Veratridine-induced oscillations of cytosolic calcium and membrane potential in bovine chromaffin cells

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1. Veratridine (VTD) induced large oscillations of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the membrane potential (V_m) in otherwise silent bovine chromaffin cells loaded with fura-2.

2. Depletion of the intracellular Ca^{2+} stores by thapsigargin or ryanodine did not affect these oscillations. Caffeine had a complex effect, decreasing them in cells with high activity but increasing them in cells with low activity.

3. The $[Ca^{2+}]_i$ oscillations required extracellular Ca^{2+} and Na^+ and were blocked by Ni^{2+} or tetrodotoxin. They were antagonized by high external concentrations of Mg^{2+} and/or Ca^{2+}.

4. The oscillations of V_m had three phases: (i) slow depolarization (20 mV in 10–40 s); (ii) further fast depolarization (30 mV in 1 s); and (iii) rapid (5 s) repolarization. $[Ca^{2+}]_i$ decreased during (i), increased quickly during (ii) with a 1 s delay with regard to the peak depolarization, and decreased during (iii).

5. Slight depolarizations increased the frequency of the oscillations whereas large depolarizations decreased it.

6. The Ca^{2+}-dependent K^+ channel blocker apamin increased the duration and decreased the frequency of the oscillations.

7. We propose the following mechanism for the oscillations: (i) the membrane depolarizes slowly by a decrease of potassium conductance (g_K), perhaps due to a gradual decrease of $[Ca^{2+}]_i$; (ii) the threshold for activation of Na^+ channels (decreased by VTD) is reached, producing further depolarization and recruiting Ca^{2+} channels, and inactivation of both Ca^{2+} and VTD-poisoned Na^+ channels is slow; and (iii) g_K increases, aided by activation of Ca^{2+}-dependent K^+ channels by the increased $[Ca^{2+}]_i$, and the membrane repolarizes. The contribution of the Na^+ channels seems essential for the generation of the oscillations.

8. Bovine chromaffin cells have the machinery required for $[Ca^{2+}]_i$ oscillations even though the more physiological stimulus tested here (high K^+, field electrical stimulation, nicotinic or muscarinic agonists) produced mainly non-oscillatory responses.

The increase of cytosolic free calcium concentration ($[Ca^{2+}]_i$) is a transduction signal for many physiological processes. When studied at the single-cell level, such $[Ca^{2+}]_i$ changes have been revealed, in many cases, to be oscillatory rather than maintained, both in excitable and non-excitable cells (Tsien & Tsien, 1990). Such a behaviour has obvious advantages regarding the gradation of physiological responses, which are easier to code in terms of frequency or duration of the Ca^{2+} oscillations than in terms of the amplitude of the $[Ca^{2+}]_i$ increase. Regarding the mechanisms responsible for the generation of $[Ca^{2+}]_i$ oscillations, two main categories have been distinguished. In one, the

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oscillations arise from periodic changes in the membrane potential and Ca^{2+} permeability of the plasma membrane (Gorman & Thomas, 1978; Schlegel *et al.* 1987). The other involves a dynamic Ca^{2+} exchange between the cytosol and internal stores (Woods, Cuthbertson & Cobbold, 1986; Tsien & Tsien, 1990). In some cases both mechanisms could co-operate, the entry of Ca^{2+} through the plasma membrane triggering a massive Ca^{2+} release from the stores, which amplifies the $[Ca^{2+}]_i$ signal (Friel & Tsien, 1992).

Catecholamine secretion by adrenal chromaffin cells is a Ca^{2+}-dependent process (Douglas & Rubin, 1961). Chromaffin cells possess voltage-dependent Na^+ and Ca^{2+} channels (Fenwick, Marty & Neher, 1982) as well as intracellular Ca^{2+} stores. Ca^{2+} release from the stores can be induced by caffeine (Cheek, O'Sullivan, Moreton, Berridge & Burgoyne, 1990) or by inositol 1,4,5-trisphosphate (Stoher, Smollen, Holz & Agranoff, 1986; Robinson & Burgoyne, 1991), which is generated on interaction of several agonists with plasma membrane receptors (Eberhard & Holz, 1987; Plevin & Herz, 1989; Stauderman & Pruss, 1990). With a few exceptions (Stauderman & Pruss, 1989; O'Sullivan & Burgoyne, 1989; Malgaroli, Fesce & Meldolesi, 1990; Dandrea, Zacchetti, Meldolesi & Grohovaz, 1993), reported $[Ca^{2+}]_i$ responses of chromaffin cells to physiological or pharmacological stimuli have been of the non-oscillatory type. However, we find here that the Na^+ channel poison veratridine (VTD) (Ohta, Narahashi & Keeler, 1973) causes large $[Ca^{2+}]_i$ oscillations in otherwise silent bovine chromaffin cells. These oscillations are generated by cyclic Ca^{2+} entry through the plasma membrane with little or no contribution of Ca^{2+} release from the intracellular Ca^{2+} stores. Ca^{2+} entry is subsequent to large oscillations of membrane potential triggered by activation of Na^+ channels.

METHODS

Bovine adrenal glands were obtained from the local slaughterhouse. Medullary chromaffin cells were isolated as described previously (Livett, 1984), with the modifications introduced by Moro, López, Gandía, Michelena & García (1990). Cells were suspended in Dulbecco's modified minimal Eagle's medium, plated on 11 mm diameter poly-L-lysine-coated (0·01 mg ml^{-1}, 5 min) glass coverslips and incubated at 37 °C under 95% O_2–5% CO_2 for 2–4 days before use. $[Ca^{2+}]_i$ measurements and digital image analysis were performed as described before (Villalobos, Fonteriz, López, García & García-Sancho, 1992). Briefly, the cell-coated coverslips were washed with standard medium containing (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; Na-Hepes, 10; pH, 7·4. The cells were loaded with fura-2 by incubation with 5 μM fura-2 AM at room temperature for about 1 h. The coverslips were mounted under the microscope (Nikon Diaphot) in a chamber thermostated at 37 °C and epi-illuminated alternately at 340 and 380 nm. Light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertbridge, East Sussex, UK) and analysed using an Applied Imaging Magical image processor (Sunderland, UK) with 32 Mbyte video RAM. Four video frames of each wavelength were averaged by hardware, with an overall time resolution of about 3 s for each pair of images at alternate wavelengths. Consecutive frames obtained at 340 and 380 nm excitation were ratioed pixel by pixel and $[Ca^{2+}]_i$ was estimated from this ratio (F_{340}/F_{380}) by comparison with fura-2 standards (Grynkiewicz, Poenie & Tsien, 1985). Quantification of the single-cell $[Ca^{2+}]_i$ oscillations was performed using two parameters, mean $[Ca^{2+}]_i$ increase and oscillation index. Mean $[Ca^{2+}]_i$ increase was computed as the average of all the $[Ca^{2+}]_i$ values minus the 'resting' $[Ca^{2+}]_i$ over the whole integration period. The 'resting' $[Ca^{2+}]_i$ was arbitrarily chosen as the smallest $[Ca^{2+}]_i$ value in the whole trace. Thus, the mean $[Ca^{2+}]_i$ increase is equivalent to the area under the $[Ca^{2+}]_i$ peaks. The increase of this parameter reflects increases of $[Ca^{2+}]_i$, irrespective of whether or not they are oscillatory. The oscillation index was computed as the average of all the differences (in absolute value) between each $[Ca^{2+}]_i$ value and the next along the whole integration period. Units are nanomolar per three seconds. The increase of this parameter reflects increased oscillations (either in frequency or amplitude) and it is largely independent of the actual $[Ca^{2+}]_i$ values.

Simultaneous measurements of membrane potential and $[Ca^{2+}]_i$ were made in single cells by combining whole-cell patch clamp and fura-2 fluorescence (Neher, 1989). Patch pipettes, prepared using Kimax glass and having a resistance of 2·5–4 MΩ, were used for loading the cells with the pentapotassium salt of fura-2. The dye was alternately excited with monochromatic light at 360 and 390 nm and the emitted fluorescence, high-pass filtered above 540 nm, was measured using a photomultiplier (Type R928; Hamamatsu Photonics, Hamamatsu City, Japan). The ratio of both signals was calibrated to provide estimates of $[Ca^{2+}]_i$ (Neher, 1989). The experimental set-up allowed simultaneous recording of membrane potential under current-clamp conditions. All the experiments were performed at 37 °C. The extracellular solution contained (mM): NaCl, 145; KCl, 2·8; $CaCl_2$, 1; $MgCl_2$, 2·5; glucose, 11; Na-Hepes, 10; pH 7·4. Veratridine was applied by pressure ejection from a puffer pipette with an opening of about 2 μm, which was placed close (20–50 μm) to the cell under investigation. The intracellular solution contained (mM): potassium glutamate, 145; NaCl, 8; MgCl, 1; MgATP, 0·5; Li_2GTP, 0·3; fura-2, 0·1; K-Hepes, 10; pH 7·2. The solutions were filtered (pore size 0·2 μm). The liquid junction potential was corrected by subtracting 8 mV from all the voltage readings obtained during the experiments. The resting cell resistance was typically larger than 10 GΩ. The monitored series resistance remained below 11 MΩ. Experimental data were sampled at intervals of about 20 ms. Mean values over 0·5 s were calculated and plotted *versus* the mean segment time.

Fura-2 AM and fura-2 pentapotassium salt were obtained from Molecular Probes, Eugene, Oregon, USA. Thapsigargin, ryanodine and charybdotoxin were obtained from Alomon Laboratories, Jerusalem, Israel. Tetrodotoxin was from Calbiochem, Spain. Apamin and veratridine were from Sigma, Spain. Other chemicals were obtained from Sigma, or from E. Merck, Darmstadt, Germany.

Unless otherwise stated, all values are given as means ± S.E.M.

RESULTS

Veratridine induces $[Ca^{2+}]_i$ oscillations in chromaffin cells, and extracellular Ca^{2+} and Na^+ are required

Treatment of bovine adrenal chromaffin cells with veratridine (5–30 μM) induced an increase in $[Ca^{2+}]_i$ (Fig. 1*A* and *B*). The proportion of cells that were responsive varied with the cell batch, but usually approached 80%. In most of the cells, $[Ca^{2+}]_i$ began to increase a few seconds after application of VTD and a regular oscillatory pattern was established within 1–3 min. These $[Ca^{2+}]_i$ oscillations remained stable for at least 40 min (the longest period tested) and were not modified by switching perfusion to VTD-free medium (see for example Fig. 1*B*, where oscillations begin on removal of VTD). In most of the experiments to be described below, the cells were treated with VTD for 5 min and then perfusion was switched to medium containing the same VTD concentration, but with the additions or the ionic substitutions described in each case.

Veratridine-induced $[Ca^{2+}]_i$ oscillations were dependent on the presence of Ca^{2+} and Na^+ in the extracellular medium (Fig. 1*C*). On Ca^{2+} removal the oscillations were immediately stopped and they reappeared on restoration of extracellular Ca^{2+}. Similar results were obtained by preventing Ca^{2+} entry by addition to the external medium of Ni^{2+}, an inorganic Ca^{2+} channel blocker (Fonteriz, García-Sancho, Gandia, López & García, 1992) (Fig. 1*D*). Sodium removal also reversibly prevented $[Ca^{2+}]_i$ oscillations (Fig. 1*C*). The addition of tetrodotoxin (TTX, 1 μM), a Na^+ channel blocker, had similar results (Fig. 1*E*). Figure 1*F* and *G* illustrates the average behaviour of all the individual cells analysed. Oscillations were quantified as the mean $[Ca^{2+}]_i$ (Area, Fig. 1*F*) or the oscillation index (Fig. 1*G*, see Methods for details). It is clear that all the conditions tested (Ca^{2+} or Na^+ removal, Ni^{2+} or TTX addition) decreased both area and oscillation indexes towards the values observed before treatment with VTD (column labelled Cont). Ca^{2+} removal or Ni^{2+} addition decreased the mean $[Ca^{2+}]_i$ even to values below the control value. This was due to the fact that these manoeuvres, apart from abolishing the $[Ca^{2+}]_i$ oscillations (reflected by the oscillation index), also decreased the basal $[Ca^{2+}]_i$ over which the oscillations take place.

Effects of membrane depolarization

The effects of membrane potential on VTD-induced $[Ca^{2+}]_i$ oscillations were studied by depolarizing the cells with either different K^+ concentrations or by field electrical stimulation. Representative results are illustrated in Fig. 2. Depolarization with increasing K^+ concentrations (replacing equimolar amounts of Na^+) produced in control cells, not treated with VTD, a graded increase of $[Ca^{2+}]_i$, which showed little or no oscillations (see below). This is illustrated in one cell not treated with VTD (Fig. 2*A*) and in one cell which had not responded to VTD (Fig. 2*D*). The responses were similar in both kinds of cells. The lower K^+ concentrations (10–18 mM) produced a sustained $[Ca^{2+}]_i$ increase, whereas with the higher K^+ concentrations (35 and 70 mM) the $[Ca^{2+}]_i$ decreased quickly after its initial rise (Fig. 2*D*). This decrease is likely to reflect the time-dependent inactivation of the Ca^{2+} channels both by maintained depolarization and by increased $[Ca^{2+}]_i$ (Garrido, López, Moro, de Pascual & García, 1990; Fonteriz *et al.* 1992). Field electrical stimulation (5 ms pulses, 10 Hz, labelled ES) produced an increase of $[Ca^{2+}]_i$ which was usually more sustained than the increase obtained by depolarization with high K^+ (Fig. 2*D*). Again the behaviour was similar in control cells, non-treated with VTD (not shown), and in cells insensitive to VTD. The $[Ca^{2+}]_i$ increases produced by high K^+ or by electrical stimulation in control cells were usually non-oscillatory. Although fluctuations of $[Ca^{2+}]_i$ were observed sometimes (see 15 mM K^+ in Fig 2*A* and ES in Fig 2*D*), these were relatively small, the peaks of $[Ca^{2+}]_i$ took place over an increased plateau and they were not followed by a decrease to the resting $[Ca^{2+}]_i$ level before stimulation.

When the cells had been treated with VTD before depolarization, a very different response was obtained. Slight depolarization with 10 mM K^+ reversibly increased the VTD-induced oscillations (Fig. 2*B*). Moderate depolarization with 18 mM K^+ seemed to decrease somewhat the oscillatory behaviour, but oscillations could still be clearly seen on top of the $[Ca^{2+}]_i$ increase directly induced by depolarization (Fig. 2*C*). Large depolarizations with 35 or 70 mM K^+ largely decreased or abolished $[Ca^{2+}]_i$ oscillations (Fig. 2*C*). Field electrical stimulation did not seem to upset $[Ca^{2+}]_i$ oscillations (Fig. 2*C*), even though it was able to produce by itself a maintained increase of $[Ca^{2+}]_i$ comparable to that obtained with the high K^+ concentrations (Fig. 2*D*).

Figure 2*E* and *F* summarizes the average behaviour of all the individual cells analysed, quantified either as mean $[Ca^{2+}]_i$ increases (*E*) or as oscillation indexes (*F*). In Fig. 2*E*, the contribution of the maintained effects of the depolarizing agents and of the $[Ca^{2+}]_i$ oscillations cannot be separated. Note, however, that the highest K^+ concentration produced a mean $[Ca^{2+}]_i$ increase that was comparatively small, not very different from that observed in the VTD-treated cells which had not been depolarized (first column in Fig. 2*E*). Field stimulation, on the other hand, increased the mean $[Ca^{2+}]_i$ increase above the value obtained with VTD alone, reflecting the increase of resting $[Ca^{2+}]_i$ by electrical stimulation. The oscillation indexes shown in Fig. 2*F* should not be affected by changes in resting $[Ca^{2+}]_i$, but reflect pure modifications of the oscillatory

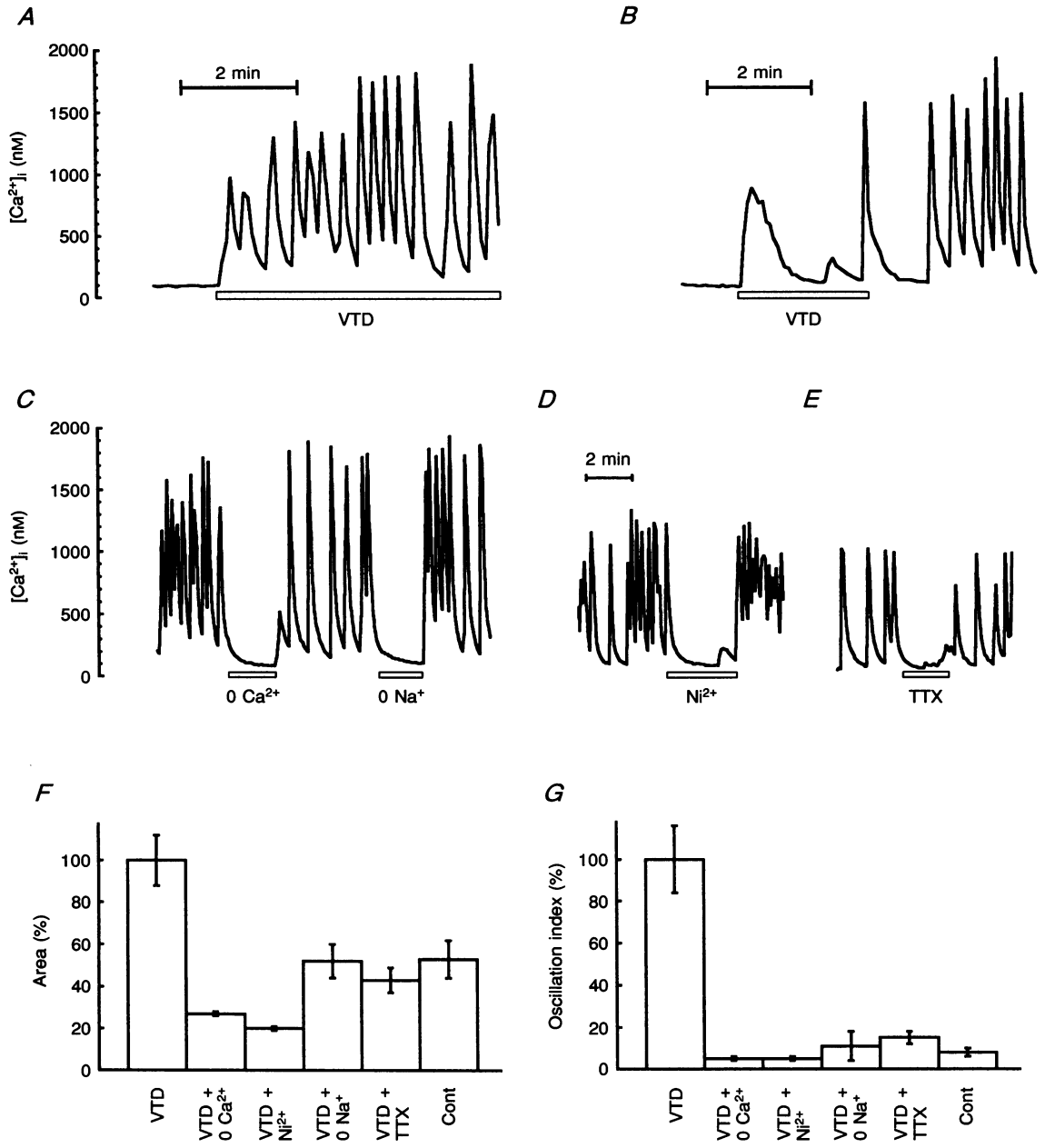

Figure 1. Effects of removal of Na^+ and Ca^{2+}, Ni^{2+} and tetrodotoxin on veratridine-induced oscillations

A and *B* illustrate the effects of veratridine (VTD, 5 μM in *A* and 30 μM in *B*) in two representative single cells. In *B*, VTD was removed after a 2·5 min treatment. In traces *C–E*, cells were treated with 5 μM veratridine for 5 min to start the $[Ca^{2+}]_i$ oscillations (not shown in the figure) and then perfusion was switched to Ca^{2+}-free (0·2 mM EGTA), Na^+-free (Na^+ replaced by equimolar concentrations of *N*-methyl-D-glucamine), Ni^{2+}-containing (1 mM) or tetrodotoxin-containing (TTX, 1 μM) medium, as shown in the figure. *F* and *G* show average values of the mean $[Ca^{2+}]_i$ (Area) and the oscillation index (see Methods) obtained in 19–31 individual cells, expressed as a percentage of the mean value obtained with VTD only. A 2 min integration period was used for these calculations, avoiding the transients by starting the integration period 1 min after the changes in perfusion. Control (Cont) refers to the period before VTD addition (not shown in traces *C–E*). Vertical bars represent S.E.M. The inhibition by 0 Ca^{2+}, Ni^{2+}, 0 Na^+ and TTX was, in all the cases, statistically significant ($P < 0·001$ when compared with VTD alone in all the cases except 0 Na^+ and TTX in panel *E*, in which P was $< 0·01$; Student's *t* test).

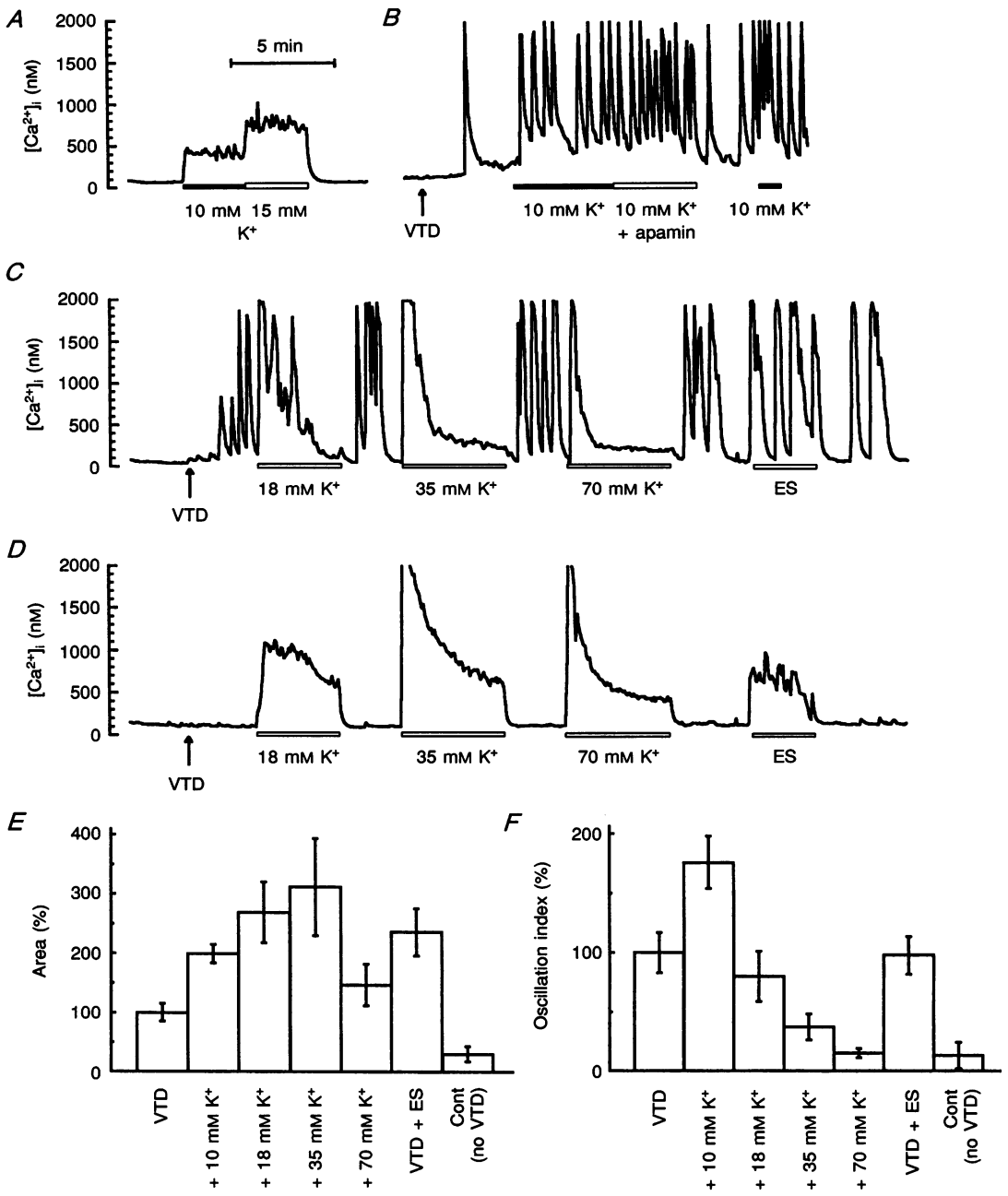

Figure 2. Effects of depolarization by high K^+ or by electrical stimulation on the $[Ca^{2+}]_i$ oscillations induced by VTD

A–D correspond to representative single cells. *A* illustrates the response of control (VTD-untreated) cells to depolarization with 10 and 15 mM K^+. *B* illustrates the stimulating effect of 10 mM K^+ on a single cell showing moderate activity after treatment with VTD; the effect of apamin (10^{-7} M) is also shown. *C* illustrates the response to different K^+ concentrations and to field electrical stimulation (ES) on the oscillations induced by VTD. *D* shows the effects of the same stimuli in a cell in which VTD had not induced $[Ca^{2+}]_i$ oscillations. For electrical stimulation, alternating (positive–negative) square pulses of 66 V and 5 ms were passed at 10 Hz through a pair of chlorided silver electrodes separated by 5 mm and positioned 1 mm above the cells. *E* and *F* show the average values of the mean $[Ca^{2+}]_i$ increase (Area) and the oscillation index (see Methods) obtained in 13–34 individual cells, expressed as a percentage of the mean value obtained with VTD only. A 3 min integration period was used for these calculations, avoiding the transients by starting the integration period 1 min after the changes in perfusion. Control (Cont, no VTD) refers to the period before VTD addition. Vertical bars represent S.E.M. The statistical significance of the differences between VTD alone and the other conditions (Student's *t* test) were as follows: panel *E*, + 10, 18 and 35 mM K^+ and + ES, $P < 0.001$; + 70 mM K^+, not significant. Panel *F*, + 35 and 70 mM K^+, $P < 0.005$; + 10 mM K^+, $P < 0.02$; + 35 mM K^+, $P < 0.05$; + 18 mM K^+, not significant.

behaviour. It is clear that slight depolarization with 10 mM K^+ increased oscillations whereas moderate or large depolarizations produced by higher K^+ concentrations decreased oscillations. The highest K^+ concentration (70 mM) fully abolished oscillations, as shown by the fact that the oscillation index was the same as in the controls not treated with VTD (last column in Fig. 2*F*). Field stimulation did not modify the oscillation index (compare with VTD-treated non-depolarized cells; first column in Fig. 2*F*). This suggests that, contrary to that observed with K^+ depolarization, the oscillatory behaviour was not essentially modified by electrical stimulation, except that oscillations did usually take place over a larger basal $[Ca^{2+}]_i$, as suggested by the increase of the mean $[Ca^{2+}]_i$ documented in Fig. 2*E*.

Effects of drugs acting on the intracellular calcium stores

Figure 3*A–C* illustrates the effects of two drugs known to deplete intracellular Ca^{2+} stores, thapsigargin and ryanodine. Thapsigargin acts on most of the stored Ca^{2+} pools, including the IP_3-sensitive ones (Thastrup, 1990; Robinson, Cheek & Burgoyne, 1992; Berridge, 1993). Ryanodine blocks the channel associated with the ryanodine receptors in an open subconductance state, thus promoting a 'use-dependent' depletion of this Ca^{2+} pool (Sorrentino & Volpe, 1993; Berridge, 1993). Thapsigargin had little or no effect on VTD-induced $[Ca^{2+}]_i$ oscillations (Fig. 3*A*), although it produced Ca^{2+} release from the intracellular stores, as indicated by the fact that it was able to increase $[Ca^{2+}]_i$ (Fig. 3*B*, obtained from a VTD-insensitive cell). The average values for the mean $[Ca^{2+}]_i$ increases and the oscillation indexes in forty-one cells were 172 ± 20 nM and 68 ± 13 nM $(3\ s)^{-1}$, respectively, after a 5 min thapsigargin treatment, compared with 158 ± 19 nM and 65 ± 11 nM $(3\ s)^{-1}$ before thapsigargin treatment. Ryanodine was also unable to prevent the VTD-induced oscillations, as illustrated in Fig. 3*C* for a representative cell. The average values for the mean $[Ca^{2+}]_i$ increase and the oscillation index in twenty-five cells were 305 ± 38 nM

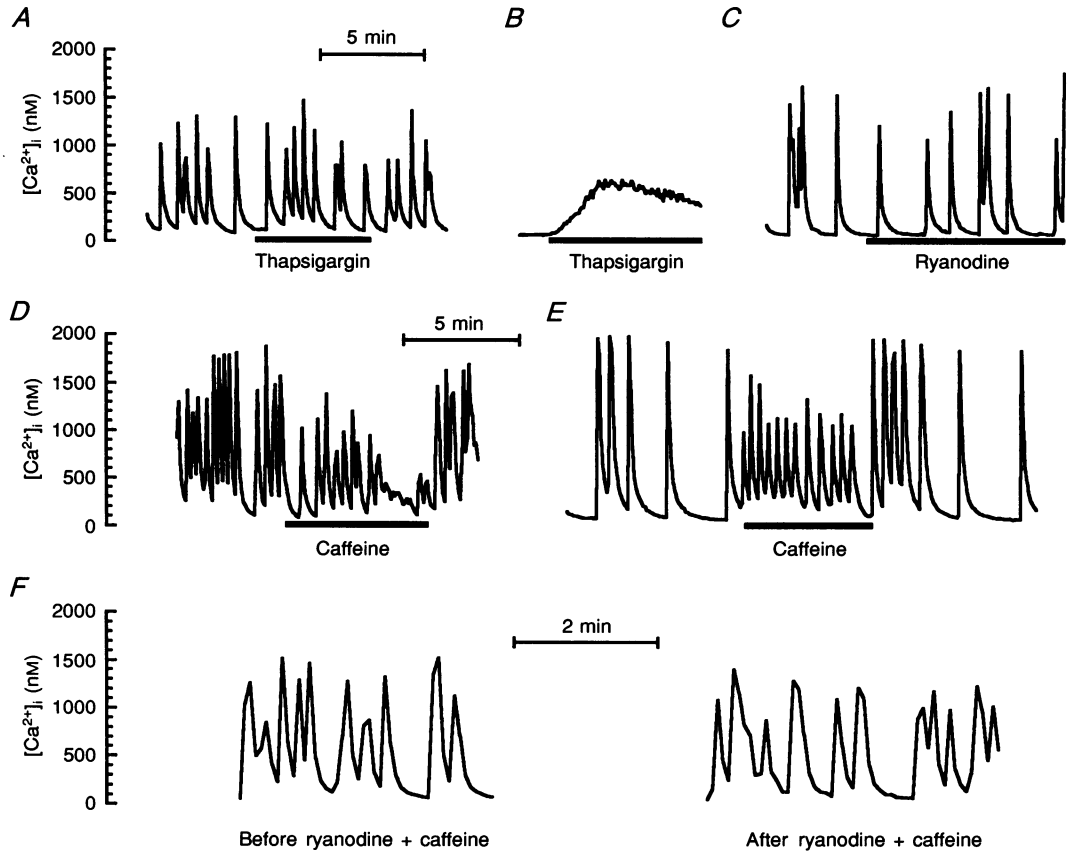

Figure 3. Effects of thapsigargin, ryanodine and caffeine on the VTD-induced $[Ca^{2+}]_i$ oscillations

Representative single-cell traces are shown. The oscillations were started with 5 μM VTD at least 5 min before the start of the traces shown here. The concentrations used were 500 nM for thapsigargin, 10 μM for ryanodine and 20 mM for caffeine. In *B*, the increase of $[Ca^{2+}]_i$ in a cell not responding to VTD is shown. In *F*, the two traces correspond to the same cell (in control solution containing 5 μM VTD) before and after a 5 min treatment with 10 μM ryanodine and 20 mM caffeine.

and 156 ± 19 nM $(3\ s)^{-1}$, respectively, after a 5 min ryanodine treatment, compared with 304 ± 31 nM and 176 ± 22 nM $(3\ s)^{-1}$ before ryanodine treatment.

Caffeine is thought to release Ca^{2+} from the stores responsible for Ca^{2+}-activated Ca^{2+} release, a subclass within the ryanodine-sensitive Ca^{2+} pools (Sorrentino & Volpe, 1993; Berridge, 1993). Caffeine had a complex effect in bovine chromaffin cells. In cells showing prominent VTD-induced oscillations it tended to have an inhibitory effect (Fig. 3*D*). In contrast, in cells showing moderate-to-low-intensity oscillations, caffeine had a stimulatory effect, usually increasing the frequency of the $[Ca^{2+}]_i$ oscillations (Fig. 3*E*). In all the cases, the effects of caffeine were reversed on perfusion with control medium.

The correlation between the mean $[Ca^{2+}]_i$ increase observed before and after caffeine in VTD-treated cells was studied in 132 single cells from four different cell batches. Most of the cells showing low activity were stimulated by caffeine whereas the reverse applied to cells with high activity. For example, in twenty-nine VTD-treated cells in which the mean $[Ca^{2+}]_i$ increase was below 100 nM, caffeine increased activity from 50 ± 4 to 200 ± 9 nM ($P < 0.001$). In contrast, in a second group of fifty-one cells selected by having a mean $[Ca^{2+}]_i$ increase before caffeine > 300 nM, activity was decreased by caffeine from 587 ± 34 to 344 ± 23 nM ($P < 0.001$). We also tested the effects of caffeine on the $[Ca^{2+}]_i$ increase induced by field electrical stimulation in control, VTD-untreated cells. Caffeine (20 mM) produced a 46% inhibition of the mean $[Ca^{2+}]_i$ increase induced by electrical stimulation (results representative of sixteen single cells; not shown).

Joint treatment with ryanodine and caffeine has been reported to produce irreversible depletion of the ryanodine-sensitive Ca^{2+} stores (Stauderman & Murawsky, 1991; Cheek, Moreton, Berridge, Stauderman, Murawsky & Bootman, 1993). $[Ca^{2+}]_i$ oscillations of VTD-treated cells were not significantly modified after a 5 min treatment with 10 μM ryanodine and 20 mM caffeine (Fig. 3*F*). In twenty-five single cells analysed, the values of the oscillation index before and after treatment with ryanodine and caffeine were 175 ± 19 and 172 ± 19 nM $(3\ s)^{-1}$, respectively.

Effects of Ca^{2+}-dependent K^+ channel inhibitors

Two different Ca^{2+}-dependent K^+ channel inhibitors, charybdotoxin (ChTX) and apamin (Dreyer, 1990), were tested for their effects on the VTD-induced oscillations. Representative traces are shown in Fig. 4. Charybdotoxin had little or no effect on the VTD-induced oscillations (Fig. 4*B*). In thirty-five single cells studied, the mean $[Ca^{2+}]_i$ and the oscillation index were 469 ± 48 nM and 244 ± 22 nM $(3\ s)^{-1}$, respectively, after 5 min treatment with ChTX, compared with 502 ± 55 nM and 264 ± 27 nM $(3\ s)^{-1}$ before treatment with the toxin. Apamin had a clear effect in about 50% of the cells, in which it tended to increase the duration of the $[Ca^{2+}]_i$ oscillations and to decrease their frequency (Fig. 4*A* and *B*; but sometimes it had other effects, see for example Fig. 2*B*). This effect was not reversed by washing, at least within 10 min periods (results not shown). These effects of apamin were not clearly reflected in the parameters used above to quantify other actions, like the mean $[Ca^{2+}]_i$ increase or the oscillation index. The frequency and duration of the $[Ca^{2+}]_i$ oscillations were measured in a selected population of VTD-treated cells showing a regular oscillatory pattern. Frequency was decreased by apamin from 2.15 ± 0.15 to 1.46 ± 0.11 min ($n = 50$; $P < 0.001$; paired t test). Duration, estimated as the time in seconds elapsed since $[Ca^{2+}]_i$ surpassed 700 nM until it dropped again below this value, was increased by apamin from 19.3 ± 1.2 to 39.9 ± 2.3 s ($n = 25$; $P < 0.001$; paired t test).

Effects of variations of the extracellular Ca^{2+} and Mg^{2+} concentrations on the VTD-induced oscillations

In a series of experiments, the effects of variations of external $[Ca^{2+}]$ on the VTD-induced oscillations were measured. When $[Mg^{2+}]$ was held constant at 1 mM, the

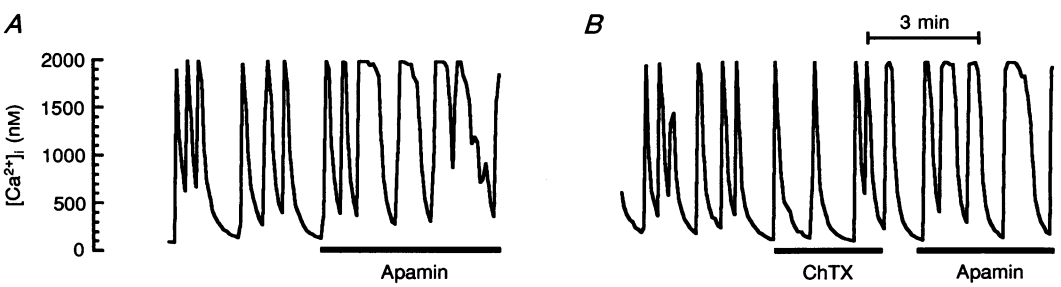

Figure 4. Effects of apamin and charybdotoxin on the VTD-induced $[Ca^{2+}]_i$ oscillations
The oscillations were started with 5 μM VTD 5 min before the start of the traces shown here. The concentrations of apamin and charybdotoxin (ChTX) were 10^{-7} and 10^{-8} M, respectively.

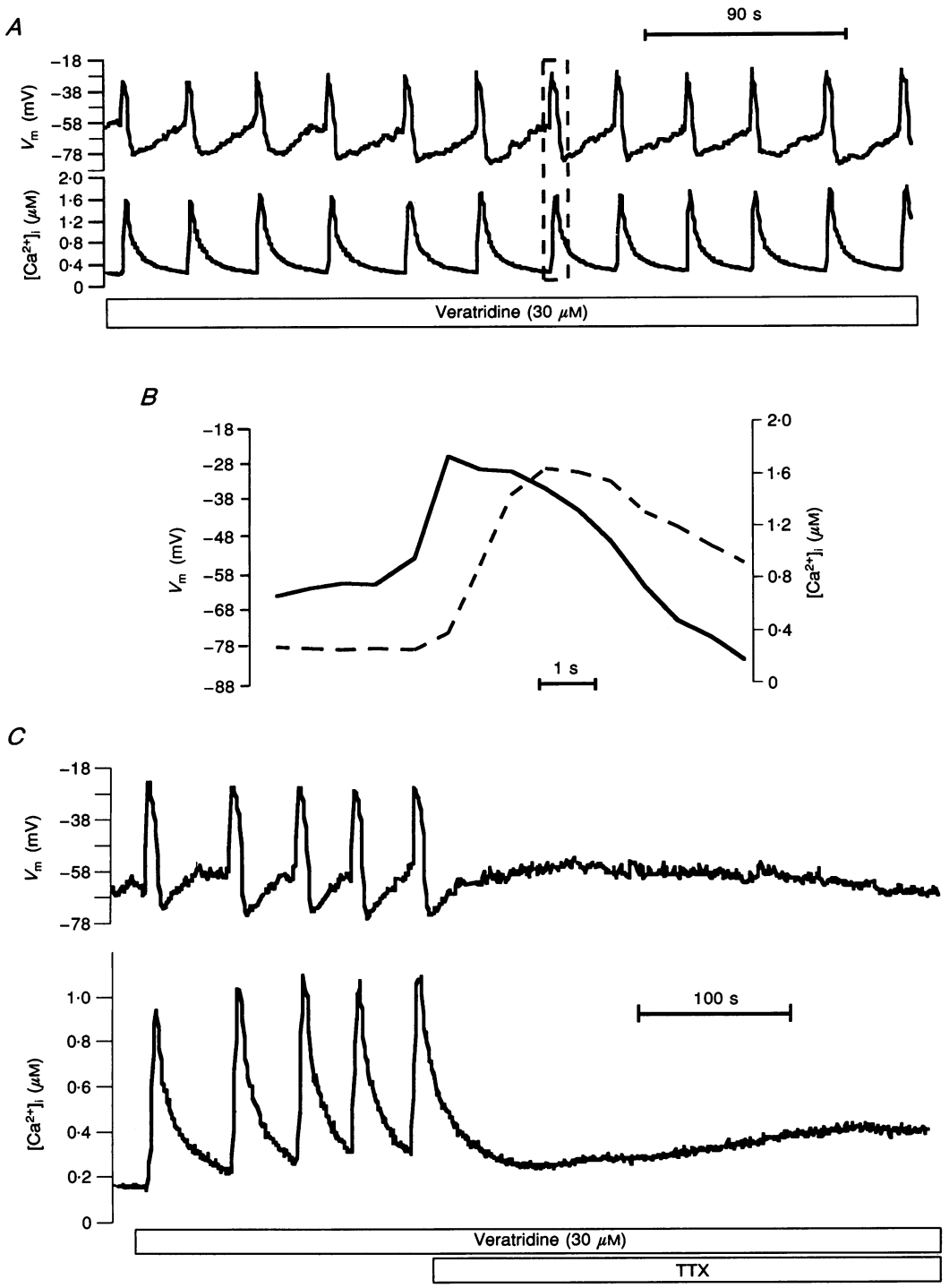

Figure 5. Simultaneous recordings of membrane potential (upper traces) and $[Ca^{2+}]_i$ (lower traces) under current-clamp conditions of single bovine chromaffin cells loaded with fura-2 through the patch pipette

A shows the oscillatory pattern of the membrane potential and of $[Ca^{2+}]_i$ in a cell continuously exposed to veratridine (30 μM) applied with a puffer pipette whose tip was placed 30 μm away from the cell. In *B*, the oscillation enclosed in the box in *A* has been magnified to increase time resolution. Membrane potential (V_m), continuous line; $[Ca^{2+}]_i$, dashed line. In *C*, the effects of tetrodotoxin (TTX, 1 μM) are shown. After washing out the TTX, the oscillations of both membrane potential and $[Ca^{2+}]_i$ started again (not shown).

activity (measured either as mean $[Ca^{2+}]_i$ or as the oscillation index) increased slightly by decreasing external $[Ca^{2+}]$ from 1 to 0·5 mM and it was strongly depressed by increasing external $[Ca^{2+}]$ to 5 mM. In sixteen cells analysed, the mean $[Ca^{2+}]_i$ increase dropped from 419 ± 50 to 60 ± 30 nM and the oscillation index from 159 ± 22 to 5 ± 1 nM $(3\ s)^{-1}$ by increasing the external $[Ca^{2+}]$ from 1 to 0·5 mM. In a second series of experiments in which the total extracellular divalent cation (Ca^{2+} + Mg^{2+}) concentration was held constant at 3·7 mM, the predominant effect of a Ca^{2+} concentration decrease from 1 to 0·5 mM was a reversible decrease in the amplitude and frequency of the $[Ca^{2+}]_i$ peaks together with an increase of the duration. When external $[Ca^{2+}]$ was increased from 1 to 2·5 mM, the predominant effect was a decrease in frequency with little modification of peak morphology (results not shown). The effect of increasing external $[Mg^{2+}]$ from 1 to 2·7 mM, holding constant external $[Ca^{2+}]$ at 1 mM, was a decrease of the $[Ca^{2+}]_i$ activity (results not shown)

Effects of veratridine on the membrane potential and $[Ca^{2+}]_i$ under current-clamp conditions

In order to study the possible relationship between the oscillatory changes of $[Ca^{2+}]_i$ caused by VTD and oscillations of membrane potential (V_m), patch-clamp experiments were done in the whole-cell configuration under current-clamp conditions. Changes in voltage and $[Ca^{2+}]_i$ were monitored simultaneously in single bovine chromaffin cells loaded with fura-2 through the patch pipette. In control cells, not treated with VTD, both the V_m and $[Ca^{2+}]_i$ remained stable, with no indication of oscillations during the whole recording period (up to 30 min; not shown). In a few cases, occasional spontaneous action potentials were seen which were not accompanied by significant $[Ca^{2+}]_i$ rises (see below).

The application of veratridine (30 μM) through a puffer pipette led to the establishment, within 1–2 min, of large synchronic oscillations of both V_m and $[Ca^{2+}]_i$ (Fig. 5*A*). The oscillations were rhythmic and remained unaltered for at least 30 min. The most negative value of V_m was about −80 mV. From this value, the membrane depolarized to −55 mV within about 30 s. This slow depolarization coincided with a slow decrease in $[Ca^{2+}]_i$. On reaching −55 mV, the membrane depolarized much faster (within about 1 s) to −30 mV, and then repolarized to −80 mV within 4–5 s to start the cycle again. The rise in $[Ca^{2+}]_i$ began at about the same time as the fast depolarization and the peak $[Ca^{2+}]_i$ was delayed about 1 s with regard to the peak depolarization (Fig. 5*B*). Repolarization preceded the downstroke of the $[Ca^{2+}]_i$ oscillation. Except for the difference in time scale (seconds *versus* milliseconds), the profile of V_m oscillations resembled the spontaneous action potentials occasionally seen in control (not treated with VTD) slightly depolarized (V_m about −50 mV) bovine chromaffin cells (not shown).

Figure 5*C* shows the oscillations of V_m and $[Ca^{2+}]_i$ in a cell, in the current-clamp mode, exposed to VTD (continuous puff application of 30 μM). The addition of TTX (1 μM) to

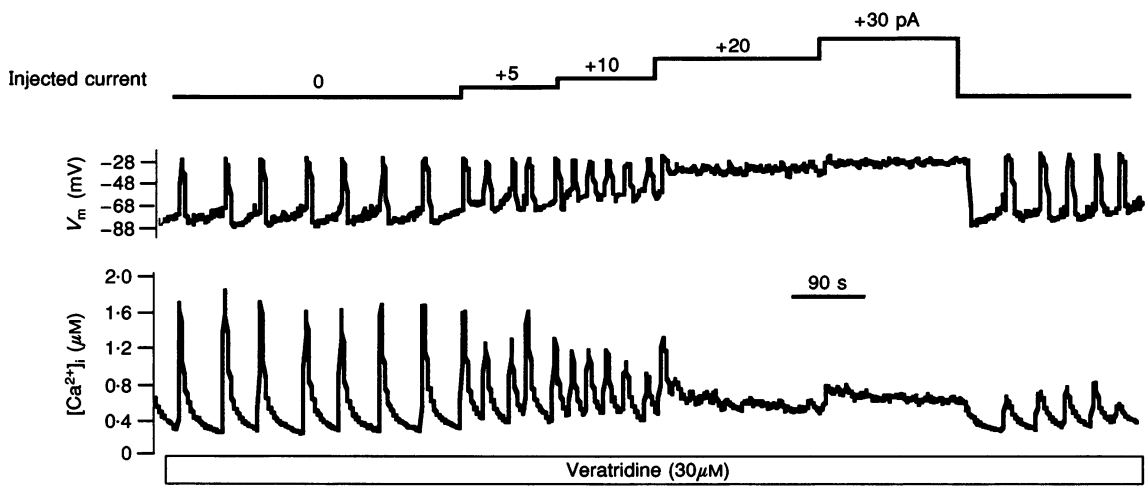

Figure 6. Effects of current injection on the VTD-induced oscillations of the membrane potential and $[Ca^{2+}]_i$

In this cell, the oscillations of membrane potential and $[Ca^{2+}]_i$ were evoked by veratridine as in Fig. 5. Injection of depolarizing current (as shown in the protocol at the top of the figure) led first (+5 and +10 pA) to a decrease of the amplitude and an increase of frequency of the oscillations of membrane potential (middle trace) and $[Ca^{2+}]_i$ (bottom trace) and then (+20 and +30 pA) to their suppression. On cessation of current injection and return to the cell 'resting potential', the oscillations of both membrane potential and $[Ca^{2+}]_i$ started again.

the bath suppressed the oscillations of V_m as well as those of $[Ca^{2+}]_i$. The V_m remained at about −60 mV during perfusion with TTX, a value well above the peak hyperpolarization reached during the oscillations, before TTX application (about −75 mV). $[Ca^{2+}]_i$ remained near the minimum value reached during the preceding oscillations. One minute after removal of TTX, the oscillations of V_m and $[Ca^{2+}]_i$ began again (not shown). Figure 6 shows the effects of injection of depolarizing current on the VTD-induced oscillations. Injection of a current of 5 pA caused the oscillations of V_m and $[Ca^{2+}]_i$ to become smaller and more frequent. An additional 5 pA of current injection caused a further reduction of the $[Ca^{2+}]_i$ peaks. After another 10 pA step of current injection, the oscillations of V_m and $[Ca^{2+}]_i$ disappeared completely. The V_m stabilized at about −45 mV, a value not far from the peak depolarization reached during oscillations, and $[Ca^{2+}]_i$ remained at about 450 nM, a value above the minimum but well below the peak $[Ca^{2+}]_i$ reached during oscillations. A final 10 pA step of current injection (+30 pA in Fig. 6) produced a further depolarization (to −40 mV) and $[Ca^{2+}]_i$ increase (to 550 nM), but again both parameters remained steady, with no large low-frequency oscillations, during the whole test period. On termination of current injection and return to the initial 'resting potential', the oscillations of V_m and $[Ca^{2+}]_i$ reappeared.

Changes in $[Ca^{2+}]_i$ caused by stimuli other than VTD

We investigated whether other kinds of stimulus, such as high K^+, cholinergic agonists or field electrical stimulation, could induce oscillations of $[Ca^{2+}]_i$ similar to those observed with VTD. Special attention was paid to the actions of submaximal stimulus. Low concentrations of K^+ (10–15 mM) produced $[Ca^{2+}]_i$ fluctuations in a minor proportion of the cells, but they were much smaller in amplitude than those observed with VTD (see for example Fig. 2*A*, compare with 2*C*). Acetylcholine (tested concentrations, from 10^{-8} to 10^{-5} M) always produced a transient increase of $[Ca^{2+}]_i$ with little or no indication of $[Ca^{2+}]_i$ oscillations. Apamin potentiated the effects of acetylcholine when submaximal doses of the agonist were used, but again no oscillations were found. Similar results were obtained with the pure nicotinic agonist dimethylphenylpiperazinium (results not shown). Field electrical stimulation produced a maintained increase of $[Ca^{2+}]_i$ in most of the cells. $[Ca^{2+}]_i$ fluctuations were observed in some cells, but, again, they were much smaller than those observed with VTD (Fig. 2*D* and results not shown).

DISCUSSION

In this paper, we have studied the mechanisms responsible for the $[Ca^{2+}]_i$ oscillations induced by VTD in bovine adrenal chromaffin cells, and particularly the relative contributions of Ca^{2+} entry through the plasma membrane and of Ca^{2+} release from the intracellular stores. The intracellular Ca^{2+} stores are responsible for the generation of $[Ca^{2+}]_i$ oscillations in several cell types and could also amplify $[Ca^{2+}]_i$ oscillations initiated by Ca^{2+} entry through the plasma membrane (Friel & Tsien, 1992). Known mechanisms for Ca^{2+} mobilization from the stores include second-messenger induced Ca^{2+} release through IP_3-sensitive channels and Ca^{2+}-induced Ca^{2+} release (CICR) through ryanodine-sensitive channels (Tsien & Tsien, 1990; Friel & Tsien, 1992; Irvine, 1992; Berridge, 1993). Both kinds of Ca^{2+} stores, IP_3-sensitive and ryanodine-sensitive, have been found in chromaffin cells (Cheek *et al.* 1990; Robinson & Burgoyne, 1991). Treatment with the endomembrane ATPase inhibitor thapsigargin (Thastrup, 1990) empties the IP_3-sensitive stores and, in many cases, also the ryanodine-sensitive stores (Berridge, 1993). On the other hand, ryanodine stabilizes an open subconductance state of the ryanodine-sensitive channels which results in an 'use-dependent' emptying of this kind of Ca^{2+} store. Caffeine also facilitates the opening of two out of the three known classes of ryanodine-sensitive channels (Sorrentino & Volpe, 1993) and that results in depletion of the stores.

We find that neither thapsigargin nor ryanodine affected significantly the VTD-induced $[Ca^{2+}]_i$ oscillations in bovine chromaffin cells (Fig. 3), suggesting that the Ca^{2+} stores do not contribute to them. The effects of caffeine were complex. Cells with little Ca^{2+} activity were stimulated by caffeine whereas oscillations were depressed in the cells with high activity. Effects of caffeine other than Ca^{2+} release from the intracellular stores may complicate the interpretation of the results obtained with this drug. Caffeine inhibits phosphodiesterase (Cheung, 1979) and may thus then modify cyclic nucleotide levels. On the other hand, we find that caffeine decreased Ca^{2+} entry in electrically stimulated control (VTD-untreated) cells, suggesting that this drug may interfere with activation of Ca^{2+} channels. Inhibitory effects of caffeine on voltage-gated Ca^{2+} channels have been reported in other tissues (Zahradnik & Palade, 1993; Varro, Hester & Papp, 1993). Our results caution the use of caffeine for selective emptying of the stores involved in CICR. A similar conclusion has recently been reached in liver cells (McNulty & Taylor, 1993).

Overall, our results indicate that stored Ca^{2+} does not play a central role in the generation of VTD-induced $[Ca^{2+}]_i$ oscillations in bovine chromaffin cells. This conclusion is consistent with the results of Neher & Augustine (1992) in bovine chromaffin cells depolarized by current injection. These authors found close quantitive agreement between the Ca^{2+} influx, as measured by current, and that detected by fura-2 fluorescence, leaving little room for participation of Ca^{2+} release from the stores in the generation of the depolarization-induced $[Ca^{2+}]_i$ signal. In contrast, other

reports attribute an important role to either CICR (Malgaroli *et al.* 1990) or to IP_3-sensitive stores (Dandrea *et al.* 1993) in the generation of spontaneous and bradykinin-induced oscillations in rat chromaffin cells. These discrepancies may be due to differences between species or between the mechanisms involved in the spontaneous and VTD-induced oscillations. We never observed spontaneous oscillations of $[Ca^{2+}]_i$ in the bovine chromaffin cells used in this work.

We find that the VTD-induced $[Ca^{2+}]_i$ oscillations are prevented by external Ca^{2+} removal or by blocking Ca^{2+} entry with Ni^{2+} (Fig. 1). This suggests that Ca^{2+} entry is responsible for the upstroke of the $[Ca^{2+}]_i$ oscillations. Veratridine is known to increase Na^+ permeability by shifting the voltage dependence for activation of Na^+ channels towards more negative values and by decreasing their rate of inactivation (Ohta *et al.* 1973; Hille, 1992). Removal of external Na^+ or block of the Na^+ channels with TTX also prevented $[Ca^{2+}]_i$ oscillations (Fig. 1). This suggests that the $[Ca^{2+}]_i$ oscillations are started by membrane depolarization due to the increased Na^+ permeability resulting from the VTD treatment. This depolarization would shift the membrane potential close to the threshold for activation of voltage-dependent Ca^{2+} channels, which would be ultimately responsible for Ca^{2+} entry.

The results obtained with the simultaneous recording of membrane potential and $[Ca^{2+}]_i$ provide further support for this interpretation. Cyclic and rhythmic oscillations of V_m were observed in cells treated with VTD and each wave of depolarization was immediately followed by a large increase of $[Ca^{2+}]_i$, which declined again to basal levels upon repolarization (Fig. 5). The shape of the V_m oscillations was reminiscent of action potentials, although with a much slower time course. The fast depolarization was preceded by a slow one reminiscent of pacemaker potentials. This slow depolarization was not able to activate Ca^{2+} entry, as it was coincident with a decrease of $[Ca^{2+}]_i$, but when the V_m reached a threshold value (about −55 mV in Fig. 5) the fast depolarization and the ensuing Ca^{2+} entry began abruptly.

A sensible interpretation of the above results may be outlined as follows. The 'pacemaker potential' should arise as the result of a gradual increase of sodium conductance (g_{Na}) and/or a decrease of potassium conductance (g_K), perhaps as a result of the decrease of $[Ca^{2+}]_i$ (see below). Membrane depolarizes slowly to reach the threshold for fast regenerative activation of Na^+ channels and then of Ca^{2+} channels. This leads to the generation of an 'action potential' which is extremely wide (4–5 s) because of the slow inactivation of both Ca^{2+} channels and VTD-treated Na^+ channels. The presence of slowly inactivating Ca^{2+} channels in bovine chromaffin cells has been documented before (Fenwick *et al.* 1982). On the other hand, the effect of VTD decreasing the rate of inactivation of Na^+ channels from the millisecond to the second scale is well known (Ohta *et al.* 1973; Hille, 1992). Slight depolarization, either by current injection (Fig. 6) or by increasing the external K^+ concentration to 10 mM (Fig. 2), approached the threshold V_m for regenerative activity and increased the frequency of the oscillations of V_m and $[Ca^{2+}]_i$. In contrast, large and sustained depolarizations decreased or abolished oscillations. This may be due to trapping of the Na^+ and Ca^{2+} channels in the inactivated state, thus precluding the establishment of an oscillatory pattern of opening and closing of these channels. The observation that cyclic (10 Hz) field electrical stimulation did not upset the VTD-induced $[Ca^{2+}]_i$ oscillations (Fig. 2*C* and *F*) is consistent with this interpretation. As depolarization is not sustained in this case, transition of the Na^+ and Ca^{2+} channels from the refractory to the closed state at the end of each action potential is not prevented.

The contribution of Na^+ channels seems essential for the generation of the oscillations, as suggested by the drastic action of TTX, preventing the electrical as well as the $[Ca^{2+}]_i$ activity (Figs 1*E* and 5*C*). In the same line, the inhibitory effects of increasing the extracellular concentrations of Mg^{2+} and/or Ca^{2+} can be rationalized in terms of the membrane-stabilizing action of these cations, shifting the voltage dependence for activation of Na^+ channels towards more positive values (Frankenhauser & Hodgkin, 1957; Hille, 1992).

The downstroke of the Ca^{2+} oscillation must result from active Ca^{2+} extrusion from the cytosol coinciding with low Ca^{2+} entry. Both inactivation of Ca^{2+} channels, either by depolarization (Garrido *et al.* 1990) or by high $[Ca^{2+}]_i$ (Fonteriz *et al.* 1992), and deactivation of Ca^{2+} channels by repolarization of the plasma membrane may co-operate to terminate the $[Ca^{2+}]_i$ oscillation. A possible role for Ca^{2+}-dependent K^+ channels in membrane repolarization has been tested here. The increased $[Ca^{2+}]_i$ during the upstroke of the oscillation could activate these channels, thus promoting repolarization and closing of Ca^{2+} channels. Two classes of Ca^{2+}-dependent K^+ channels have been identified in chromaffin cells (Marty & Neher, 1985; Artalejo, García & Neher, 1993), which can be blocked by ChTx and by apamin, respectively (Dreyer, 1990). We find that ChTx has no significant effects on VTD-induced oscillations, whereas apamin increased the length and decreased the frequency of the $[Ca^{2+}]_i$ oscillations (Fig. 4). These results indicate that the apamin-sensitive Ca^{2+}-dependent K^+ channels, although not essential for the $[Ca^{2+}]_i$ oscillations, could contribute to their modulation. Several features of the V_m oscillations might also be attributed to the action of Ca^{2+}-dependent K^+ channels. For example, the observation that, after abolition of oscillations with TTX, V_m becomes less negative than the peak hyperpolarization value reached during oscillations (Fig. 5*C*) suggests that an additional g_K is activated during the downstroke of the V_m oscillation, when $[Ca^{2+}]_i$ reaches its highest value. The slow depolarization during the 'pacemaker potential' may also

be interpreted as resulting from a decrease of g_K due to the gradual decrease of $[Ca^{2+}]_i$ seen during this period.

The $[Ca^{2+}]_i$ oscillations induced by VTD may contribute towards explaining the Ca^{2+}-dependent cytotoxic effects reported for this drug (Pauwels, Van-Assouw, Peeters & Leysen, 1990). It is not clear, however, whether $[Ca^{2+}]_i$ oscillations such as the ones described here may be involved in the activation of the chromaffin cell secretory cycle during physiological responses. Oscillatory $[Ca^{2+}]_i$ responses have been demonstrated for many cell kinds and it is clear that gradation of the $[Ca^{2+}]_i$ stimuli in terms of frequency rather than amplitude offers obvious codification advantages (see Introduction). Although small oscillatory $[Ca^{2+}]_i$ responses have been reported in rat chromaffin cells in response to muscarinic agonists and bradykinin (Neely & Lingle, 1992; Dandrea *et al.* 1993), the 'physiological' stimuli tested here (electrical stimulation, nicotinic or muscarinic agonists) did not produce a net oscillatory $[Ca^{2+}]_i$ response in bovine chromaffin cells. However, our results with VTD indicate that the machinery required for oscillations is present in these cells and that 'adequate' stimuli, perhaps acting on Na^+ rather than on Ca^{2+} channels, might be identified in the future.

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