5337 Journal of Physiology (1996), 494.3, pp.687–695

The mechanism of calcium channel facilitation in bovine chromaffin cells

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1. This study was planned to clarify the mechanism of Ca^{2+} channel facilitation by depolarizing prepulses given to voltage-clamped bovine chromaffin cells. The hypothesis for an autocrine modulation of such channels was tested by studying the effects of a soluble vesicle lysate (SVL) on whole-cell Ba^{2+} currents (I_{Ba}).

2. SVL was prepared from a bovine adrenal medullary homogenate. The ATP content in this concentrated SVL amounted to $3{\cdot}18 \pm 0{\cdot}12$ mM ($n = 4$). The concentration of noradrenaline and adrenaline present in the SVL was $11{\cdot}2 \pm 0{\cdot}97$ and $15{\cdot}2 \pm 2$ mM, respectively ($n = 5$). A 1:1000 dilution of SVL in the external solution halved the magnitude of I_{Ba} and produced a 7-fold slowing of its activation kinetics. The blocking effects of SVL were concentration dependent and quickly reversed upon washout.

3. Inhibition and slowing of the kinetics of I_{Ba} by SVL could be partially reversed by strong depolarizing prepulses (+90 mV, 45 ms). This reversal of inhibition, called Ca^{2+} channel facilitation, persisted in the presence of 3 μM nifedipine.

4. Intracellular dialysis of GDP-β-S (0·5 mM) or pretreatment of the cells with pertussis toxin (100 ng ml^{-1} for 18–24 h) prevented the reduction in peak current caused by a 1:100 dilution of SVL; no prepulse facilitation could be observed under these conditions.

5. The receptor blockers naloxone (10 μM) or suramin (100 μM) and PPADS (100 μM) largely antagonized the effects of SVL. Treatment of SVL with alkaline phosphatase or dialysis against a saline buffer to remove low molecular mass materials (< 10 kDa) considerably reduced the activity of SVL.

6. Stopping the flow of the external solution (10 mM Ba^{2+}) gradually reduced the size, and slowed down the activation phase, of the current. Prepulse facilitation of I_{Ba} was absent or weak in a superfused cell, but was massive upon flow-stop conditions in the presence or absence of 3 μM nifedipine.

7. Our experiments suggest that facilitation by prepulses of whole-cell current through Ca^{2+} channels is due to the suppression of an autoinhibitory autocrine loop present in bovine chromaffin cells. By acting at least on purinergic and opiate receptors, the exocytotic release of ATP and opiates will cause a tonic inhibition of the current through a G-protein-mediated mechanism. Such a mechanism will be removed by strong depolarizing prepulses, and will involve preferentially non-L-type channels. In the light of these and other recent results, previously held views on the selective recruitment by prepulses of dihydropyridine-sensitive Ca^{2+} channels are not tenable.

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Fenwick, Marty & Neher (1982) first showed that whole-cell Ca^{2+} currents in bovine chromaffin cells could be facilitated by strong depolarizing prepulses. Since then, various attempts have been made to reveal the underlying mechanism. Increased external Ca^{2+} (Ca^{2+}_o) entry through the same channel (Hoshi, Rothlein & Smith, 1984; Hoshi & Smith, 1987) was first suggested to explain the facilitation.

In 1991, Artalejo, Dahmer, Perlman & Fox found two types of Ca^{2+} current in bovine chromaffin cells and suggested that facilitation was due to selective recruitment of the L-type, dihydropyridine (DHP)-sensitive Ca^{2+} channel. This hypothesis was stressed in studies by the same group showing that activation of a D_1 dopamine receptor coupled to adenylate cyclase caused the recruitment of DHP-sensitive, but not of DHP-resistant, Ca^{2+} channels (Artalejo, Ariano, Perlman & Fox, 1990). This facilitation of the whole-cell Ca^{2+} current by recruitment of L-type Ca^{2+} channels led to a sharp increase of catecholamine release, as reflected in capacitance measurements of exocytosis in single bovine chromaffin cells (Artalejo, Adams & Fox, 1994).

Facilitation of Ca^{2+} channels by prepulses has also been seen in cat chromaffin cells; however, we found that facilitation disappeared in the presence of ω-conotoxin GVIA (a selective blocker of N-type Ca^{2+} channels) but not in the presence of nifedipine (an L-type Ca^{2+} channel blocker) (Albillos, Artalejo, López, Gandía, García & Carbone, 1994). This facilitation was mediated by G-proteins and thus may be related to the well-established neurotransmitter-induced inhibition of Ca^{2+} channels through a G-protein-coupled mechanism (Carbone & Swandulla, 1989; Hille, 1994). Thus, facilitation of Ca^{2+} channels by prepulses might simply reflect the removal of a tonic autoinhibition of the current, resulting from the activation of autoreceptors by materials released from chromaffin vesicles. This release of materials is caused by depolarizing pulses applied to the cell to elicit and measure the currents flowing through Ca^{2+} channels. In fact, various of the soluble components of chromaffin vesicles (Winkler, Sietzen & Schober, 1987) such as protons (Callewaert, Johnson & Morad, 1991), ATP (Gandía, García & Morad, 1993*b*) and opiates (Albillos, Carbone, Gandía, García & Pollo, 1995, 1996) inhibit the whole-cell currents through Ca^{2+} channels in bovine chromaffin cells.

Indirect evidence for this autocrine inhibitory loop modulating Ca^{2+} channels in bovine chromaffin cells has been provided by the recent flow-stop experiments of Doupnik & Pun (1994). In the present study we followed a more direct approach and show that low molecular mass materials present in a soluble vesicle lysate (SVL), obtained from an extract of bovine adrenal medulla, caused a strong voltage-dependent inhibition of whole-cell currents mainly through non-L-type Ca^{2+} channels of bovine chromaffin cells. In addition, in this paper we performed flow-stop experiments, similar to those reported by Doupnik & Pun (1994), by analysing the voltage dependence of autocrine modulation and its sensitivity to DHPs. The present data are incompatible with previous views that implicated a selective recruitment of DHP-sensitive Ca^{2+} channels as the mechanism for the facilitation of Ca^{2+} channel currents by depolarizing prepulses.

METHODS

Bovine adrenal medullary chromaffin cells were isolated from the adrenal glands of young calves, obtained from a local abattoir, and maintained in culture as previously described (Moro, López, Gandía, Michelena & García, 1990). Experiments were performed on cells in culture for 1–3 days at room temperature (22–25 °C). The Ba^{2+} current (I_{Ba}) was recorded using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Current recordings were made with fire-polished electrodes (resistance 2–5 MΩ) mounted on the headstage of a DAGAN 8900 patch-clamp amplifier, allowing cancellation of capacitative transient and compensation of series resistance. Cells were voltage clamped at a holding potential (V_h) of −70 mV. Unless otherwise indicated, two different protocols were used. The double-pulse protocol (Fig. 1*E* inset) consisted of a first control pulse of 30 ms to 0 or +10 mV from V_h, followed by a second pulse to the same potential preceded by a conditioning pulse of 45 ms to +90 mV (trace 2 in Fig. 1*E*). The single-pulse protocol was based on 50 ms step depolarizations to 0 or +10 mV from V_h, repeated every 10 s. Stimulation and data acquisition were performed using a Labmaster board and pCLAMP software (Axon Instruments). Off-line data analysis and curve fittings were made using AUTES P programs (Garching Instruments, Munich, Germany) (Pollo, Lovallo, Sher & Carbone, 1992). Capacitative transients and leakage currents were minimized by a preliminary electronic compensation and by subtracting Cd^{2+}-insensitive currents off-line (Carbone, Sher & Clementi, 1990). The external solution contained (mM): 10 $BaCl_2$, 155 NaCl, 1 $MgCl_2$, 10 Hepes–NaOH, and 0·002 tetrodotoxin. Cells were internally dialysed with a standard intracellular solution containing (mM): 10 NaCl, 100 CsCl, 20 TEACl, 5 MgATP, 14 EGTA, 0·3 NaGTP, 20 Hepes–CsOH, pH 7·2. Solutions were exchanged using electronically driven miniature solenoid valves coupled to a multibarrelled concentration-clamp device, the common outlet of which was placed within 100 μm of the cell to be patched. The flow rate was low (<1 ml min^{-1}) and regulated by gravity to achieve complete replacement of the cell surroundings within less than 1 s.

In one set of experiments, currents through nicotinic receptor channels, Na^+ channels and Ca^{2+} channels were recorded sequentially using the whole-cell configuration of the patch-clamp technique. Nicotinic currents were elicited by fast application of 100 μM dimethylphenylpiperazinium (DMPP; 250 ms). External solution contained (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$ (or 10 $BaCl_2$), 11 glucose, 10 Hepes–NaOH, pH 7·4. The holding potential was −80 mV and electrode resistances ranged from 2 to 5 MΩ.

To prepare a soluble vesicle lysate (SVL), chromaffin vesicles were isolated from ten adrenal medullae of ten bovine adrenal glands (Smith & Winkler, 1967). The pellet was resuspended in a 10 mM based Tris solution to release the SVL. After centrifugation of this lysate at 27 000 *g* for 20 min, the supernatant (SVL) was aliquoted and kept frozen at −70 °C until used. Protein contents of SVL were estimated according to Bradford (1976) and ATP content was estimated by the method of Ogilvie (1985). Adrenaline and noradrenaline contents were electrochemically estimated after their separation using HPLC techniques (Borges, Sala & García, 1986).

Pyridoxal-phosphate-6-azophenyl-2′,4′disulphonic acid tetrasodium (PPADS), methionine enkephalin and suramin were purchased from RBI. DMPP was purchased from Sigma.

Results are given as means ± S.E.M. with n the number of cells given in parenthesis. Differences between means were analysed by Student's t test, taking $P < 0.05$ as the limit of statistical significance.

RESULTS

Characteristics of the soluble vesicle lysate

The initial experiments were designed to test whether the soluble contents of purified chromaffin vesicles (SVL) affected the size and time course of I_{Ba} of bovine chromaffin cells. Protein content of SVL was 9·27 mg ml^{-1}. The ATP concentration was 3·18 ± 0·12 mM ($n = 4$). The concentration of noradrenaline and adrenaline in the SVL was 11·2 ± 0·97 and 15·2 ± 2 mM, respectively ($n = 5$). Thus, a 1:1000 dilution of the lysate in the external solution gave final concentrations of 11·2 μM for noradrenaline, 15·2 μM for adrenaline and 3 μM for ATP. According to Viveros, Diliberto, Hazum & Chang (1979), the molar ratio of catecholamine to opiate-like materials (OLM) in the bovine large granular fraction of the adrenal medulla is 283–817:1. Since the concentration of catecholamines within the chromaffin vesicle is considered to be close to 0·5 M, the concentration of OLM (if all were equipotent with methionine enkephalin) would be between 0·6 and 1·8 mM. Thus, we calculated a concentration of 34–101 μM of OLM in our SVL, given that its concentration of total catecholamines was 28·2 mM.

Effects of SVL on Ba^{2+} currents

I_{Ba} were evoked by test depolarizations to 0 mV in voltage clamped chromaffin cells from a V_h of −70 mV (see protocol in Fig. 1*B* inset). Test pulses were repeatedly applied at 10 s intervals, to produce current traces whose magnitude was stable for at least 15–30 min. In thirty-nine cells (1–3 days in culture), the currents peaked between 0 and +10 mV in 10 mM Ba^{2+} (700 ± 57 pA; mean ± S.E.M.)

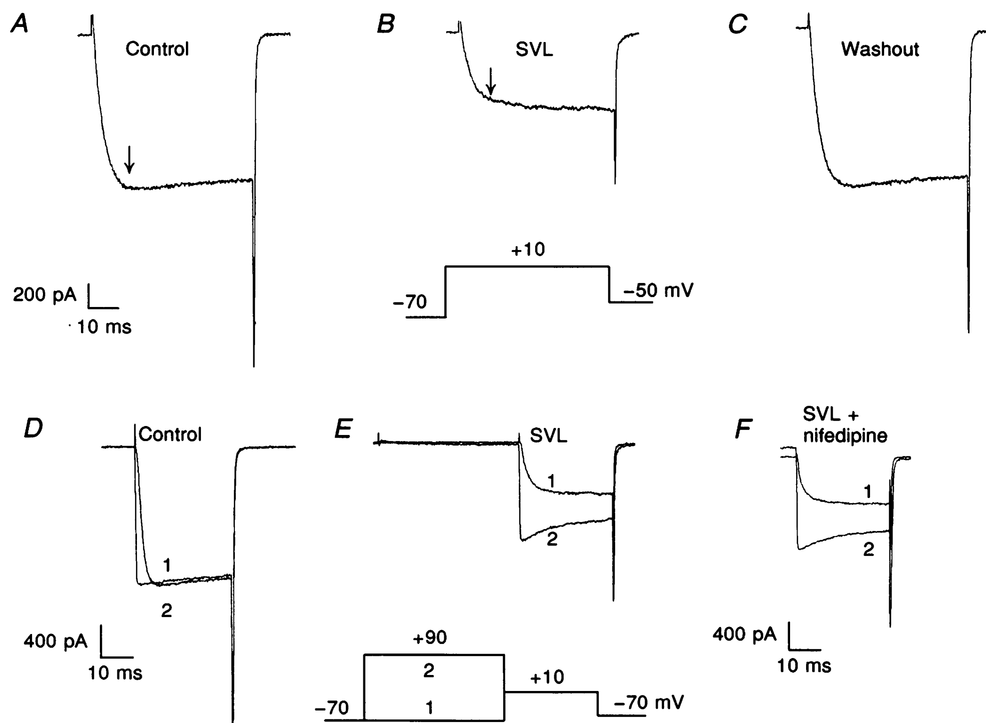

Figure. 1. Effects of SVL on I_{Ba} in voltage-clamped chromaffin cells

A pulse of 50 ms to 10 mV from a V_h of −70 mV was used to depolarize the cells. Top panels show I_{Ba} recorded before (*A*), during (*B*), and after (*C*) the perfusion of a 1:1000 dilution of SVL. The vesicle lysate depression of I_{Ba} was accompanied by a dramatic slow down of activation. The percentage of SVL inhibition was maximal at the peak of control currents (arrows in *A* and *B*) and decreased gradually towards the end of the 50 ms pulse. Voltage-dependent recovery from SVL inhibition was proven using the double-pulse protocol illustrated in the inset of panel *E* (Grassi & Lux, 1989). In control conditions (*D*), pulses of 30 ms to +10 mV with (trace 2) and without (trace 1) a prepulse of 45 ms to +90 mV produced similar current amplitudes and inactivation rates. On the contrary, SVL-modified currents, which showed a pronounced slowing down in the absence of prepulse (*E*, trace 1), became larger and fast decaying after the conditioning pulse (*E*, trace 2). This recruitment of the inhibited currents with voltage (facilitation) disappeared after washing out SVL (not shown). In a different cell treated with nifedipine (3 μM) and SVL (1:100 dilution), the facilitation of I_{Ba} by the prepulse (*F*) was similar to that seen in the absence of nifedipine (not shown).

Figure 1*A–C* shows the reversible effects of applying a 1:1000 dilution of SVL to a normally superfused cell. There were two clear effects of SVL on I_{Ba}: reduction to nearly half of the magnitude of the current and a marked slowing of its activation kinetics. In nine cells the activation of the control current was well fitted with single exponential functions, with a mean time constant of 1·9 ± 0·16 ms (τ_{fast}). In the presence of SVL, the activation phase was best fitted by a double exponential, with τ_{fast} of 1·55 ± 0·3 ms and τ_{slow} of 14 ± 3 ms ($n = 9$). Inhibition of I_{Ba} by SVL was dose dependent. I_{Ba} was depressed by: 27 ± 1·5% ($n = 5$) with a 1:10000 dilution of SVL; 38 ± 3·3% ($n = 9$) with a 1:1000 dilution; and 46 ± 3·8% ($n = 13$) with a 1:100 dilution. Inhibition of I_{Ba} by SVL was fully reversible; 20 s after the washout of the 1:1000 dilution, I_{Ba} completely recovered its fast activation and its initial magnitude (Fig. 1*C*).

Inhibition and slowing of the kinetics of I_{Ba} by SVL could be partially reversed by facilitatory depolarizing prepulses (Fig. 1*D* and *E*). Test depolarizations to +10 mV, preceded by a prepulse of 45 ms to + 90 mV, caused no changes to control currents (Fig. 1*D*, traces 1 and 2) but induced a marked increase (facilitation) of the SVL inhibited current (Fig. 1*E*, trace 2). The current in the presence of SVL without prepulse (Fig. 1*E*, trace 1) was smaller and slower compared with control (Fig. 1*D*, trace 1). The percentage of facilitation amounted to 53 ± 5% with respect to the total inhibition. Another component of the inhibition was not recruited by voltage, and represented 47 ± 5% of the total inhibition ($n = 11$). These results are in good agreement with previous data obtained on modulation by opioids of Ca^{2+} currents in bovine chromaffin cells. Here, the voltage-dependent (facilitation) and voltage-independent components of the inhibition by 10 μM methionine enkephalin were 56 and 44%, respectively (Albillos *et al.* 1995, 1996). Twenty seconds after washout of SVL, the test current recovered its original kinetics and magnitude and prepulse facilitation disappeared (not shown).

We also studied the effect of a 1:100 dilution of SVL in the presence of the dihydropyridine (DHP) nifedipine, a selective blocker of L-type Ca^{2+} channels. Figure 1*F* shows the original I_{Ba} induced by depolarizing prepulses in the presence of 3 μM nifedipine. In this cell, the percentage of I_{Ba} facilitation in the presence of SVL (1:100 dilution) was similar before (not shown) and during nifedipine application (about 60%). In four cells, the facilitation before nifedipine was 55% and in the presence of nifedipine it was 49 ± 4%.

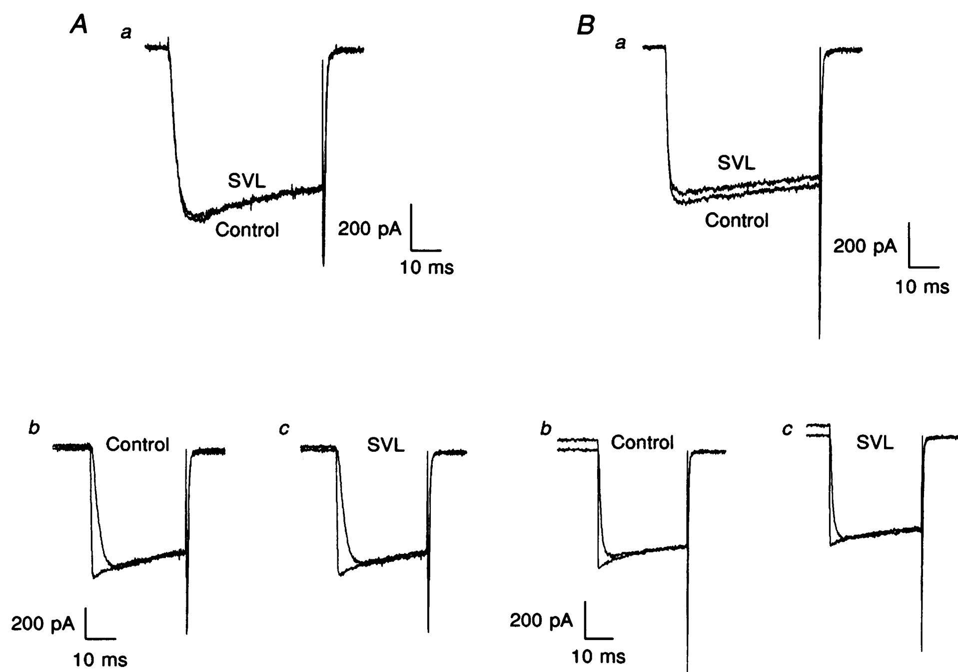

Figure 2. Inhibition of I_{Ba} by SVL is mediated by G-proteins

The protocols for panels *Aa* and *Ba* are the same as those for Fig. 1*B*. The double-pulse protocols of panels *Ab* and *c*, and *Bb* and *c*, are the same as those for Fig. 1*E*. *A* shows the lack of effect of SVL (dilution 1:100) on I_{Ba} after 15 min dialysis with 0·5 mM GDP-β-S in the pipette. The control trace was recorded 15 min after the establishment of whole-cell recording conditions. Double-pulse protocols before (*Ab*) and during (*Ac*) application of SVL on the same cell as in *Aa* showed no evidence of current facilitation. *B*, when chromaffin cells were treated for 18–24 h with pertussis toxin (100 ng ml^{-1}), SVL inhibition was largely prevented although some residual inhibition by the SVL (dilution 1:100) was preserved (*Ba*). However, the prepulse-induced facilitation completely disappeared (*Bc*). Thus, the voltage-dependent inhibition of I_{Ba} by SVL is mostly mediated by pertussis toxin-sensitive G-proteins.

Effects of GDP-β-S and pertussis toxin on the modulation of I_{Ba} by SVL

The voltage dependency of SVL action is similar to the inhibitory effects of several neurotransmitters on neuronal Ca^{2+} channels (Dunlap & Fischbach, 1978; see Carbone & Swandulla, 1989, for a review). As these effects are mediated by G-proteins, it was interesting to test whether the SVL effects were also mediated by these molecules. Figure 2 shows that this was indeed the case. Intracellular application of 0·5 mM GDP-β-S abolished the effects of a 1:100 dilution of SVL on the size and the activation kinetics of I_{Ba} (Fig. 2*Aa*). In six cells, the average inhibition of peak I_{Ba} was $2 \pm 1{\cdot}4$%. Also, pretreatment of the cells with pertussis toxin (100 ng ml^{-1} for 18–24 h) drastically reduced the modulatory effects of SVL on I_{Ba} (Fig. 2*Ba*). Thus, a 1:100 dilution of SVL reduced I_{Ba} by only $12 \pm 1{\cdot}8$% in pertussis toxin-treated cells ($n = 4$). GDP-β-S (Fig. 2*Ab* and *c*) or pertussis toxin treatment (Fig. 2*Bb* and *c*) were also very effective in preventing prepulse-induced facilitation of I_{Ba}.

Comparison of the effects of SVL on I_{Ba} with those of ATP and opiates

ATP and opioids have been shown to cause kinetic slowing and Ca^{2+} channel depression in bovine chromaffin cells similar to those reported here for SVL (Kleppisch *et al.* 1992; Twitchell & Rane, 1993; Gandía *et al.* 1993*b*; Albillos *et al.* 1995, 1996). It was, therefore, interesting to compare the effects of SVL, ATP, opiates and adrenaline on I_{Ba}. Figure 3 shows the results of such comparison. The current modifications induced by SVL products (Fig. 3*A*) were indeed mimicked by either 10 μM methionine enkephalin (Fig. 3*B*) or by 10 μM ATP (Fig. 3*C*). Adrenaline caused mainly a reversible depression of I_{Ba} with no changes to the current activation kinetics (Fig. 3*D*); inhibition amounted to 13 ± 3% ($n = 4$). The effects of adrenaline were prevented by 1 μM propranolol.

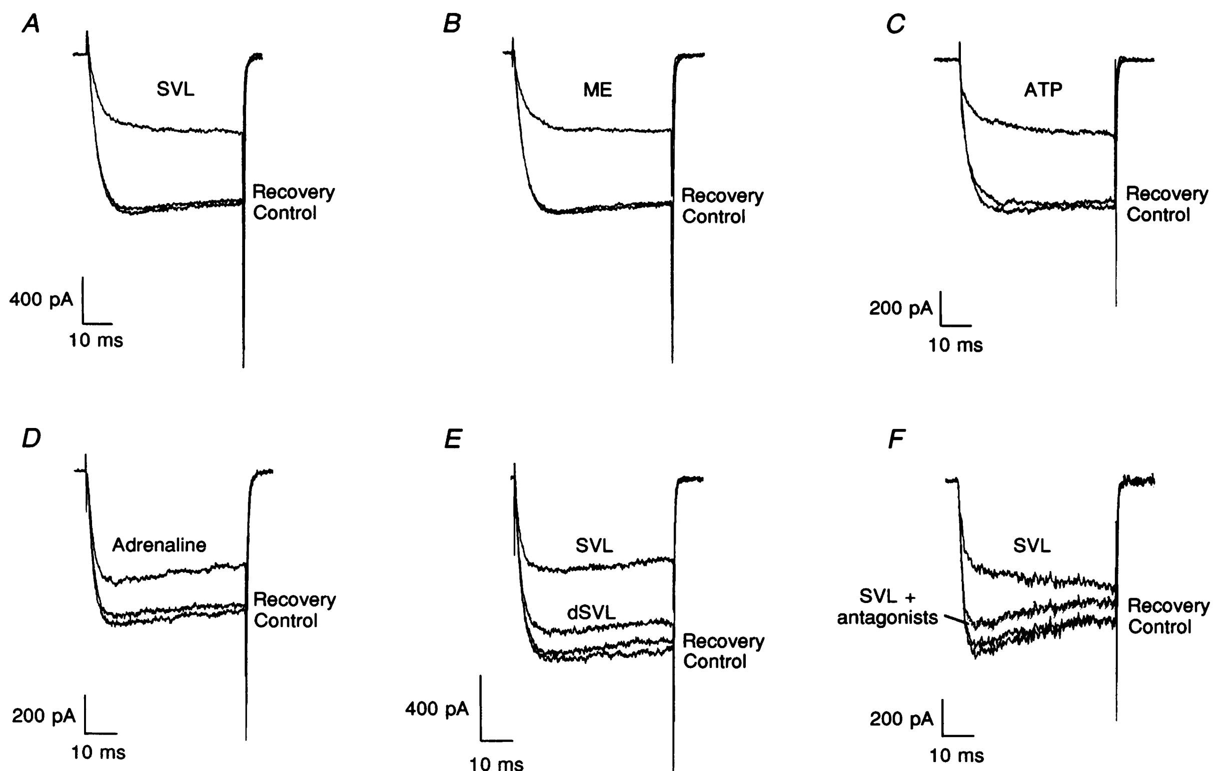

Figure 3. I_{Ba} inhibition by SVL is mainly due to the ATP and opiates contained in the vesicles

SVL (*A*) modified I_{Ba} in the same way as methionine enkephalin (ME, *B*) and ATP (*C*), but in a different way from adrenaline (*D*). Ba^{2+} currents were elicited by step depolarizations from −70 to +10 mV in control conditions, during application of either SVL (dilution 1:1000), methionine enkephalin (10 μM) or ATP (10 μM) and after washing out each compound (recovery). Traces in *A* and *B* are from the same cell. Adrenaline was tested on a different cell and did not modify the kinetics of control currents, though some decrease of I_{Ba} amplitude was apparent. *E*, dialysed SVL caused a less pronounced inhibition of I_{Ba}. The control current was depressed by 49% during perfusion of intact SVL (dilution 1:100; trace SVL), but only by 11% with dialysed SVL (trace dSVL). The current recovery was complete after washout. *F*, purinergic and opioid receptor antagonists (SVL + antagonists) prevented the slowing down and part of the amplitude depresssion induced by SVL (1:3000 dilution). The mixture of antagonists contained: naloxone (10 μM), suramin (100 μM) and PPADS (100 μM). The sequence of the recording was as follows: control (1); SVL (2); recovery (3), and SVL + antagonists (4). Test depolarization was to +10 mV from −70 mV.

Additional information on the molecular constituents responsible for the I_{Ba} modifications were obtained by manipulating the SVL contents in various ways. For instance, heating SVL at 100 °C for 15 min reduced the action of a 1:100 dilution by only 3·7 + 2% ($n = 6$). On the other hand, a 1:100 dilution of SVL pretreated with excess alkaline phosphatase blocked I_{Ba} by 25·3 ± 8% ($n = 6$). In other experiments, a 1:100 dilution of SVL (diluted in 5 ml of external solution) was dialysed against 500 ml of the external solution to elute molecules of molecular mass smaller than 10 kDa. The dialysis was performed during two periods of 2 h each; the solution in which the dialysis was performed was replaced by a new solution during the second period of dialysis. The dialysed SVL reduced I_{Ba} by only 17·5 ± 6% ($n = 4$), while the non-dialysed SVL blocked I_{Ba} by 53·5 ± 8% in the same four cells (see Fig. 3*E*). Thus, the modulatory effects of SVL are likely to derive from molecules of low molecular mass (< 10 kDa) such as ATP, catecholamines and opiates that are lost during dialysis from the initial SVL mixture. The fact that some inhibitory effect remained after dialysis suggests that a non-dialysable protein of molecular weight greater than 10 kDa had an inhibitory effect on I_{Ba}; possible candidates are the acidic proteins chromogranins, that have been shown to inhibit Ca^{2+} channels in bovine chromaffin cells (Galindo *et al.* 1992).

The involvement of ATP and opioid agonists in the autocrine modulation of Ca^{2+} channels by SVL is supported also by the observation that opiate and purinergic receptor antagonists prevented the action of SVL. External solutions containing 10 μM naloxone (a wide-spectrum blocker of opiate receptors), 100 μM suramin (a blocker of $P_{2x,y}$ purinergic receptors) and PPADS, a blocker of P_{2x} purinergic receptors) were sufficient to largely prevent the action of SVL. An example of this is shown in Fig. 3*F*. A short application of SVL (50 s at 1:3000 dilution) caused marked depression and pronounced slowing of the kinetics of the control current that was fully reversed after washing out the SVL solution. Cell superfusion with SVL and the mixture of antagonists caused, on the contrary, only a

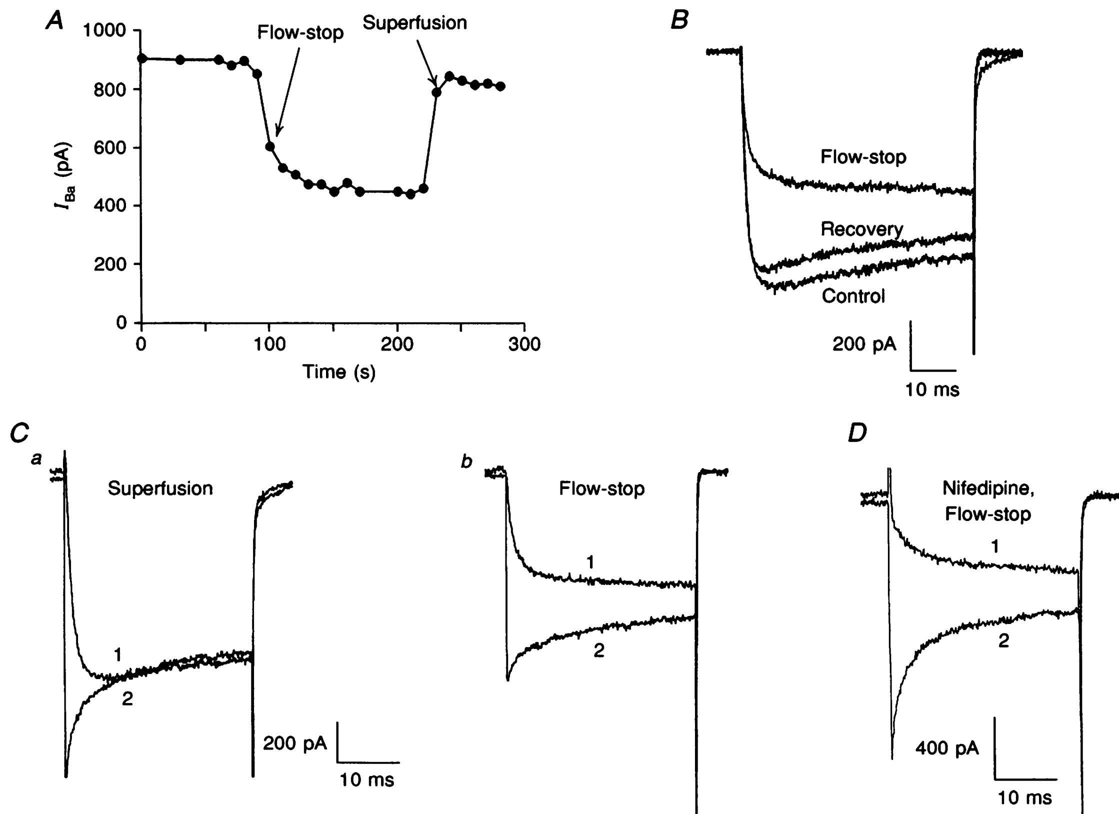

Figure 4. Flow-stop decreases I_{Ba}

Flow-stop experiments were performed with 10 mM Ba^{2+} in the bath and 10 mM Ba^{2+} in the superfusion solution. *A*, time course of I_{Ba} when stopping the flow of the superfusion system. A voltage-clamped cell was superfused with 10 mM Ba^{2+}, then the flow was stopped for 2 min and finally the cell was superfused again with the external solution. *B*, original recordings when perfusing the same cell before (Control), and after the flow-stop. Under flow condiitons (*Ca*) no facilitation of I_{Ba} was observed after giving a prepulse; in contrast, under flow-stop conditions I_{Ba} became greater and suffered rapid inactivation after the prepulse (*Cb*). In a different cell treated with 3 μM nifedipine, flow-stop revealed the same facilitation pattern (*D*). (See legend to Fig. 1 for the facilitation protocols and how traces 1 and 2 in *C* and *D* were obtained.)

partial depression and hardly any changes to the current activation kinetics. In ten cells, the initial control I_{Ba} was 909 ± 51 pA and it decreased to 453 ± 54 pA with SVL (1:3000 dilution). In the presence of antagonists, SVL reduced I_{Ba} by only $25{\cdot}1 \pm 3{\cdot}3$% ($P < 0{\cdot}005$, compared with SVL alone).

Flow-stop mimics the effects of SVL on I_{Ba}

Although it has a 100-fold less potency than Ca^{2+}, Ba^{2+} behaves as a powerful secretagogue, causing long-lasting secretory activity in single bovine adrenal chromaffin cells (Von Rüden, García & López, 1993). Therefore, we expected significant exocytosis to occur under the ionic conditions used here while recording I_{Ba} for several minutes. This implies that already in control conditions, SVL compounds are likely to be released in response to Ba^{2+} entry during repeated depolarizations; hence the degree of removal of these compounds from the immediate vicinity of surface receptors could strongly affect the shape and size of Ba^{2+} currents. To test this, I_{Ba} was recorded from single cells during periods of continuous superfusion with the external solution, alternated by periods of flow-stop. In the example of Fig. 4, the initial I_{Ba} under fast cell superfusion amounted to 903 pA. Flow-stop produced a quick reduction of I_{Ba} to 52% of its initial value (Fig. 4*A*); in eleven cells the mean blockade of I_{Ba} amounted to 42 ± 7%. What seemed most interesting was the pronounced slowing of the activation of I_{Ba} observed with flow-stop (Fig. 4*B*). This slowing of Ca^{2+} channel activation was associated with a pronounced voltage-dependent facilitation of I_{Ba} by strong depolarizing prepulses during flow-stop; the current facilitation was almost completely lost when the cell was superfused continuously (compare Fig. 4*Ca* and *b*). In eight cells, the prepulse protocol was also studied in the presence of nifedipine (3 μM). As for the SVL action (Fig. 1*F*), the facilitation of I_{Ba} under flow-stop conditions was preserved in the presence of nifedipine (see in Fig. 4*D* the traces obtained in a cell treated with nifedipine).

SVL modifies I_{Ca} and I_{Ba}, but leaves I_{Na} and I_{DMPP} unaffected

Finally, to investigate whether the SVL modulatory effects were selective for Ca^{2+} channels, SVL was tested on three different ionic currents in the same cell. Transient currents through the nicotinic acetylcholine receptor (I_{DMPP}) were elicited by applying brief (250 ms) pulses of DMPP (100 μM) to cells held at −80 mV. A 30 ms depolarizing pulse to +10 mV was applied 2 s later, to elicit both voltage-dependent Na^+ (I_{Na}) and Ca^{2+} (I_{Ca}) currents. Superfusion of the cell with SVL (1:3000) led to a 45% blockade of I_{Ca} without significantly altering either I_{DMPP} or I_{Na}. The same occurred when Ca^{2+} was replaced with Ba^{2+}, despite the fact that the size of I_{DMPP} in 10 mM Ba^{2+} was drastically decreased and I_{Ba} nearly doubled (Fig. 5).

DISCUSSION

Chromaffin cells from the bovine adrenal medulla possess three main features that make them suitable for testing the hypothesis that an autocrine regulatory loop for Ca^{2+} channels is present in neurosecretory cells: (i) its catechol-amine storing vesicles contain a mixture of opiates, nucleotides and various peptides which are co-released with adrenaline and noradrenaline during cell activity (Winkler

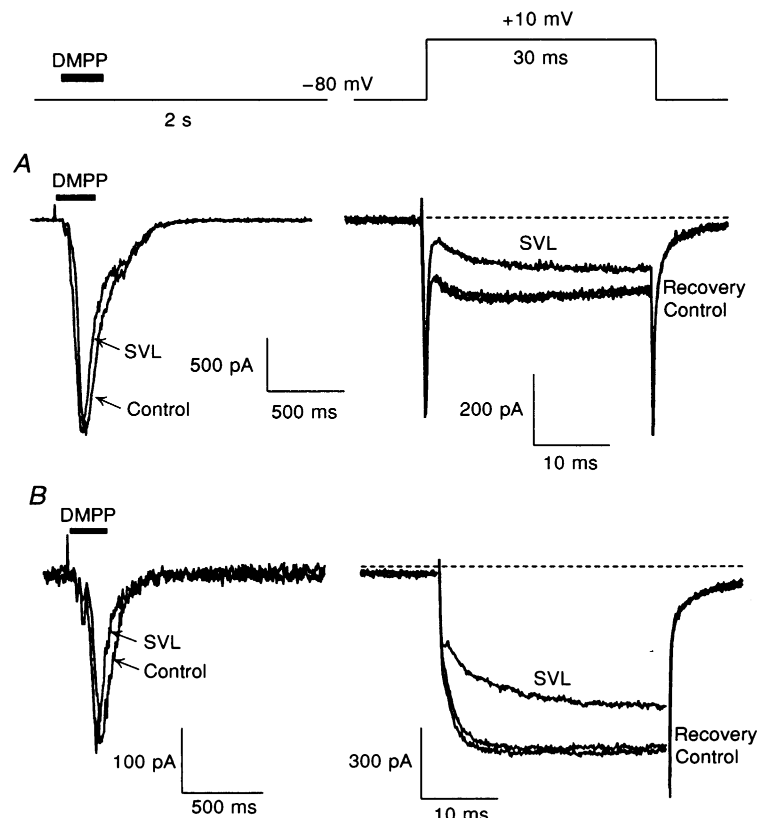

Figure 5. SVL blocks I_{Ba} but not Na^+ or nicotinic receptor currents

Experiments showing the selectivity of a 1:3000 dilution of SVL in blocking the voltage-dependent Ca^{2+} current (*A*) or the Ba^{2+} current (*B*), but not the voltage-dependent Na^+ current or the nicotinic receptor current (I_{DMPP}). A voltage-clamped chromaffin cell (holding potential, −80 mV) was subjected to the stimulation protocol shown above panel *A*. First, a pulse of DMPP (100 μM, 250 ms) was applied; 2 s later a depolarizing pulse to +10 mV, of 50 ms duration was given. This alternating pattern of chemical and electrical stimulation was repeated at 30 s intervals, continuously in every cell studied. In *A*, currents were elicited in the presence of 2 mM Ca^{2+} and in *B*, in 10 mM Ba^{2+} (see Methods for the composition of the external solutions). SVL, current seen after 60 s of superfusion of the cell with a 1:3000 dilution of SVL; Recovery, 60 s after washout of SVL.

et al. 1987); (ii) chromaffin vesicles can be isolated in large quantities and in highly purified form, from bovine adrenal medulla homogenates (Smith & Winkler, 1967); and (iii) bovine chromaffin cells contain an array of well-characterized voltage-dependent Ca^{2+} channels: the L-type (García *et al.* 1984; Artalejo *et al.* 1991), N-type (Ballesta *et al.* 1989; Hans, Illes & Takeda, 1990; Artalejo, Perlman & Fox, 1992), P-type (Gandía, Albillos & García, 1993*a*; Albillos, García & Gandía, 1993; Artalejo *et al.* 1994), and Q-type (López *et al.* 1994), contributing in different degrees to exocytosis.

Ever since Fenwick *et al.* (1982) showed that Ca^{2+} currents in bovine chromaffin cells can be facilitated by strong depolarizing prepulses, various attempts have been made to reveal the underlying mechanism. Either more Ca^{2+} entry through the same channel (Hoshi *et al.* 1984; Hoshi & Smith, 1987), selective recruitment of otherwise silent L-type channels (Artalejo *et al.* 1990, 1991, 1994) or removal of tonic inhibition on Ca^{2+} channels (Callewaert *et al.* 1991; Gandía *et al.* 1993*b*; Albillos *et al.* 1994; Doupnik & Pun, 1994) have been proposed. Our present data clarify the conflicting views held during the last 13 years. We have observed that the time course and size of I_{Ba} in bovine chromaffin cells depend critically on the cell superfusion conditions and are likely to derive from the G-protein-mediated coupling between ATP- or opioid-autoreceptors and the high-threshold Ca^{2+} channels. Cell activity under flow-stop conditions (unperfused cell) favours the local rise of secreted products outside the plasmalemma, the subsequent activation of membrane autoreceptors and the rapid inhibition of spatially localized Ca^{2+} channels. This tonic inhibition, induced by low molecular mass SVL compounds released during cell stimulation, is minimal under cell superfusion and can be partially reversed (facilitated) by strong depolarizations. Under these conditions, I_{Ba} accelerates and acquires its fast activation time course and maximal amplitude. Superfusion and flow-stop conditions may thus account for the extreme variability of the time course and voltage-dependent facilitation of I_{Ba} reported, either using static incubation systems (Fenwick *et al.* 1982; Hoshi *et al.* 1984; Hoshi & Smith, 1987; Artalejo *et al.* 1991, 1992, 1994) or different superfusion systems (Callewaert *et al.* 1991; Gandía *et al.* 1993*b*; Doupnik & Pun, 1994; Albillos *et al.* 1994, 1995).

Our findings are in good agreement with the flow-stop experiments of Doupnik & Pun (1994) in bovine chromaffin cells that showed a marked reduction of I_{Ba} with respect to superfused cells and we interpreted this finding as feedback inhibition of Ca^{2+} channels by released material. Support for this idea comes also from the similar effects that protons (Callewaert *et al.* 1991), ATP (Gandía *et al.* 1993*b*; Currie & Fox, 1995) and opioids (Albillos *et al.* 1996) are shown to induce on I_{Ba} of the same cell type. The fact that modulation of Ca^{2+} channels by SVL opioids and ATP is clearly seen under different experimental conditions, suggests that protons might play a secondary role in such modulation. Otherwise, protons should have blunted the effects of opioids and ATP, as well as those of their antagonists for their receptors.

An interesting finding of our work concerns the role that non-L-type channels play in the overall voltage-dependent mechanism of autocrine inhibition by secreted products. We showed clearly that I_{Ba} facilitation during SVL application (Fig. 1*F*) or flow-stop conditions (Fig. 4*D*) is preserved in the presence of DHP antagonists, making previously held views that L-type channels are primarily responsible for the 'facilitation current' of bovine chromaffin cells (Artalejo *et al.* 1990) unlikely to be correct.

In conclusion, to our knowledge, the experiments reported here constitute the most direct evidence yet provided for the presence of a voltage-dependent autocrine loop capable of regulating neuronal high-threshold Ca^{2+} channels and hence, neurotransmitter release. In chromaffin cells and sympathetic neurons, such a regulatory mechanism might involve G-protein-coupled receptors for at least opiates and ATP. This mechanism could constitute the basis for the regulation of transmitter release by presynaptic autoreceptors present on sympathetic nerve terminals and in many other synapses (Kirpekar & Puig, 1971; Langer, 1980; Lipscombe, Kongsamut & Tsien, 1989).

Albillos, A., Artalejo, A. R., López, M. G., Gandía, L., García, A. G. & Carbone, E. (1994). Calcium channel subtypes in cat chromaffin cells. *Journal of Physiology* **477**, 197–213.

Albillos, A., Carbone, E., Gandía, L., García, A. G. & Pollo, A. (1995). Selective voltage-dependent and voltage-independent inhibition by opioids in bovine chromaffin cells. *Society for Neuroscience Abstracts* **21**, 1575.

Albillos, A., Carbone, E., Gandía, L., García, A. G. & Pollo, A. (1996). Opioids inhibition of Ca^{2+} channel subtypes in bovine chromaffin cells: selectivity of action and voltage-dependence. *European Journal of Neuroscience* (in the Press).

Albillos, A., García, A. G. & Gandía, L. (1993). ω-Agatoxin-IVA-sensitive calcium channels in bovine chromaffin cells. *FEBS Letters* **336**, 259–262.

Artalejo, C. R., Adams, M. E. & Fox, A. P. (1994). Three types of Ca^{2+} channels trigger secretion with different efficacies in chromaffin cells. *Nature* **367**, 72–76.

Artalejo, C. R., Ariano, M. A., Perlman, R. L. & Fox, A. P. (1990). D_1 dopamine receptors activate facilitation calcium channels in chromaffin cells via a cAMP/protein kinase A mechanism. *Nature* **348**, 239–242.

Artalejo, C. R., Dahmer, M. K., Perlman, R. L. & Fox, A. P. (1991). Two types of Ca^{2+} currents are found in bovine chromaffin cells: facilitation is due to the recruitment of one type. *Journal of Physiology* **432**, 681–707.

Artalejo, C. R., Perlman, R. L. & Fox, A. P. (1992). ω-Conotoxin GVIA blocks a Ca^{2+} current in chromaffin cells that is not of the 'classic' N type. *Neuron* **8**, 85–95.

BALLESTA, J. J., PALMERO, M., HIDALGO, M. J., GUTIÉRREZ, L. M., REIG, J. A., VINIEGRA, S. & GARCÍA, A. G. (1989). Separate binding and functional sites for ω-conotoxin and nitrendipine suggest two types of calcium channels in bovine chromaffin cells. *Journal of Neurochemistry* **53**, 1050–1056.

BORGES, R., SALA, F. & GARCÍA, A. G. (1986). Continuous monitoring of catecholamine release from perfused cat adrenals. *Journal of Neuroscience Methods* **16**, 289–300.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

CALLEWAERT, G., JOHNSON, R. G. & MORAD, M. (1991). Regulation of the secretory response in bovine chromaffin cells. *American Journal of Physiology* **260**, C851–860.

CARBONE, E., SHER, E. & CLEMENTI, F. (1990). Ca currents in human neuroblastoma IMR32 cells: kinetics, permeability and pharmacology. *Pflügers Archiv* **416**, 170–179.

CARBONE, E. & SWANDULLA, D. (1989). Neuronal calcium channels: kinetics, blockade and modulation. *Progress in Biophysics and Molecular Biology* **54**, 31–58.

CURRIE, K. P. M. & FOX, A. P. (1995). Voltage dependent inhibition of N- and P-type calcium channel currents in cultured bovine adrenal chromaffin cells. *Society for Neuroscience Abstracts* **21**, 515.

DOUPNIK, C. A. & PUN, R. Y. K. (1994). G-protein activation mediates prepulse facilitation of Ca^{2+} channel currents in bovine chromaffin cells. *Journal of Membrane Biology* **140**, 47–56.

DUNLAP, K. & FISCHBACH, G. D. (1978). Neurotransmitters decrease the calcium component of sensory neurone action potentials. *Nature* **276**, 837–838.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982). Sodium and calcium channels in bovine chromaffin cells. *Journal of Physiology* **331**, 599–635.

GALINDO, E., MÉNDEZ, M., CALVO, S., GONZÁLEZ-GARCÍA, C., CEÑA, V., HUBERT, P., BADER, M.-F. & AUNIS, D. (1992). Chromostatin receptors control calcium channel activity in adrenal chromaffin cells. *Journal of Biological Chemistry* **267**, 407–412.

GANDÍA, L., ALBILLOS, A. & GARCÍA, A. G. (1993*a*). Bovine chromaffin cells possess FTX-sensitive calcium channels. *Biochemical and Biophysical Research Communications* **194**, 671–676.

GANDÍA, L., GARCÍA, A. G. & MORAD, M. (1993*b*). ATP modulation of calcium channels in chromaffin cells. *Journal of Physiology* **470**, 55–72.

GARCÍA, A. G., SALA, F., REIG, J. A., VINIEGRA, S., FRÍAS, J., FONTERÍZ, R. & GANDÍA, L. (1984). Dihydropyridine Bay K 8644 activates chromaffin cell calcium channels. *Nature* **308**, 69–71.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HANS, M., ILLES, P. & TAKEDA, K. (1990). The blocking effects of ω-conotoxin on Ca current in bovine chromaffin cells. *Neuroscience Letters* **114**, 63–68.

HILLE, B. (1994). Modulation of ion-channel function by G-protein-coupled receptor. *Trends in Neurosciences* **17**, 531–536.

HOSHI, T., ROTHLEIN, J. & SMITH, S. J. (1984). Facilitation of Ca^{2+} channel currents in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the USA* **81**, 5871–5875.

HOSHI, T. & SMITH, S. J. (1987). Large depolarization induces long openings of voltage-dependent calcium channels in adrenal chromaffin cells. *Journal of Neuroscience* **7**, 571–580.

KIRPEKAR, S. M. & PUIG, M. (1971). Effects of flow-stop on noradrenaline release from normal spleens and spleens treated with cocaine, phentolamine or phenoxybenzamine. *British Journal of Pharmacology* **43**, 359–369.

KLEPPISCH, T., AHNERT-HILGER, G., GOLLASCH, M., SPICHER, K., HESCHELER, J., SCHULTZ, G. & ROSENTHAL, W. (1992). Inhibition of voltage-dependent Ca^{2+} channels via α_2-adrenergic and opioid receptors in cultured bovine adrenal chromaffin cells. *Pflügers Archiv* **421**, 131–137.

LANGER, S. Z. (1980). Presynaptic regulation of the release of catecholamines. *Pharmacological Reviews* **32**, 337–362.

LIPSCOMBE, D., KONGSAMUT, S. & TSIEN, R. W. (1989). α-adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type Ca^{2+}-channel gating. *Nature* **340**, 639–642.

LÓPEZ, M. G., VILLARROYA, M., LARA, B., MARTÍNEZ-SIERRA, R., ALBILLOS, A., GARCÍA, A. G. & GANDÍA, L. (1994). Q- and L-type Ca^{2+} channels dominate the control of secretion in bovine chromaffin cells. *FEBS Letters* **349**, 331–337.

MORO, M. A., LÓPEZ, M. G., GANDÍA, L., MICHELENA, P. & GARCÍA, A. G. (1990). Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. *Analytical Biochemistry* **185**, 243–248.

OGILVIE, A. (1985). Diadenosine tetraphosphate (Ap_4A). In *Methods of Enzymatic Analysis*, vol. VII, 3rd edn, ed. BERGMEYER, H. U., BERGMEYER, I. & GRASSE, M., pp. 332–339. VCH, Weinheim, Germany.

POLLO, A., LOVALLO, M., SHER, E. & CARBONE, E. (1992). Voltage-dependent noradrenergic modulation of ω-conotoxin-sensitive Ca^{2+} channels in human neuroblastoma IMR32 cells. *Pflügers Archiv* **422**, 75–83.

SMITH, A. D. & WINKLER, H. (1967). A simple method for the isolation of adrenal chromaffin granules on a large scale. *Biochemical Journal* **103**, 480–482.

TWITCHELL, W. A. & RANE, S. G. (1993). Opioid peptide modulation of Ca^{2+}-dependent K^+ and voltage-activated Ca^{2+} currents in bovine adrenal chromaffin cells. *Neuron* **10**, 701–709.

VIVEROS, O. H., DILIBERTO, E. J., HAZUM, E. & CHANG, K. J. (1979). Opiate-like materials in the adrenal medulla: evidence for storage and secretion with catecholamines. *Molecular Pharmacology* **16**, 1101–1108.

VON RÜDEN, L., GARCÍA, A. G. & LÓPEZ, M. G. (1993). The mechanism of Ba^{2+}-induced exocytosis from single chromaffin cells. *FEBS Letters* **336**, 48–52.

WINKLER, H., SIETZEN, M. & SCHOBER, M. (1987). The life cycle of catecholamine-storing vesicles. *Annals of the New York Academy of Sciences* **493**, 3–19.

Acknowledgements

This work has been supported by European Science Fund travel grant No. 125 to E.C. and A.G.G. Also grants from Fundación Ramón Areces, and Dirección General de Investigación Científica y Tècnica (No. PB94-0150), Spain. We thank M.-A. Günther-Sillero and A. Sillero for helpful discussions and suggestions. We also thank Mrs Carmen Molinos for typing this manuscript.

Received 4 January 1996; accepted 1 April 1996.