyt}}$ induced by concentrations of vasopressin or adrenaline that induce repetitive Ca^{2+} oscillations in the presence of Ca^{2+}_o ; nor was there any evidence that the kinetics of adrenaline- or vasopressin-induced increase or decrease in $[Ca^{2+}]_{\text{cyt}}$ were affected by 2-APB. These observations indicate that the inhibition of Ca^{2+} oscillations by 2-APB observed in the presence of Ca^{2+}_o is unlikely to be due to inhibition of a step or steps in the intracellular mechanisms by which Ca^{2+} oscillations are generated and propagated.

2-APB (75 μM) inhibits Ca^{2+} inflow through CaT1 Ca^{2+} channels expressed in mast cells [50], and through magnesium-nucleotide-regulated metal ion (MagNum/LTRPC7) channels (now known as TRPM7 channels; activated by a decrease in the intracellular concentration of $MgATP^{2-}$) in rat basophilic leukaemia cells [49]. However, there is no evidence for the expression of CaT1 mRNA in rat liver ([54]; cf. human liver [55]). Moreover, the conditions of the present experiments in which Ca^{2+} inflow is measured using fura-2 (i.e. where the intracellular concentrations of Mg^{2+} and ATP^{4-} are not changed by dialysis of the cell through a patch pipette) make it unlikely that MagNum channels would be activated. Thus, in the present experiments with rat hepatocytes, the inhibitory effect of 2-APB is unlikely to be due to the inhibition of Ca^{2+} inflow through CaT1 or MagNum/LTRPC7 channels.

In addition to SOC, Gd^{3+} inhibits several types of plasma-membrane Ca^{2+} channels, including voltage-operated Ca^{2+} channels [56], maitotoxin-activated NSCCs [19], Ca^{2+} inflow through heterologously expressed human transient receptor potential canonical 3 ('hTRPC3') channels [57] and Ca^{2+} inflow through MagNum/LTRPC7 channels [49]. In the present exper-

iments, the concentration of Gd^{3+} which gave 50% inhibition of vasopressin-stimulated Ca^{2+} inflow was 0.07 μM , and a concentration of 1 μM Gd^{3+} inhibited hormone-induced Ca^{2+} oscillations. On the basis of the dose-response curve for Gd^{3+} , it can be predicted that 1 μM Gd^{3+} should selectively inhibit CRAC channels in hepatocytes [17,32] with little detectable effect on other Ca^{2+} channels and transporters [13,19,58]. Taken together, the results obtained with 2-APB, Gd^{3+} , SK&F 96365 and LOE-908 indicate that the inhibition of hormone-induced Ca^{2+} oscillations is due to the inhibition of Ca^{2+} inflow through CRAC channels, and provide evidence that Ca^{2+} inflow through these channels is required for the maintenance of hormone-induced Ca^{2+} oscillations.

In some cell types, arachidonic-acid-activated NSCCs can supply Ca^{2+}_o to maintain Ca^{2+} oscillations [13,14,16,59]. However, recent studies in our laboratory have so far provided no evidence for arachidonic-acid-activated channels in rat liver cells (R. B. Gregory, G. Rychkov and G. Barritt, unpublished work). Moreover, the arachidonic-acid-activated Ca^{2+} inflow implicated in the maintenance of Ca^{2+} oscillations is not inhibited by 2-APB [58], whereas 2-APB essentially completely inhibits hormone- and thapsigargin-induced Ca^{2+} inflow in hepatocytes [23]. Thus it is considered unlikely that arachidonic-acid-activated Ca^{2+} channels contribute to the maintenance of hormone-induced Ca^{2+} oscillations in hepatocytes.

Maitotoxin could not restore Ca^{2+} oscillations when added to cells in which oscillations had been initiated by addition of a low concentration of adrenaline, and then inhibited by addition of 2-APB. This suggests that Ca^{2+} inflow through one type of NSCC (the maitotoxin-activated NSCC) cannot substitute for Ca^{2+} inflow through CRAC channels in supporting the maintenance of Ca^{2+} oscillations in rat hepatocytes. However, the possibility that maitotoxin initiates the generation of an intracellular messenger that interferes with the propagation of Ca^{2+} oscillations cannot be excluded.

It is concluded from the present work that Ca^{2+} inflow through CRAC channels is necessary for the maintenance of hormone-induced Ca^{2+} oscillations in rat hepatocytes. Moreover, an implication of the observation that maitotoxin-activated NSCCs cannot substitute for CRAC channels in maintaining Ca^{2+} oscillations is that CRAC channels have the ability to deliver Ca^{2+} to appropriate regions of the cell that permit the generation and maintenance of Ca^{2+} oscillations, whereas maitotoxin-activated NSCCs deliver Ca^{2+} to the cytoplasmic space at inappropriate locations and/or concentrations.

Knowledge of the roles of different plasma-membrane Ca^{2+} channels in maintaining Ca^{2+} oscillations in animal cells is at present incomplete. Thus there is evidence that CRAC channels are required to maintain Ca^{2+} oscillations in lymphocytes [60] and rat cortical astrocytes [61] (cf. the present studies with hepatocytes), but in avian nasal glands [14] and HEK293 cells transfected with muscarinic receptors [16], arachidonic-acid-activated NSCCs, but not CRAC channels, appear to be required. From other studies, it has been concluded that the pathway of Ca^{2+} inflow to HEK cells is complex, and neither CRAC channels nor arachidonic-acid-activated Ca^{2+} channels are required for the maintenance of Ca^{2+} oscillations [58]. Different cell types may utilize different types of plasma-membrane Ca^{2+} channels for the maintenance of Ca^{2+} oscillations. Further experiments are required to elucidate the functions of the different channels.

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