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Different types of calcium channels and secretion from bovine chromaffin cells

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Abstract

Bovine chromaffin cells possess several types of Ca^{2+} channels, and influx of Ca^{2+} is known to trigger secretion. However, discrepant information about the relative importance of the individual subtypes in secretion has been reported. We used whole-cell patch-clamp measurements in isolated cells in culture combined with fura-2 microfluorimetry and pharmacological manipulation to determine the dependence of secretion on different types of Ca^{2+} channels. We stimulated cells with relatively long depolarizing voltage-clamp pulses in a medium containing 60 mM $CaCl_2$. We found that, within a certain range of pulse parameters, secretion as measured by membrane capacitance changes was mainly determined by the total cumulative charge of Ca^{2+} inflow and the basal $[Ca^{2+}]$ level preceding a stimulus. Blocking or reducing the contribution of specific types of Ca^{2+} channels using either 20 μM nifedipine plus 10 μM nimodipine or 1 μM ωCTxGVIA (omega-conotoxin GVIA) or 2 μM ωCTxMVIIC (omega-conotoxin MVIIC) reduced secretion in proportion to Ca^{2+} charge, irrespective of the toxin used. We conclude that for long-duration stimuli, which release a large fraction of the readily releasable pool of vesicles, it is not so important through which type of channels Ca^{2+} enters the cell. Release is determined by the total amount of Ca^{2+} entering and by the filling state of the readily releasable pool, which depends on basal $[Ca^{2+}]$ before the stimulus. This result does not preclude that other stimulation patterns may lead to responses in which subtype specificity of Ca^{2+} channels matters.

Introduction

Details regarding the mechanisms of Ca^{2+}-dependent exocytosis are being studied with increasing precision. One of the important questions is the significance of the fact that several subtypes of Ca^{2+} channels exist in many secretory cells. It has been known for a long time that a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) near the plasma membrane due to Ca^{2+} influx through the membrane Ca^{2+} channels is the main mechanism inducing exocytosis (Katz, 1969; Burgoyne, 1995; see Neher, 1998 for review). However, a great variation seems to exist with respect to which types of Ca^{2+} channels are expressed in secretory cells and to what degree they participate in eliciting secretion. It also remains to be determined if the involvement of a given subtype of Ca^{2+} channel in secretion depends on the particular stimulation pattern employed. In nerve terminals (Sitges & Chiu, 1995), lung cancer cells (Viglione *et al*., 1995) and cortical synaptosomes (Turner & Dunlap, 1995), mainly P-type channels seem to be involved in secretion. Both L- and N-type channels can participate in secretion from human neuroblastoma SH-SY5Y cells (Vaughan *et al*., 1995), whereas L-type channels are responsible for secretion from photoreceptor cells (Barnes & Hille, 1989) and some other retinal cells (Heidelberger & Matthews, 1992).

Due to several shared characteristics and a common origin with neurons, chromaffin cells have been extensively used to study the fundamental mechanisms of Ca^{2+}-dependent exocytosis (Douglas & Rubin, 1961; Neher & Marty, 1982; Neher & Augustine, 1992; Chow *et al*., 1992, 1996; Steyer *et al*., 1997). Chromaffin cells secrete catecholamines and accompanying substances (VIP, substance P, opioids) in response to activity of the splanchnic nerve. Several types of high-voltage-activated Ca^{2+} channels (N-, L-, Q- or P-types) have been identified in chromaffin cells by means of selective blockers of Ca^{2+} channels (Artalejo *et al*., 1994; Albillos *et al*., 1994, 1996b; Kitamura *et al*., 1997). However, the individual contribution of all the identified channel subtypes in secretion from chromaffin cells remains unclear. For example, involvement of Q- or L- but not N-type channels in cells from bovine origin has been shown by K^+-induced depolarizations and electrochemical detection of secretion (Lopez *et al*., 1994a,b). However, in voltage-clamped cells, Engisch & Nowycky (1996) did not find any specific differences in the secretory efficacy of Ca^{2+} currents contributed by different types of channels. Likewise, in a study on rat chromaffin cells, it was reported that both N- and L-type channels contribute to secretion in proportion 60% and 40%, respectively, which was in accordance with their overall representation (Kim *et al*., 1995). Finally, *in situ* experiments on perfused adrenal glands, in which catecholamine release from bovine chromaffin cells and acetylcholine release from splanchnic nerve terminals were electrochemically detected, showed that N-type channels are largely responsible for catecholamine release induced by nerve stimulation but not for release stimulated by exogenously applied acetylcholine. At the same time, these experiments showed that L-type channels did not play a major role in catecholamine release (O'Farrell *et al*., 1997).

Due to the existence of such discrepant information regarding the role of Ca^{2+} channel subtypes in secretion, which probably reflects differences in stimulation protocols used, we made an effort to estimate the involvement of different types of Ca^{2+} channels in Ca^{2+}-

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dependent secretion of bovine chromaffin cells under conditions as well controlled as possible. To this end, we studied cultured cells under voltage-clamp with simultaneous fluorimetric recording of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We find that, for depolarizations between 50 and 200 ms, secretion is strictly proportional to cumulative Ca^{2+} influx (represented by the Ca^{2+} current integral or charge flowing during the stimulus). We find that another important factor influencing secretion is the basal $[Ca^{2+}]_i$ level preceding the stimulus. This is consistent with recent data which show that the number of vesicles available for immediate release through a strong stimulus (the 'readily releasable pool' of vesicles) is strongly increased by long-term elevation of basal $[Ca^{2+}]_i$ (von Rüden & Neher, 1993, Neher & Zucker, 1993; Smith *et al*., 1998). Part of these findings has been published in abstract form (Lukyanetz & Neher, 1998).

Methods

Cell culture

Bovine adrenal chromaffin cells were prepared as described by Zhou & Neher (1993). In short, glands were obtained from a local abattoir, and cells were prepared by enzymatic dissociation. They were maintained in tissue culture for 2–5 days. Cells were plated on poly-L-lysine-coated glass coverslips and cultured in Medium 199, supplemented with 10% foetal calf serum, 1% bovine serum albumin and 2 mM glutamine. We did not separate adrenaline- and noradrenaline-secreting cells.

Electrophysiological measurements

Cells were voltage-clamped using the whole-cell patch-clamp technique (Hamill *et al*., 1981). Whole-cell Ca^{2+} currents were recorded at high time resolution with a computer-controlled patch-clamp amplifier EPC-9 and 'Pulse' software (HEKA Electronic, Lambrecht, Germany). The 'P/4' pulse protocol was used to subtract the linear leak and capacitance currents. We used Sylgard-coated patch pipettes with resistances in the range 2–4 MΩ. Standard bath solution contained (in mM): NaCl, 140; KCl, 2.8; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10; TEACl, 10; tetrodotoxin, 0.01; pH 7.2. In most experiments we used bath solutions with high-Ca^{2+} concentration to induce a large Ca^{2+} current by short depolarizing pulses (in mM): NaCl, 40; $CaCl_2$, 60; $MgCl_2$, 2; HEPES, 10; TEACl, 40; tetrodotoxin, 0.01; pH 7.2. The intracellular solution was designed to block K^+ currents. It contained (in mM): CsCl, 64; Cs_2SO_4, 28; MgATP, 2; $MgCl_2$, 2; EGTA, 0.5; HEPES, 10; *N*-methyl-D-glucamine, 10; GTP, 0.3; pH 7.4. The osmolarity of the solutions was adjusted to 320 mOsM with glucose. All internal solutions also contained 100 μM fura-2 (Molecular Probes, Eugene, USA). No liquid junction potential correction was applied. Channel blockers were applied just before experiments to the bath solution of dishes containing coverslips with cells. All compounds were obtained from Sigma (St. Louis, MO, USA), with the exception of ωCTxMVIIC (omega-conotoxin MVIIC, Bachem, Feinchemikalien AG, Switzerland). All experiments were performed at room temperature (21–24 °C).

Capacitance measurements

Membrane capacitance (C_m) is directly proportional to the surface area of the membrane; therefore changes in C_m reflect the progress of exocytosis. We used the method of Lindau & Neher (1988) for capacitance measurement, applying a sine wave stimulus superimposed on a DC holding potential of –70 mV in the whole-cell recording mode. A 1-kHz, 10-mV peak-to peak sine wave was generated by an ITC-16 multichannel interface (Instrutech, Port Washington, NY, USA) controlled by a MacIntosh Quadra 700 computer. The sine generation as well as occasional compensation of the major part of cell capacitance and phase sensitive detection of C_m were accomplished using a software 'Lock-in amplifier', controlled by 'Pulse' software (HEKA Electronics, Lambrecht, Germany). Differences in C_m (ΔC_m) before and after depolarizing pulses were used to infer exocytosis during the pulses (Neher & Marty, 1982; Gillis, 1995; Chow *et al*., 1996). Specifically, ΔC_m was calculated off-line as the difference between the average prepulse C_m over a 50-ms window before depolarization and the C_m average taken over a 500-ms window after the end of a depolarization. This protocol should result in values free of endocytosis artefact, as for the stimulus strengths used in our study only slow forms of endocytosis occur (Smith & Neher, 1997). Analysis of the data was performed with a program written in IgorPro (WaveMetrics, Lake Oswego, OR, USA) macro code by E.A.L. also including code written by K. D. Gillis.

Fluorescence measurements

Patch-clamp and capacitance records were combined with fura-2 fluorescence measurements to allow simultaneous on-line monitoring of Ca^{2+} currents, surface area and two fura-2 fluorescence signals (F_{360} and F_{390}, excited at 360 and 390 nm wavelengths, respectively) to measure $[Ca^{2+}]_i$. Fura-2 (100 μM) was loaded into the cells via patch pipettes. The fluorescence signal was sampled at 2 Hz throughout the experiment together with pipette current. Ca^{2+} concentration was displayed on-line using a system by Luigs and Neumann (Ratingen, Germany). The spatially averaged $[Ca^{2+}]_i$ was determined using the microfluorimetric ratio technique with *in vivo* calibration, as described previously (Neher, 1989; Augustine & Neher, 1992). $[Ca^{2+}]_i$ was calculated from the ratios (R) of light emission produced by the two wavelengths 360 and 390 nm, using the equation (Grynkiewicz *et al*., 1985) $[Ca^{2+}]_i = K_{eff}(R - R_{min})/(R_{max} - R)$

where R is the fluorescence ratio F_{360}/F_{390}, K_{eff} is the effective dissociation constant of fura-2, R_{min} is the minimum R-value, and R_{max} the maximum. F_{360} and F_{390} are fluorescence intensities at 360 and 390 nM, respectively. Our calibration is based on a value for $K_{D,EGTA} = 0.15$ μM at pH 7.2 (Grynkiewicz *et al*., 1985). EGTA used during the calibration was found not to contribute significantly to fluorescence. In order to evaluate differences among group means, data were analysed statistically using Student's paired *t*-test or one factor analysis of variance (ANOVA), as indicated in the text.

Results

Depolarization-induced capacitance changes

Single chromaffin cells were stimulated with a series of depolarizing pulses in order to elicit secretory responses evoked by Ca^{2+} current (I_{Ca}). Changes in membrane capacitance (ΔC_m) were recorded and taken as a measure of secretion (Neher & Marty, 1982). In our experiments, we used high bath Ca^{2+} concentration (60 mM) to induce secretion by 50 or 200 ms depolarizations. I_{Ca} was suppressed to various degrees by Ca^{2+} channel blockers. Most experiments required long-lasting series of sparsely spaced depolarizing pulses, during which some rundown occurred. Also, the basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) had a tendency to increase over prolonged times probably due to high external $[Ca^{2+}]$. We chose a protocol of experiments which allowed us to correct for such slow changes. To do so, we held the membrane potential at –70 mV and started each series of depolarizations with a pulse to a reference potential of +40 mV. This corresponded to the maximum of the current–voltage relationship (I–V). Subsequently, depolarizations to different membrane potentials were given to activate I_{Ca} to various degrees. Pulses

to the reference potential were randomly repeated several times within a series such that during the analysis both Ca^{2+} currents and ΔC_m values could be normalized to the last reference value. On a given cell, we used several series with different sequences of test potentials, e.g.: +40, +35, +45, +60, +20, +40, +30, +50, +25, +40, +50, +30, +40, +20, +60, +40, +45 mV, or else: +40, +50, +30, +40, +20, +60 mV, etc. All series were applied automatically by using macro commands of 'Pulse' software. Interpulse intervals of 40 s were used to allow $[Ca^{2+}]_i$ to decay to a steady-state level (Fig. 1A; note that the steady-state is somewhat higher than the basal $[Ca^{2+}]$ before the first pulse in this example). Capacitance compensation was adjusted during series using the 'autotrack' option of the software. We also evaluated the integral of I_{Ca}, the Ca^{2+} charge (Q), as a measure of Ca^{2+} inflow. Figure 1 demonstrates typical records of $[Ca^{2+}]_i$ transients, membrane capacitance (C_m), as well as Ca^{2+} current traces (on an extended time scale). Series conductance remained constant throughout this recording. As can be seen from the figure, C_m increased rapidly as a consequence of I_{Ca} activation. Subsequently, C_m declined somewhat slower than the Ca^{2+} transient. We attributed the decline in C_m to endocytosis (Smith & Neher, 1997). Such a response pattern was observed in most of our experiments.

It can be seen from Fig. 1 that the peaks of Ca^{2+} transients correlate with Q evoked by I_{Ca}. This impression is confirmed by quantitative analysis, as shown in Fig. 2A, where both Q and the peak of Ca^{2+} transients normalized to their maximal values are plotted against the similarly normalized peaks of I_{ca}.

Dependence of capacitance responses on basal $[Ca^{2+}]_i$

In our experiments, we observed that secretory responses to reference stimuli (+40 mV) were quite stable, even during prolonged recordings (up to 20 or 30 min) if the basal $[Ca^{2+}]_i$ was stable during such measurements. This could be achieved by including 0.5 mM EGTA in the pipette solution and by using relatively rare stimulation (0.025 Hz), which allowed $[Ca]_i$ to fully settle to baseline before the subsequent stimulus. Figure 2B shows values of basal $[Ca^{2+}]_i$ (dots) from one cell which were measured just before a given reference depolarizing pulse (+40 mV), and values of C_m-increase (squares) measured during such reference potential stimuli from the same cell. It can be seen that under these conditions Q (triangles) and the C_m responses changed only very little throughout the recording. More often, however, the basal level of $[Ca^{2+}]_i$ increased during the experiment (see Fig. 1A and Smith *et al.*, 1998). This led to large changes in the secretory properties and the value of secretion calculated as ΔC_m was significantly increased (Fig. 2D). In this figure, data from 10 cells were pooled. Responses to depolarizations to various membrane potentials are shown that induced different values of Q. ΔC_m values are plotted against basal $[Ca^{2+}]_i$, before a given stimulus, with different test potentials represented by different types of symbols, as indicated. It is seen that responses for a given test potential are larger for basal $[Ca]_i$ values between 250 and 500 nM, compared with lower $[Ca]_i$. This occurs, in spite of the fact that Q values (Fig. 2C) are largest for low $[Ca^{2+}]_i$ and decrease for $[Ca^{2+}]_i > 300$ nM. Increases in basal $[Ca^{2+}]_i$ above 600 nM often produced much smaller ΔC_m responses (Fig. 2D) which can be explained by depletion of a readily releasable pool of vesicles due to continuous release in between stimuli (Heinemann *et al.*, 1993; Smith *et al.*, 1998). At the same time, the relatively high $[Ca^{2+}]_i$ seemed to prevent large I_{Ca} values. We attribute this to Ca^{2+}-dependent rundown of I_{Ca} and Ca^{2+}-dependent inactivation. The data in Fig. 2C, representing the dependence of Q on the level of basal $[Ca^{2+}]_i$, were obtained from the same 10 cells as that of Fig. 2D. Figure 3 shows a summary graph three-dimensional plot demonstrating the influence of basal level of $[Ca^{2+}]_i$ and Q on secretory responses (ΔC_m). The data points represent those of Fig. 2C and D for $[Ca^{2+}]_i < 500$ nM.

In spite of appreciable scatter, it can be seen that, depending on the level of basal $[Ca^{2+}]_i$, the same Q can induce quite different changes in ΔC_m, and different Q-values can induce similar ΔC_m changes. Therefore, in subsequent experiments all data were evaluated by

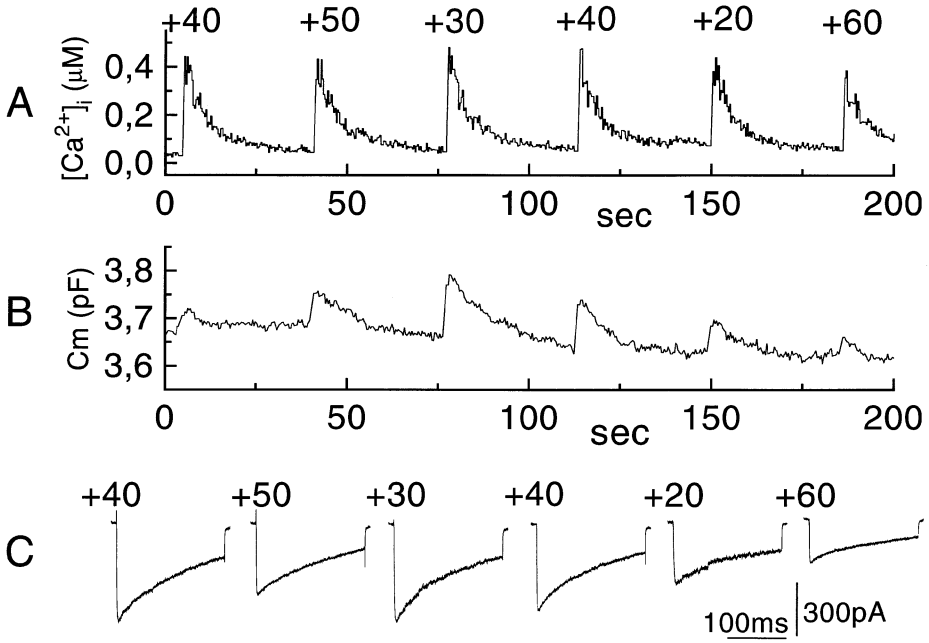

FIG. 1. Cellular responses induced by depolarizations. (A) Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). (B) Membrane capacitance (C_m). (C) Ca^{2+} current (I_{Ca}) traces induced by 200-ms depolarizations from a holding potential of –70 mV to test potentials, as indicated near the corresponding current traces shown. The bath solution here and in other figures contained 60 mM Ca^{2+}.

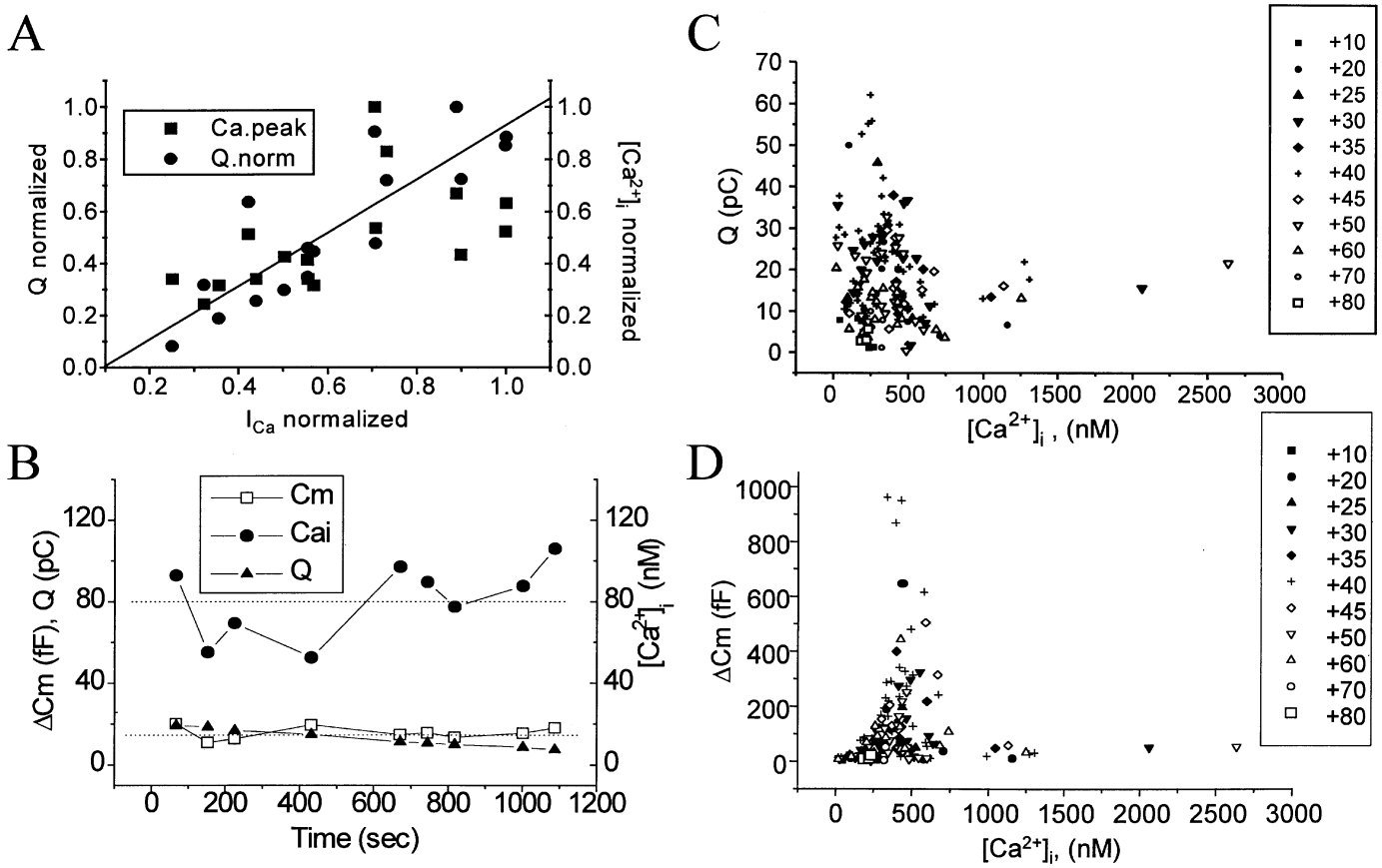

FIG. 2. Capacitance changes at different levels of basal $[Ca^{2+}]_i$. (A) Relationship between Ca^{2+} charge, Q (circles) and the peak of Ca^{2+} transients measured by fura-2 (squares) normalized to their maximal values and plotted against the similarly normalized peak of I_{Ca}; data are from one cell. (B) Membrane capacitance changes (ΔC_m, squares), Q (triangles) and basal $[Ca^{2+}]_i$ (circles) measured at reference potential (+40 mV) versus time from another single cell. Dependence of Ca^{2+} charge, Q (C) and ΔC_m (D) on basal $[Ca^{2+}]_i$; pooled data recorded from 10 cells of the same set. Basal $[Ca^{2+}]_i$ was measured by fura-2 as the resting value immediately preceding a given depolarization. I_{Ca} was evoked by 200-ms depolarizations to different membrane potentials from a holding potential of –70 mV. The straight line in A is a linear regression to all data points.

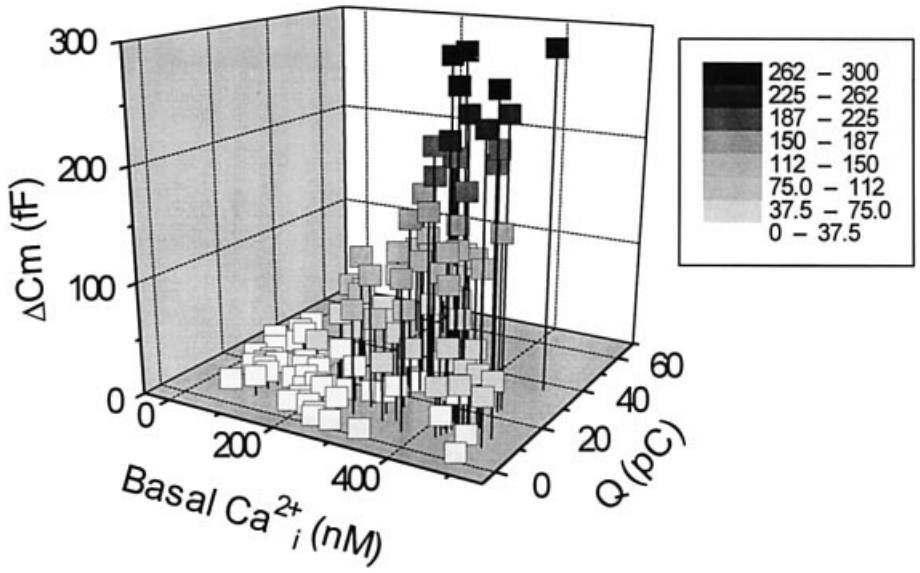

FIG. 3. Joint influences of basal $[Ca^{2+}]_i$ and the charge Q of Ca^{2+} entering through channels on capacitance changes (ΔC_m) in the form of a three-dimensional scatter graph. The data points ($n = 131$) are those of Fig. 2C and D, restricted to the ranges $[Ca^{2+}]_i < 500$ nM and $\Delta C_m < 300$ fF. Different grey-scale symbols represent one of the eight ranges of ΔC_m values (each range equals 36.5 fF) as shown in the inset.

normalizing a given ΔC_m to the most recent reference value (see above). This way, the influence of slow changes in $[Ca^{2+}]_i$ occurring over the course of an experiment, and other slow trends of rundown could be largely eliminated, because mostly only a small change occurred between the test and nearest reference value.

Voltage dependence of secretion

To determine the role of different types of Ca^{2+} channels in secretion from chromaffin cells, we first tested the voltage dependence of secretion under control conditions. Cells were depolarized from

V_{hold} = –70 mV for periods of 200 ms to values between 0 and +70 mV in order to evoke I_{Ca}. The average values of Q and corresponding changes in C_m were plotted against membrane test potentials. Figure 4A gives a summary of 107 records from different cells. The current–voltage relationship (I–V) is shifted to the right in comparison with I–V relationships obtained in 2 mM $[Ca^{2+}]_0$. This may be induced by the high concentration of divalent ions in the bath solution (Kostyuk *et al*., 1982). The ΔC_m values as well as Q-values were normalized to their reference values (see above). It can be seen from Fig. 4A, that C_m step size (squares) precisely follows values in Q (circles) at all potentials tested.

This points to Ca^{2+} channel activity as the main source of Ca^{2+}-evoked secretion, and shows that influences of basal $[Ca]_i$ can be eliminated by our normalization procedure. As a rule, a given cell showed ΔC_m values within a narrow range when tested at potentials between –25 and +45 mV, whereas another cell may show values in a different range. Therefore, after normalization their values did not differ significantly. We also plotted the changes in average C_m (ΔC_m) as a function of Q induced at different depolarizing voltages (Fig. 4B). The dependence of normalized ΔC_m on normalized Q was approximated by a linear function with a slope $B = 0.98 \pm 0.06$ ($n = 10$, Fig. 4B). The potential dependence after normalization is somewhat different from results previously reported where some hysteresis was observed (Augustine & Neher, 1992). Part of this hysteresis might have been caused by changes in basal $[Ca^{2+}]_i$ which, in the present approach, were eliminated by normalization and randomization of depolarizing sequences. Given the mean normalization values (see Fig. 8, below), our data agree within a factor of 1.72 with the 'standard curve' of Engisch *et al*. (1997).

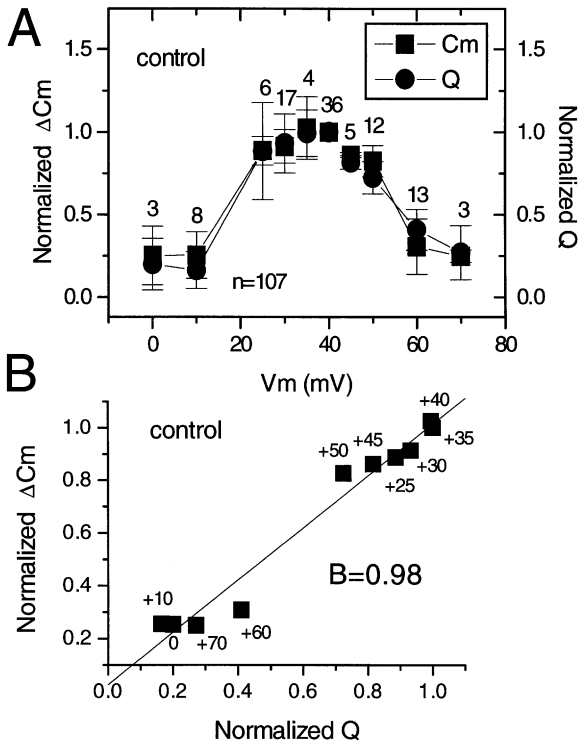

FIG. 4. Voltage dependence of secretion under control conditions. (A) Dependence of Q (circles) and ΔC_m (squares) on membrane potential induced by depolarizing 200-ms pulses from V_{hold} = –70 mV. Points represent mean ± SD values normalized to reference values. They are obtained from 107 records from different cells. (B) The same data presented as dependence of ΔC_m on Q normalized to the reference values. The number of measurements is indicated near the points. Normalized values used for this and the following graphs were calculated according to every preceding reference value within a series of stimuli. Absolute values of selected runs are presented in Fig. 8.

Effects of dihydropyridines on exocytosis

Nifedipine and nimodipine are well-known selective blockers of dihydropyridine-sensitive Ca^{2+} channels termed L-type channels (reviewed by Kostyuk, 1989). We used the combination of two dihydropyridines, nifedipine and nimodipine, to suppress activity of L-type channels selectively, and thereby eliminate this channel type from participation in secretion. The same pulse protocols as for control conditions were applied for cells kept in 20 μM nifedipine + 10 μM nimodipine. Eighty-six records from different cells were obtained. Despite the partial suppression of Q_{Ref} (38.2 ± 6.1%, $n = 72$) by the blockers cocktail, we still could observe depolarization-induced exocytosis (Fig. 5). Figure 5A presents the dependence of Q (squares) and ΔC_m (circles) normalized to corresponding reference values on membrane potentials evoked by 200 ms depolarizing pulses from a holding potential of –70 mV.

We observed the same characteristic voltage dependence of secretion as in control conditions, whereby changes in ΔC_m precisely followed changes in Q. The experimental points from Fig. 5B could be well approximated by a linear function with slope B = 0.92 ± 0.12 (n = 7). Thus, we can conclude that the voltage and Ca^{2+} dependence of secretion are not drastically changed when L-type channels were eliminated.

In our experiments, we also intended to test the involvement of 'facilitation' calcium current (Artalejo *et al*., 1994) in secretion by using distinct sets of pulse protocols to invoke the 'facilitation' by prepulses. However, under no circumstances were we able to observe facilitation of a similar magnitude as reported for calf chromaffin cells by Artalejo *et al*. (1994).

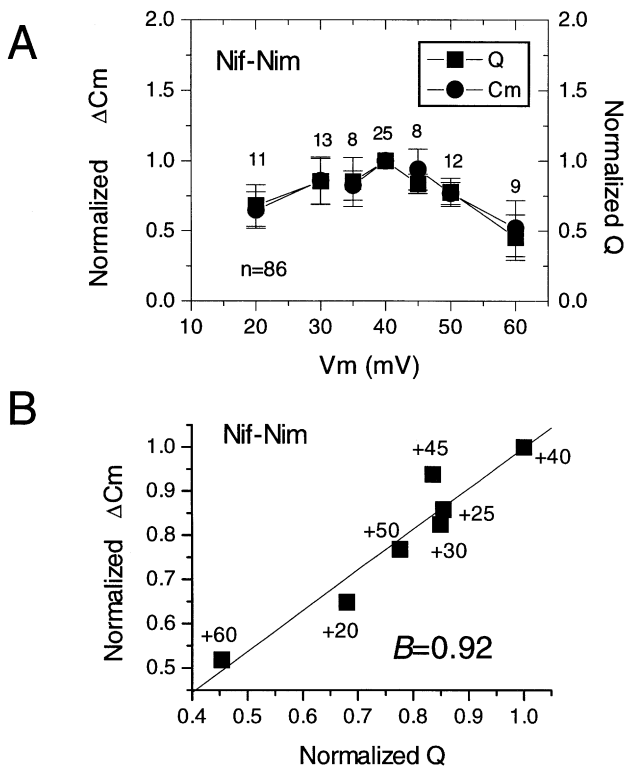

FIG. 5. Secretion in the presence of dihydropyridines. Data are presented as in Fig. 4, but cells were bathed in a solution with 20 μM nifedipine + 10 μM nimodipine (averages of 86 records from different cells).

Effects of ωCTxGVIA on exocytosis

In the next series of experiments, we used ωCTxGVIA (omega-conotoxin GVIA), a widely used selective blocker of N-type Ca^{2+} channels in distinct types of nerve cells. Taking into account the finding that action of ωCTxGVIA on N-type channels is Ca^{2+} dependent (Abe *et al*., 1986), we pretreated cells for 5 min in Ca^{2+}-free solution containing 1 μM ωCTxGVIA just before the experiment. Assuming irreversibility of ωCTxGVIA action (McClesky *et al*., 1987; Lukyanetz, 1998), cells were placed after pretreatment in recording (60 mM Ca^{2+}) solution, which contained the ωCTxGVIA concentration. Otherwise, experimental conditions were the same as in previous cases. Q_{Ref} measured in a number of chromaffin cells was decreased by 49.4 ± 7% ($n = 65$) under ωCTxGVIA. Figure 6 shows that the value of Q which was not blocked by ωCTxGVIA was still capable of inducing secretion in chromaffin cells under these conditions.

The peak of the *I–V* curve and hence the maximum position of Q and ΔC_m were somewhat shifted to the left in comparison with control as a result of N-current component suppression (Fig. 6A). Otherwise, the dependence of Q and ΔC_m on membrane potential obtained from 95 records was similar to previous cases. The experimental points also could be well approximated by a linear function with slope $B = 0.82 \pm 0.1$ ($n = 8$, Fig. 6B). The decrease of B by 18% in the presence of the blocker of N-type channels might indicate a shallower dependency of secretion upon Q in the absence of N-channel activity which, in turn, would suggest that non-N-channels can induce close to maximum secretion even at submaximum Ca^{2+} inflow. However, an analysis of the differences between these and control means of B-values showed that the difference was not statistically significant, $P = 0.189$.

Effects of ωCTxMVIIC on exocytosis

It was reported that chromaffin cells also express Q-type Ca^{2+} channels (Albillos *et al*., 1994). We therefore used ωCTxMVIIC to test the role of this type of Ca^{2+} channel in secretion. ωCTxMVIIC is known as a non-selective blocker of N-, P- and Q-types, with Q-type channels being most sensitive to ωCTxMVIIC at a blocker concentration of 2 μM (Miljanich & Ramachandran, 1995). ωCTxMVIIC as well as ωCTxGVIA were reported to be Ca^{2+} dependent in their blocking actions and the potency being lost in the presence of high concentrations of Ca^{2+}. Moreover, it was shown recently that Ca^{2+} ions can also attenuate the effectiveness of ωCTxMVIIC (Albillos *et al*., 1996b). Therefore, we also employed a 5-min pretreatment with the blocker in Ca^{2+}-free solution and subsequently tested cells in the presence of 2 μM ωCTxMVIIC. As can be seen from Fig. 7, the toxin exerted only a slight action on I_{Ca} (Q_{Ref} was suppressed by 15.1 ± 9.1%, $n = 52$). It cannot be excluded that block by ωCTxMVIIC was partly reversible under the conditions of our experiments. Thus, the 15% reduction of I_{ca} may represent partial block of Q-type channels, according to the results of Albillos *et al*. (1996b).

Figure 7A represents the voltage dependence of Q and exocytosis calculated as ΔC_m from 86 records. As in all other cases, the value of ΔC_m during reduction of the activity of Q-type Ca^{2+} channels proportionally depended on the Ca^{2+} charge with a slope $B = 0.74 \pm 0.19$ ($n = 8$, Fig. 7B). A slight deviation in the positive range of membrane potentials was statistically insignificant (Fig. 7A).

Summary of results

The results presented show that both under control conditions and with the various toxin treatments, secretion depended linearly on

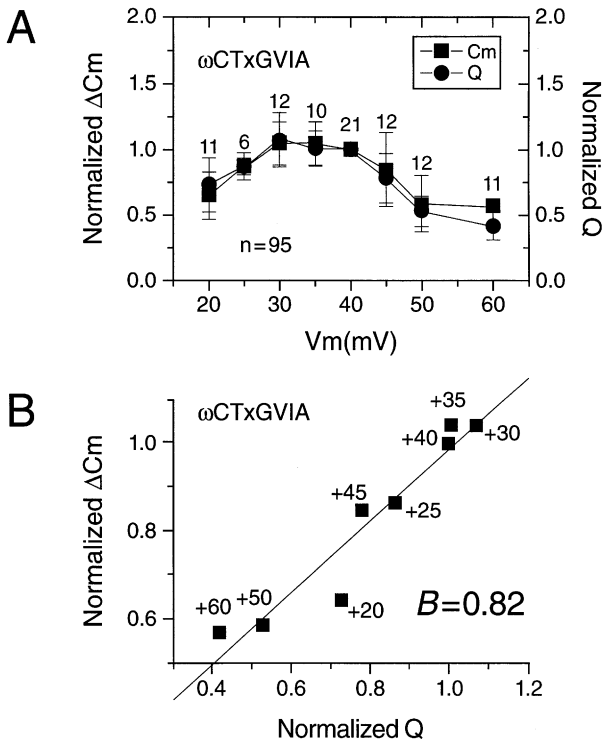

FIG. 6. Secretion in the presence of ωCTxGVIA. Data are presented as in Fig. 4, but cells were pretreated in Ca^{2+}-free solution containing 1 μM ωCTxGVIA and then recorded in a bath solution with the same concentration of the toxin. Data obtained from 95 cells.

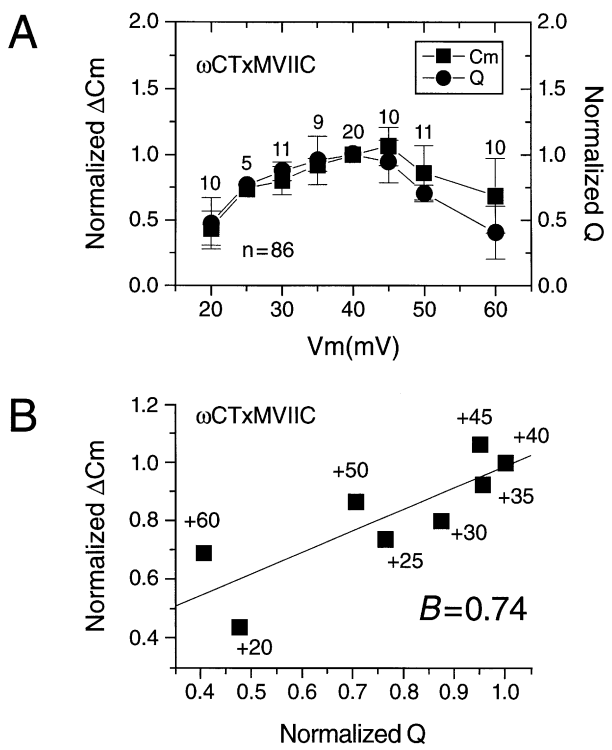

FIG. 7. Secretion in the presence of ωCTxMVIIC. Data are presented as in Fig. 4, but cells were pretreated in Ca^{2+}-free solution containing 2 μM ωCTxMVIIC and then recorded in a bath solution with the same concentration of the toxin (averaged of 42 records from different cells).

Ca^{2+} reflux. No statistically significant changes in either the shape of this relationship or in the *I–V* relationship of Ca^{2+} currents was apparent except for a small shift of the *I–V* relation under ωCTxGVIA. In the figures shown so far, all data were plotted with respect to values at the reference potential of +40 mV. Figure 8 shows a summary of reference values. It is seen that suppression of ΔC_m by toxins is very similar in all cases to suppression of *Q*. This indicates that under the condition of our experiments, a given Ca^{2+} influx (as represented by charge) can elicit secretion, irrespective of the type of Ca^{2+} channel mediating the influx.

Discussion

In the present paper, we have examined the relationship between Ca^{2+} influx through different types of Ca^{2+} channels and exocytosis in bovine chromaffin cells. We used a high external Ca^{2+} concentration and relatively long depolarizations (50, 200 ms) to evoke exocytosis under conditions when activity of distinct Ca^{2+} channel subtypes was preferentially suppressed by selective blockers. Our experiments show that L- and N-type channel blockers were effective under tested experimental conditions, as they blocked 38% and ≈50% of Ca^{2+} charge, respectively. About 15% of Ca^{2+} current was blocked by ωCTxMVIIC, which is a non-selective blocker of N-, Q- and P-channels. Taking into account that we did not use blocker combinations and that at the high external Ca^{2+} concentration (60 mM) used, the effectiveness of toxins to block channels might be partially lost due to Ca^{2+} dependence of their action, we cannot quantitatively separate all three components. However, we can conclude that participation of N-type channels is higher than that of L-type and that, taken together, they contribute more than half of all the Ca^{2+} influx. The observed N- and L-type Ca^{2+} channels' contribution is in rough agreement with previous data obtained in rat (Kim *et al*., 1995), bovine (Albillos *et al*., 1996b), and porcine (Kitamura *et al*., 1997) adrenal chromaffin cells, and on single rat pancreatic β cells (Kim *et al*., 1998). A large contribution of P/Q-type channels has been found in chromaffin cells by Albillos *et al*. (1996b).

In our experiments, we carefully tried to reproduce the 'facilitation' Ca^{2+} channel phenomenon with protocols similar to those described earlier (Artalejo *et al*., 1991; Dolphin, 1996). However, we could not achieve degrees of facilitation as high as reported for cells from young animals. Therefore, we suppose that in cells from adult animals, as used by most investigators, this phenomenon is relatively small and is mainly a consequence of tonic autoinhibition of I_{Ca} by one of the released substances (Elhamdani *et al*., 1995; Albillos *et al*., 1996a; Garcia & Carbone, 1996; Currie & Fox, 1996; Otsuguro *et al*., 1996). In fact, in a recent study comparing cells from young and old animals, marked differences were observed between cell types in both I_{ca} facilitation and secretion (Elhamdani *et al*., 1998).

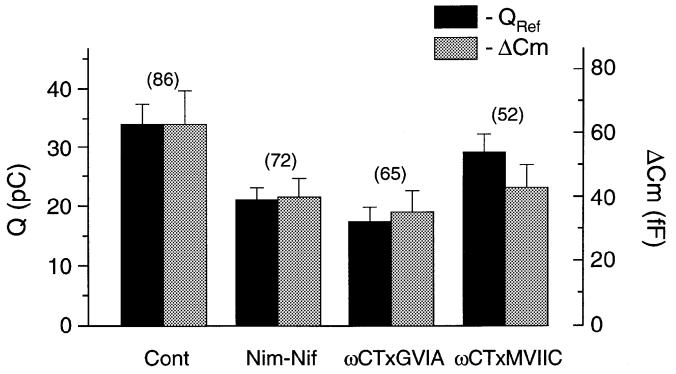

FIG. 8. Integrated Ca^{2+} current, *Q* (black) and membrane capacitance changes ΔC_m (grey) during control and after application of selective blockers of Ca^{2+} channel types are shown. The number of records on which the mean values are based are indicated near the bars. Data are mean ± SEM. Q_{Ref} was evoked by 200-ms step depolarizations of membrane to the reference potential (+40 mV) which is at the peak of the *I–V* curve. Each cell ($n = 52$) was subjected to only one antagonist (with the exception of nifedipine + nimodipine) or was recorded under control conditions.

Our experiments showed that the amount of secretion was mainly determined by two parameters: the Ca^{2+} charge estimated as the integral of Ca^{2+} current during a stimulus and the basal $[Ca^{2+}]_i$ preceding a given stimulus. We could not observe a preferential role of any Ca^{2+} channel subtype as the action of Ca^{2+} currents was proportional to Ca^{2+} charge, irrespective of channel type. Besides, elevated basal $[Ca^{2+}]_i$ increased the secretory response to Ca^{2+} currents of any subtype. Thus, pharmacological exclusion of different types of Ca^{2+} channels from participation in secretion did not reveal their specific roles in secretion in our experiments which employed relatively long depolarizing pulses (50–200 ms). This result is similar to published data on bovine (Engisch & Nowycky, 1996), rat (Kim *et al*., 1995) or porcine (Kitamura *et al*., 1997) adrenal chromaffin cells, or on single rat pancreatic β cells (Kim *et al*., 1998). It is different from findings on intact bovine chromaffin cells stimulated with K^+-depolarization, where a preferential role of Q- and L-type channels in secretion was reported (Lopez *et al*., 1994b). It also contrasts with *in situ* studies, where nerve stimulation indicated an N-type specific response (O'Farrell *et al*., 1997).

When considering these differences, one has to appreciate that secretion is the result of a chain of events including electrical excitation, Ca inflow, rise in $[Ca^{2+}]$, and exocytosis. The latter process depends not only on the Ca^{2+} stimulus, but also on the availability of release-ready vesicles, which itself is the result of another chain, including vesicle docking and priming. Ca^{2+} channel properties may manifest themselves at several steps along these chains, and secretion may be affected in different ways depending on the kind of stimulation and recording conditions. For instance, in an intact cell, Ca^{2+} current not only has a direct action on secretion; it also influences electrical excitability (both through its depolarizing action and by activating Ca^{2+}-dependent currents). Different types of Ca^{2+} current may contribute differentially to these processes due to differences in their long-term inactivation or their spatial coupling to Ca^{2+}-activated currents. Furthermore, inactivation properties determine the duration and degree of long-term Ca^{2+} elevations during long-lasting stimuli (> 1 s), which in turn influence both the secretory process itself and the recruitment of vesicles to a release-ready pool (Heinemann *et al*., 1993; von Rüden & Neher, 1993; Smith *et al*., 1998). In voltage-clamped cells, the indirect effects on cell excitability (e.g. changes in action potential waveforms due to contributions of Ca^{2+}-activated K^+-channels) are eliminated. However, the duration of a voltage-clamp stimulus will determine to what extent subtype-specific inactivation properties of Ca^{2+} channels will matter. Also, depolarizing stimuli will test the release efficiency of a given channel subtype only when they are brief enough not to deplete the release-ready pool. Long-duration stimuli, on the other hand, may deplete the release-ready pool no matter whether a more efficient or less efficient Ca^{2+} channel type is being activated. In that case, long-lasting stimuli test the size of the release-ready pool or else the recruitment of vesicles to such a pool. For short pulses, a tight association of Ca^{2+} channels with release sites will be very favourable, because the release rate of vesicles from the readily releasable pool increases steeply with $[Ca^{2+}]_i$ up to quite high values

(Chow *et al*., 1996). Thus, Ca^{2+} channels tightly coupled to release sites will be at an advantage. In the case of prolonged stimulation, this will not be important, however, as vesicle recruitment to the readily releasable pool saturates at $[Ca^{2+}]$ values low enough (Augustine & Neher, 1992; Smith *et al*., 1998), such that maximum recruitment can be obtained even if the channels are not very close to a release site.

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Abbreviations

C_m, membrane capacitance; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; I_{Ca}, calcium current; *I–V*, current–voltage relationship; *Q*, calcium charge; ωCTxGVIA, omega-conotoxin GVIA; ωCTxMVIIC, omega-conotoxin MVIIC.

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