

## All classes of calcium channel couple with equal efficiency to exocytosis in rat melanotropes, inducing linear stimulus–secretion coupling

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1. The contribution of low voltage-activated (LVA) T-type  $\text{Ca}^{2+}$  channels and four different types of high voltage-activated (HVA)  $\text{Ca}^{2+}$  channel to exocytosis, and the relationship between calcium influx and exocytosis during action potentials (APs) were studied in pituitary melanotropes.
2. Selective HVA  $\text{Ca}^{2+}$  channel blockers reduced exocytosis, monitored by membrane capacitance measurements, proportional to the reduction in  $\text{Ca}^{2+}$  influx. The efficacy of  $\text{Ca}^{2+}$  in stimulating exocytosis did not change in the presence of the  $\text{Ca}^{2+}$  channel blockers, indicating that all HVA  $\text{Ca}^{2+}$  channels act together in stimulating exocytosis.
3. The relationship between  $\text{Ca}^{2+}$  influx and exocytosis during the AP was examined using APs recorded from spontaneously active melanotropes as command templates under voltage clamp. Under voltage clamp, multiphasic  $\text{Ca}^{2+}$  currents were activated over the entire duration of the APs, i.e. during the rising phase as well as the plateau phase. The maximum amplitude of the  $\text{Ca}^{2+}$  current coincided with the peak of the AP.
4. The relationship between  $\text{Ca}^{2+}$  entry and exocytosis was linear for the different phases of the AP. Also, the influx of  $\text{Ca}^{2+}$  through LVA T-type channels stimulated exocytosis with the same efficacy as through the HVA channels.
5. APs of increasing duration (~50 to ~300 ms) evoked increasing amounts of exocytosis. The number of entering  $\text{Ca}^{2+}$  ions and the capacitance change were linearly related to AP duration, resulting in a fixed relationship between  $\text{Ca}^{2+}$  entry and exocytosis.
6. The results show that  $\text{Ca}^{2+}$  ions, entering a melanotrope, couple with equal strength to exocytosis regardless of the channel type involved. We suggest that the linear relationship between  $\text{Ca}^{2+}$  entry and secretion observed under physiological conditions (during APs), results from the equal strength with which LVA and HVA channels in melanotropes couple to exocytosis. This guarantees that secretion takes place over the entire duration of the AP.

Release of neurotransmitters and hormones from nerve terminals and neuroendocrine cells is controlled by the influx of  $\text{Ca}^{2+}$  through voltage-gated  $\text{Ca}^{2+}$  channels. The natural stimulus for opening  $\text{Ca}^{2+}$  channels and triggering exocytosis is the action potential (AP). The strength of the coupling between an AP and exocytosis depends on the spatial organization of  $\text{Ca}^{2+}$  channels and vesicles. Release-ready vesicles located in the close vicinity of  $\text{Ca}^{2+}$  channels are likely to be released with little delay when an AP is fired. This is the case in fast synapses where N- and P-type  $\text{Ca}^{2+}$  channels are located close to the release sites and actually bind to the fusion peptides syntaxin and/or synaptotagmin (Bennett *et al.* 1992; Sheng *et al.* 1996). On the other hand, vesicles that are further away are released with a considerable delay, due to a longer diffusion distance for  $\text{Ca}^{2+}$ .

In the neuroendocrine chromaffin cells, the majority of the large dense-cored vesicles (LDCVs) are thought to be docked far away from  $\text{Ca}^{2+}$  channels (200–300 nm), because the delay between stimulation and secretion is rather long and exocytosis persists after the stimulus has ended (Chow *et al.* 1992, 1994, 1996; Klingauf & Neher, 1997). Moreover, the concentration of intracellular  $\text{Ca}^{2+}$  chelator that affects exocytosis is low (Chow *et al.* 1996). This distance results in a weak coupling between the AP and exocytosis (Zhou & Mislisler, 1995). However, a small population of LDCVs are docked within 30 nm from the  $\text{Ca}^{2+}$  channel. The release of these vesicles is strongly coupled to the AP, and they are rapidly released upon firing (Klingauf & Neher, 1997; Elhamdani *et al.* 1998). In calf chromaffin cells, these ‘near-by’ vesicles are specifically docked near the L-type

facilitation  $\text{Ca}^{2+}$  channel (Artalejo *et al.* 1991, 1994). As a result,  $\text{Ca}^{2+}$  entry through this channel stimulates exocytosis about 5 times more effectively than  $\text{Ca}^{2+}$  entry through other types of  $\text{Ca}^{2+}$  channel. A similar strong coupling between L-type channels and exocytosis exists in pancreatic  $\beta$ -cells (Bokvist *et al.* 1995).

In melanotropes of the rat, the influx of  $\text{Ca}^{2+}$  through  $\text{Ca}^{2+}$  channels causes the release of predocked LDCVs (Thomas *et al.* 1990; Parsons *et al.* 1995). Exocytosis starts with a delay (Thomas *et al.* 1993*b*) and low concentrations of  $\text{Ca}^{2+}$  buffers block exocytosis, suggesting a large distance between  $\text{Ca}^{2+}$  channels and LDCVs (Mansvelder & Kits, 1998). Nevertheless, we observed a stringent coupling between depolarization and secretion in melanotropes. Exocytosis was observed upon very short depolarizations (2–5 ms), as well as upon the final stimulus in a train of 25 depolarizations of 40 ms. This indicates that in melanotropes different channel types are able to sustain secretion, and that APs should be robustly coupled to secretion. The present paper puts these two predictions to the test. Thus, we first addressed the role of the different  $\text{Ca}^{2+}$  channel types in melanotropes in exocytosis and we analysed the efficacy with which the various channel types couple to secretion. Second, we examined the relation between calcium influx and exocytosis during the AP and addressed the question of whether the shape and duration of the APs influence the strength of excitation–secretion coupling.

Melanotropes express five different types of  $\text{Ca}^{2+}$  channel: L-, N-, P-, Q- and T-type (Keja *et al.* 1991; Stack & Suprenant, 1991; Ciranna *et al.* 1996; Mansvelder *et al.* 1996). To investigate the coupling of each of these channel types to exocytosis we examined the effects of specific  $\text{Ca}^{2+}$  channel blockers on secretion, assessed by capacitance measurements. To study the coupling of APs to secretion, we recorded APs from spontaneously active cells. Subsequently we stimulated melanotropes under voltage clamp with APs which covered the range of shapes and durations encountered, and measured the resulting changes in membrane capacitance.

Part of this work has appeared in abstract form (Mansvelder & Kits, 1999).

## METHODS

### Cell culture

Pituitary melanotropes of male Wistar rats (200–300 g, Harlan CPB, The Netherlands) were isolated as described previously (Keja *et al.* 1991). Animals were killed by swift decapitation using a guillotine, without the use of anaesthetics. Killing procedures were approved by the ethical committee concerning animal experiments. The cells were cultured on poly-L-lysine-coated coverslips (7 mm  $\times$  7 mm) at a density of 0.1 intermediate lobe per coverslip. The culture medium consisted of Biorich I (Flow) supplemented with 26.2 mM  $\text{NaHCO}_3$ , 5% Ultrosor G (Gibco), 200 U  $\text{ml}^{-1}$  penicillin G (Sigma), 50  $\mu\text{g ml}^{-1}$  streptomycin (Sigma) and 1  $\mu\text{M}$  cytosine arabinosine (Sigma). Cells were maintained in a 37 °C

incubator with a humidified atmosphere comprising 5%  $\text{CO}_2$  in air. Recordings were made up to 4 days after isolation.

### Recording solutions

Coverslips bearing melanotropes were transferred to a recording chamber containing  $\sim 0.5$  ml external solution. The external solution for the experiments determining the pharmacological sensitivity of the whole-cell calcium current consisted of (mM): TEACl 142, glucose 10,  $\text{CaCl}_2$  5, Hepes 10 and 4-aminopyridine (4-AP) 1; pH adjusted to 7.4 with TEAOH. The internal solution for these experiments contained (mM): CsCl 145,  $\text{MgCl}_2$  2, EGTA (Sigma) 0.1, Hepes 10 and MgATP 2; pH adjusted to 7.4 with CsOH. For recording spontaneous electrical activity of melanotropes the external solution contained (mM): NaCl 145,  $\text{MgCl}_2$  1.2,  $\text{CaCl}_2$  2.4, KCl 3, glucose 10 and Hepes 10; pH adjusted to 7.4 with NaOH. The internal solution for these experiments contained (mM): KCl 135,  $\text{MgCl}_2$  2,  $\text{CaCl}_2$  1, Hepes 10, EGTA 11, MgATP 2 and GTP Tris salt 0.1; pH adjusted to 7.4 with KOH (free  $\text{Ca}^{2+}$  concentration, 3.8 nM). The external solution for measuring the calcium current and changes in membrane capacitance elicited by AP templates contained (mM): TEACl 145.75, glucose 10,  $\text{CaCl}_2$  2.5, Hepes 10 and 4-AP 1; pH 7.4 with TEAOH. The internal solution for these experiments contained (mM): CsCl 145,  $\text{MgCl}_2$  2, EGTA 0.1, Hepes 10 and MgATP 2; pH adjusted to 7.4 with CsOH (nominal zero calcium). The recording chamber was continuously perfused at a rate of  $\sim 1.5$   $\text{ml min}^{-1}$ , driven by a pump, while the bath volume was kept constant by continuous suction. All experiments were carried out at a temperature of 32–34 °C.

### Membrane potential recordings

Electrodes were pulled on a Flaming-Brown P-87 (Sutter Instruments, UK) horizontal microelectrode puller from thick-walled Clark GC-150 borosilicate glass (Clark Electromedical Instruments, UK). To reduce the pipette capacitance, the tips of the electrodes were covered with Sylgard. The impedance of the electrodes after fire polishing was 5–10 M $\Omega$ . Membrane voltages were monitored in the 'fast I-clamp' mode of an Axopatch 200A amplifier (Axon Instruments), filtered at 1 kHz (4-pole low-pass Bessel filter on the Axopatch 200A) and digitized with a Digidata 1200 interface (Axon Instruments) at 6 kHz. The pipette capacitance was compensated for. Membrane voltages were recorded in the whole-cell configuration. Data were stored and analysed with pCLAMP 6 software (Axon Instruments).

### Capacitance measurements

Electrodes were pulled in the same manner as for the membrane potential recordings, and the tips of the electrodes were also covered with Sylgard. Impedance of the electrodes after fire polishing was 2.5–4 M $\Omega$ . The whole-cell membrane current was monitored and digitized with an EPC 9 amplifier (HEKA, Lambrecht Germany). Capacitance measurements were made using PULSE software running on an IBM-compatible computer. The membrane capacitance, access conductance and membrane conductance were calculated according to the Lindau-Neher technique (Lindau & Neher, 1988; for review, see Gilles, 1995), implemented as the 'Sine + D.C.' feature of the PULSE lock-in module. A sine wave of 1000 Hz, 40 mV peak-to-peak, was added to a holding potential of  $-80$  mV. The reversal potential of the lock-in module was set to 0 mV. The membrane current was filtered at 2 kHz by the Bessel filter of the EPC 9 and then sampled at 10 kHz. The membrane capacitance, access conductance and membrane conductance were calculated at 1 kHz.

Cells that generated a peak calcium current smaller than  $-50$  pA upon the step depolarization from  $-80$  mV to  $+10$  mV were left out

of the analysis. The number of Ca<sup>2+</sup> ions that entered the cell during a step depolarization or during an AP was determined using the following equation:

$$\text{No. of Ca}^{2+} \text{ ions} = \frac{\int I_{\text{Ca}}(t) dt}{2F} N_A,$$

where  $t$  is time,  $F$  is Faraday's constant (96 485 C mol<sup>-1</sup>) and  $N_A$  is Avogadro's constant (6.022 × 10<sup>23</sup> mol<sup>-1</sup>). Tail currents were included in this integration. Leak currents were not corrected for.

### Data analysis

The amount of exocytosis was calculated as the difference between the mean of 100 membrane capacitance samples before and the first 10 samples following a particular depolarizing pulse or AP template. The amount of endocytosis was calculated as the difference between the first membrane capacitance sample after a pulse and the mean of the last five samples before the next pulse. The numerical values of exocytosis and endocytosis thus obtained were corrected for transient capacitance changes ( $\Delta C_i$ , amounting to  $2.0 \pm 0.29$  fF and  $-1.1 \pm 0.21$  fF, respectively) that are not related to Ca<sup>2+</sup> influx (Horrigan & Bookman, 1994; Mansvelder & Kits, 1998).  $\Delta C_i$  was determined by depolarizing the cell in the presence of Ni<sup>2+</sup> (40  $\mu\text{M}$ ) and Cd<sup>2+</sup> (100  $\mu\text{M}$ ) to block all calcium currents ( $n = 4$  cells, not shown). The statistical significance of differences of means was determined with Student's  $t$  test, using Systat software (Evanston, IL, USA). Means mentioned in the text are given with standard errors of the mean (s.e.m.) unless mentioned otherwise. Error bars presented signify s.e.m.

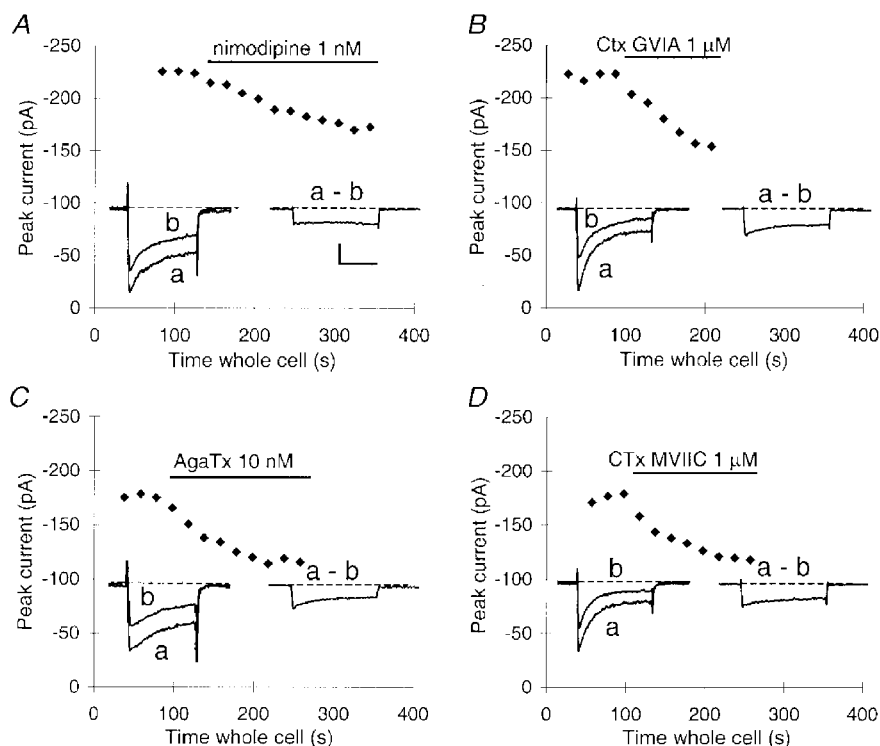
### Pharmacology

Calcium channel blockers were applied with pressure ejection from a glass pipette. Nimodipine was purchased from RBI (Natick, MA, USA),  $\omega$ -conotoxin GVIA ( $\omega$ -CgTx GVIA),  $\omega$ -agatoxin TK ( $\omega$ -AgTx) and  $\omega$ -conotoxin MVIIC ( $\omega$ -CgTx MVIIC) were obtained from Alamone Labs (Jerusalem, Israel). The toxins were stored as stock solutions at  $-20^\circ\text{C}$  in small aliquots for single use, and diluted to their final concentrations in external medium immediately before the experiments started. Nimodipine was stored as a stock solution of  $10^{-2}$  M in methanol at  $-20^\circ\text{C}$  and only diluted to the final concentration in external solution immediately before the experiments. The amount of methanol was below 0.1%, which did not affect calcium currents.

## RESULTS

### Pharmacology of the Ca<sup>2+</sup> currents

Our first goal in this study was to examine the contribution of different Ca<sup>2+</sup> channel types to exocytosis at 33 °C. Therefore, we first determined the pharmacological sensitivity of the whole-cell Ca<sup>2+</sup> current at 33 °C by measuring the amplitude and the kinetics of the block induced by specific blockers of the various HVA Ca<sup>2+</sup> channels. Ca<sup>2+</sup> currents were stable for at least 7 min of recording, before rundown began ( $n = 7$ , not shown). Control applications of bath solution did not reduce the Ca<sup>2+</sup>



**Figure 1.** Kinetics of block of the whole-cell HVA Ca<sup>2+</sup> current by different blockers

Block was induced by 1 nM nimodipine (A), 1  $\mu\text{M}$   $\omega$ -CgTx GVIA (B), 10 nM  $\omega$ -AgTx TK (C) and 1  $\mu\text{M}$   $\omega$ -CgTx MVIIC (D). Every data point represents the peak inward current reached during a 40 ms depolarization from  $-80$  mV to  $+10$  mV (holding potential,  $-80$  mV). To allow Ca<sup>2+</sup> channels to recover from inactivation, the interval between depolarizations was 20 s. All drugs were applied after three or four stable control pulses. Bars indicate the timing of drug applications. Left insets, example traces in the absence (a) and presence (b) of the Ca<sup>2+</sup> channel blocker. Right insets, means of the current blocked by the Ca<sup>2+</sup> channel blocker.

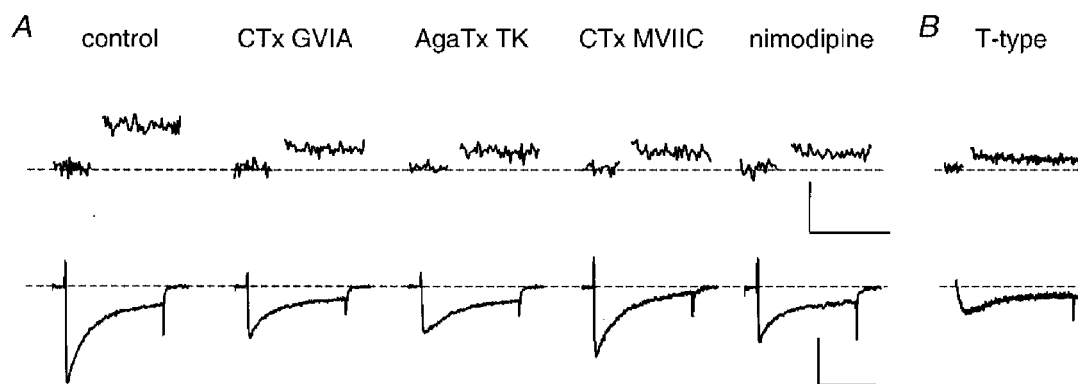
current ( $n=4$ , not shown). The mean peak  $\text{Ca}^{2+}$  current elicited by short step depolarizations from  $-80$  mV to  $+10$  mV at  $33^\circ\text{C}$  was  $-174 \pm 75$  pA (mean  $\pm$  s.d.;  $n=60$ ). We used four drugs to block the different HVA  $\text{Ca}^{2+}$  currents found in these cells (Mansvelder *et al.* 1996; Ciranna *et al.* 1996; Fig. 1). All drugs took 2–3 min to establish a steady-state level of block (Fig. 1A–D). L-type  $\text{Ca}^{2+}$  channels constituted  $21 \pm 4\%$  of the whole-cell  $\text{Ca}^{2+}$  current at  $33^\circ\text{C}$  ( $n=8$ ), N-type channels constituted  $30 \pm 2\%$  ( $n=6$ ), P-type channels constituted  $28 \pm 3\%$  ( $n=10$ ) and Q-type channels constituted  $25 \pm 4\%$  ( $n=7$ ). At  $33^\circ\text{C}$  the L-type current was non-inactivating (see insets of Fig. 1A), whereas the N-, P- and Q-type currents were all inactivating (see insets of Fig. 1B–D). These results differ somewhat from previous data obtained at room temperature and with  $\text{Ba}^{2+}$  as the charge carrier. Under these conditions, the contributions were 35% for the L-type, 26% for the N-type and 31% for the combined P- and Q-type currents (Mansvelder *et al.* 1996). The differences in percentages between these conditions might result from different temperature dependencies, or from differences in the relative conductance of  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  for different channel types.

In separate experiments we confirmed earlier data of Keja *et al.* (1992) that melanotropes express LVA T-type channels. At  $33^\circ\text{C}$ , a transient current response was obtained upon stepping from  $-80$  mV to  $-40$  mV, which was completely blocked by  $40 \mu\text{M}$   $\text{Ni}^{2+}$  ( $n=3$ , data not shown).

### Contribution of different $\text{Ca}^{2+}$ channel types to exocytosis

Next, we determined the role of each of the different  $\text{Ca}^{2+}$  channel types in secretion in melanotropes. To this end, we first examined the effects of the HVA  $\text{Ca}^{2+}$  channel blockers on changes in membrane capacitance ( $\Delta C_m$ ). Exocytosis in melanotropes is a multiphasic process involving different pools of vesicles, which differ from each other in release readiness (Thomas *et al.* 1993a; Parsons *et al.* 1995). The rate of release of these pools ranges from tens of milliseconds up to several seconds. We wanted to study the role of  $\text{Ca}^{2+}$  channels in the release of the predocked, immediately releasable pool of vesicles, because this only depends on the distance between the  $\text{Ca}^{2+}$  channels and vesicles, and not on additional priming steps. The closer a channel is to the vesicles, the more efficiently that  $\text{Ca}^{2+}$  channel couples to exocytosis.

Exocytosis of this small group of predocked release-ready vesicles in melanotropes is complete in  $\sim 50$  ms at  $30$ – $34^\circ\text{C}$  (Thomas *et al.* 1993a). To induce the release of vesicles only from this pool, we used step depolarizations to  $+10$  mV of 40 ms. Prior to the step depolarizations the cells were exposed for 3 min to either bath solution alone or bath solution containing one of the  $\text{Ca}^{2+}$  channel blockers. Under control conditions, the step depolarization elicited a  $\text{Ca}^{2+}$  current of  $-173 \pm 12$  pA which induced a  $\Delta C_m$  of  $17.1 \pm 2.1$  fF ( $n=18$ , Figs 2A, and 3A and B). This confirms earlier data from melanotropes (Mansvelder & Kits, 1998) that only a small fraction of the total immediately releasable pool (250 fF; Parsons *et al.* 1995) is



**Figure 2.** All classes of  $\text{Ca}^{2+}$  channel contribute to release, as indicated by selective activation of T-type channels or application of selective blockers of HVA channels

A, upper traces, changes in  $C_m$  in response to a step depolarization of 40 ms. The control  $C_m$  response and the responses in the presence of the  $\text{Ca}^{2+}$  channel blockers were evoked by depolarizations from  $-80$  mV to  $+10$  mV. Lower traces,  $\text{Ca}^{2+}$  currents evoked by the step depolarizations. In the presence of blockers, the  $\text{Ca}^{2+}$  currents were reduced. Single example traces are shown. B, upper trace, the  $C_m$  change in response to only T-type current activation evoked by a 40 ms step depolarization from  $-80$  mV to  $-40$  mV. Lower trace, example of the T-type current response at  $-40$  mV. Application of either control solution or solutions containing one of the blockers was started 3 min before the step depolarizations were applied. Each point in the  $C_m$  traces is a consecutive mean of 10  $C_m$  samples with a 1 ms time resolution. Thus, the resulting time resolution of the  $C_m$  traces shown here is 10 ms per sample. Each cell was subjected to only one set of five step depolarizations (1 Hz), because a second train of depolarizations always evoked less exocytosis than the first train. The traces shown here are the means of three single traces obtained from one cell per group.  $C_m$  measurements were interrupted during the depolarizations.

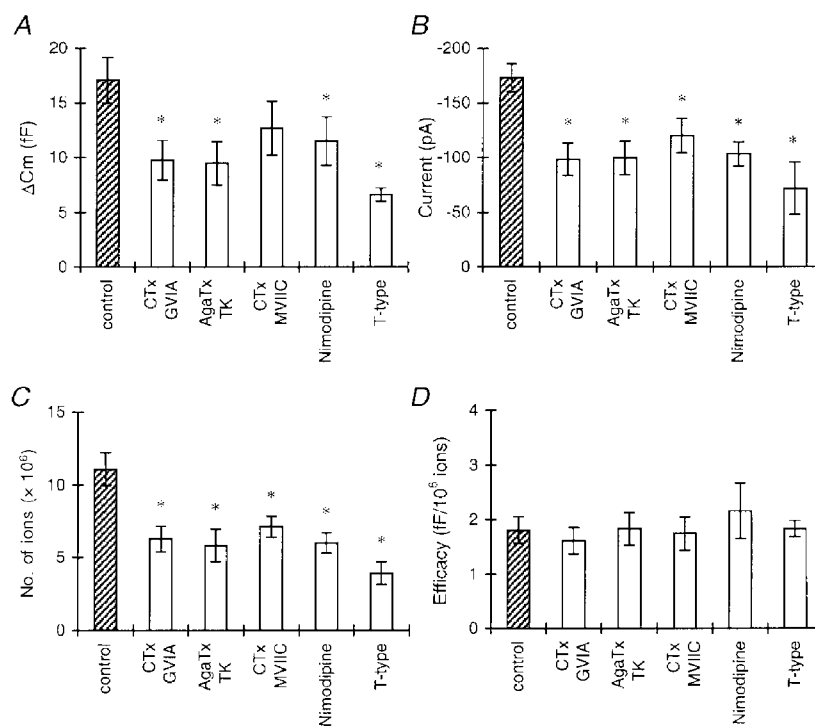
released by a 40 ms depolarization. In cells that were exposed to a Ca<sup>2+</sup> channel blocker, both the Ca<sup>2+</sup> current and the increase in capacitance in response to step depolarizations were reduced (Fig. 2A). The effects of the blockers on  $\Delta C_m$  were proportional to the effects on the Ca<sup>2+</sup> current (Fig. 3A and B). These data show that all HVA Ca<sup>2+</sup> channel types contribute to exocytosis in melanotropes.

This conclusion is substantiated when we compare the efficacy of Ca<sup>2+</sup> in stimulating exocytosis (Fig. 3D). The reduction of the Ca<sup>2+</sup> current implies a decrease in the number of Ca<sup>2+</sup> ions that enter the cell during the depolarization (Fig. 3C). Under control conditions the efficacy for the HVA channels, defined as the change in  $C_m$  per 10<sup>6</sup> Ca<sup>2+</sup> ions entering the cell, was  $1.8 \pm 0.25$  fF (10<sup>6</sup> Ca<sup>2+</sup> ions)<sup>-1</sup>. In the presence of blockers the efficacy was not significantly different from that of the control group (Fig. 3D;  $P > 0.5$ ). Thus, the efficacy of Ca<sup>2+</sup> in stimulating exocytosis is constant for all conditions and is not affected if the entry of Ca<sup>2+</sup> through a certain type of Ca<sup>2+</sup> channel is blocked. From these data, we conclude that all HVA Ca<sup>2+</sup> channel types couple to exocytosis with a similar strength.

To establish whether the LVA T-type channels also contribute to release, we recorded the capacitance change upon a 40 ms step to -40 mV. This step depolarization, which only evoked a T-type current response (see above), did induce an increase in  $C_m$  (Figs 2B and 3). In six cells, the mean  $\Delta C_m$  was  $8.6 \pm 0.61$  fF, and the efficacy was  $1.8 \pm 0.15$  fF (10<sup>6</sup> Ca<sup>2+</sup> ions)<sup>-1</sup>. Thus T-type channels also couple to exocytosis in melanotropes and do so with a similar efficacy to that of HVA channels.

### APs in melanotropes

Ca<sup>2+</sup> channels play an important role in the endogenous electrical activity of melanotropes, since the major part of the AP depends on the activation of voltage-gated Ca<sup>2+</sup> channels (Douglas & Taraskevich, 1980, 1982; Williams *et al.* 1990). Since all HVA Ca<sup>2+</sup> channels, despite their differences in activation and inactivation kinetics and voltage dependencies, couple with equal efficiency to exocytosis, a robust and constant relation between calcium influx and exocytosis during the AP is expected. This was specifically tested by asking whether the shape and duration of the APs influence the strength of excitation–secretion coupling.

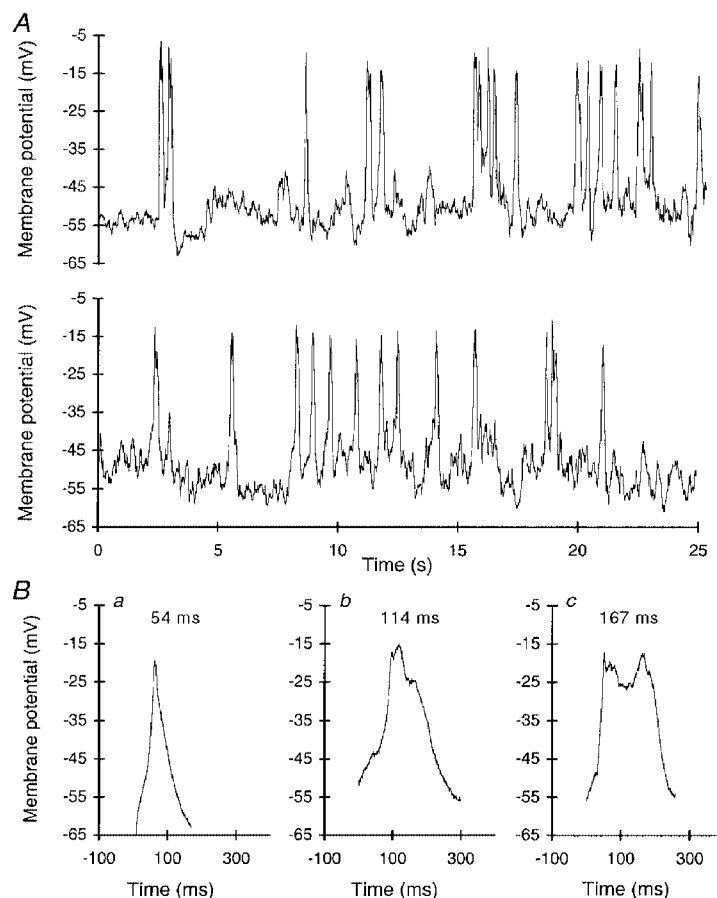


**Figure 3.** All classes of calcium channel couple with equal efficiency to exocytosis

A, the amount of exocytosis per step depolarization for control conditions (total HVA current), selective activation of the T-type current, and the HVA current in the presence of selective HVA Ca<sup>2+</sup> channel blockers. First, the mean  $\Delta C_m$  elicited by five step depolarizations was calculated per cell, then the mean for the total group was calculated. Asterisks in A–C indicate statistical differences from the control group ( $P < 0.05$ ). B, peak amplitudes of the control Ca<sup>2+</sup> current, the T-type current, and the HVA current in the presence of different Ca<sup>2+</sup> channel blockers. C, number of Ca<sup>2+</sup> ions entering the cell during a step depolarization, calculated as described in Methods. D, efficacy of Ca<sup>2+</sup> ions in stimulating exocytosis.  $\Delta C_m$  was divided by the number of Ca<sup>2+</sup> ions entering the cell for each depolarizing pulse. For each cell, the mean efficacy for five pulses was calculated. Shown are the means of a number of cells (see text) per group. There was no significant difference between the control group and the groups with the Ca<sup>2+</sup> channel blockers. (Stimulation protocols are as described for Fig. 2.)

We made membrane potential recordings from 49 melanotropes to obtain endogenously generated APs that could be used in voltage-clamp experiments as physiological stimuli to induce exocytosis. The resting membrane potential ranged from  $-35$  to  $-60$  mV, with a mean of  $-49 \pm 6$  mV ( $\pm$  s.d.), which is in accordance with earlier reports (Williams *et al.* 1989; Stack & Suprenant, 1991; Lee, 1996). Usually the membrane potential was quite unstable, showing small depolarizations of 5–10 mV. Twenty-two cells ( $\sim 45\%$ ) were spontaneously active at  $33^\circ\text{C}$ , generating APs (Fig. 4A). Mostly, the AP patterns were irregular. Spontaneous activity declined with recording time, and cells became silent after  $\sim 5$  min, possibly due to washout of a soluble component, essential for activity. The duration of the APs ranged from 50 to 300 ms (mean  $\pm$  s.d.,  $135 \pm 43$  ms, measured at  $\sim 10$  mV above resting membrane potential). This is in the range of previously reported values for stimulated APs (15–180 ms; Douglas & Taraskevich, 1978, 1980). In contrast to stimulated APs, the peak value of the spontaneous APs never exceeded 0 mV in our measurements. The maximal membrane potential that was reached during an AP was  $-14 \pm 6$  mV (mean  $\pm$  s.d.), resulting in an AP amplitude of  $\sim 35$  mV. The frequency and patterning of AP firing differed widely between cells.

Since there was considerable variation in the duration of the APs, we selected three AP waveforms that differed widely in shape and duration to cover this variation (Fig. 4B). The AP waveforms had similar peak amplitudes. The shortest waveform lasted 54 ms at half-maximal amplitude (Fig. 4Ba). The AP waveform of intermediate duration lasted 114 ms at half-maximal amplitude (Fig. 4Bb). The waveform of longest duration lasted 167 ms at half-maximal amplitude (Fig. 4Bc). These AP waveforms were slightly modified to be used as templates in voltage-clamp experiments for the following reasons. (1) To be able to monitor the membrane capacitance at high amplitude and time resolution, a sine wave of 40 mV amplitude was used. Reliable capacitance recordings require that the membrane conductance remains constant during the entire sine wave (Neher & Marty, 1982; Gilles, 1995). To achieve this a holding potential of  $-80$  mV was used, around which the sine wave fluctuates between  $-60$  and  $-100$  mV. Within this voltage range no  $\text{Ca}^{2+}$  channels were activated. The APs were adjusted to slowly ramp from  $-80$  mV to about the original resting membrane potential, without activating any  $\text{Ca}^{2+}$  current. (2) We wanted to measure the membrane capacitance as soon as the waveform ended. The use of the PULSE software allowed us to measure the membrane



**Figure 4.** AP recordings from melanotropes firing spontaneously

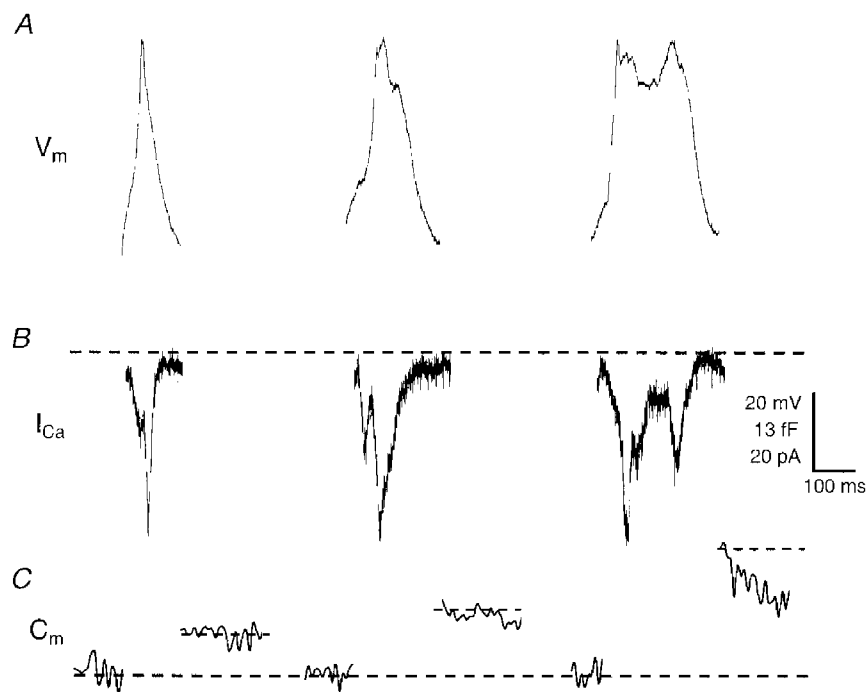
A, example of the spontaneous activity of a melanotrope (consecutive traces). B, APs that were selected to be used as templates for stimulation in voltage-clamp experiments. Durations at half-maximal amplitude are given above the traces.

capacitance with only a 5 ms delay. It required us, however, to re-construct the APs as a series of ramps.

### The efficacy of different AP waveforms in eliciting exocytosis

To determine how much exocytosis was elicited by each of the three AP waveforms, we stimulated cells 5 times in succession at 1 Hz with one of the AP waveforms (Fig. 5A). We monitored the calcium current during the AP waveform (Fig. 5B) and we recorded the membrane capacitance before and immediately after each imposed AP waveform (Fig. 5C). The Ca<sup>2+</sup> current elicited by the AP waveform was in all cases multiphasic, partly due to the successive activation of different types of Ca<sup>2+</sup> channel at increasingly depolarized membrane potentials. In most cells, a local maximum was reached by the Ca<sup>2+</sup> current during the rising phase of the AP waveform at approximately -50 mV (Fig. 5B). This Ca<sup>2+</sup> current solely flows through the LVA T-type channels, since all current at membrane potentials up to -40 mV is blocked by 40 μM Ni<sup>2+</sup> (see above and Keja *et al.* 1992). The absolute maximum of the Ca<sup>2+</sup> current (-44 ± 14 pA, mean ± s.d.) coincided with the peak of the AP waveform. This maximum is significantly lower than the -175 pA that is reached when cells are stimulated with a step depolarization (see above). During the decay of the AP waveform, the Ca<sup>2+</sup> current deactivated. The duration of the Ca<sup>2+</sup> current increased with increasing AP waveform duration.

All three AP waveforms induced exocytosis, but the amount of exocytosis increased with increasing AP waveform duration (Fig. 5C). Upon stimulation with the short AP waveform, C<sub>m</sub> increased by 5.4 ± 0.9 fF (n = 12, Fig. 6A). The intermediate and the long AP waveforms induced a change in C<sub>m</sub> of 12.1 ± 1.2 fF (n = 10) and 18.1 ± 2.3 fF (n = 8), respectively. As can be seen from Fig. 6A, C<sub>m</sub> increased linearly with AP waveform duration. The number of Ca<sup>2+</sup> ions that entered the cell during the AP waveforms also increased linearly with the AP waveform duration (Fig. 6B). This was not due to a change in the peak amplitude of the Ca<sup>2+</sup> current, since this was the same for all three AP waveforms (Figs 5B and 6C). Rather, it was caused by the increase in duration of the Ca<sup>2+</sup> current. The number of Ca<sup>2+</sup> ions entering the cell per AP waveform increased from 4.7 (± 0.6) × 10<sup>6</sup> during the short AP waveform to 15.6 (± 2.4) × 10<sup>6</sup> during the long AP waveform. As a result, the efficacy of Ca<sup>2+</sup> in stimulating exocytosis, i.e. ΔC<sub>m</sub> per 10<sup>6</sup> Ca<sup>2+</sup> ions, was constant for the three different APs, and amounted to ~1.2 fF (10<sup>6</sup> Ca<sup>2+</sup> ions)<sup>-1</sup> (Fig. 6D). This is well in accordance with the efficacy found with step depolarizations (Mansvelder & Kits, 1998; Kits *et al.* 1999; this study). Thus, the equal efficacy of the different types of Ca<sup>2+</sup> channel in inducing exocytosis in these cells results in a linear relation between AP duration and exocytosis.



**Figure 5. Exocytosis increases with increasing AP duration**

A, AP templates used with the PULSE software. For technical reasons, AP waveforms had to start from a holding potential of -80 mV (see text for explanation). B, example traces of the calcium currents elicited by the APs depicted in A. The peak currents coincided with the peak membrane voltages reached during the AP. C, examples of the membrane capacitance traces associated with the various AP templates. C<sub>m</sub> measurements were interrupted during stimulation with the AP templates. C<sub>m</sub> traces shown are the means of three such traces obtained from single cells. Note the different time scales in B and C.

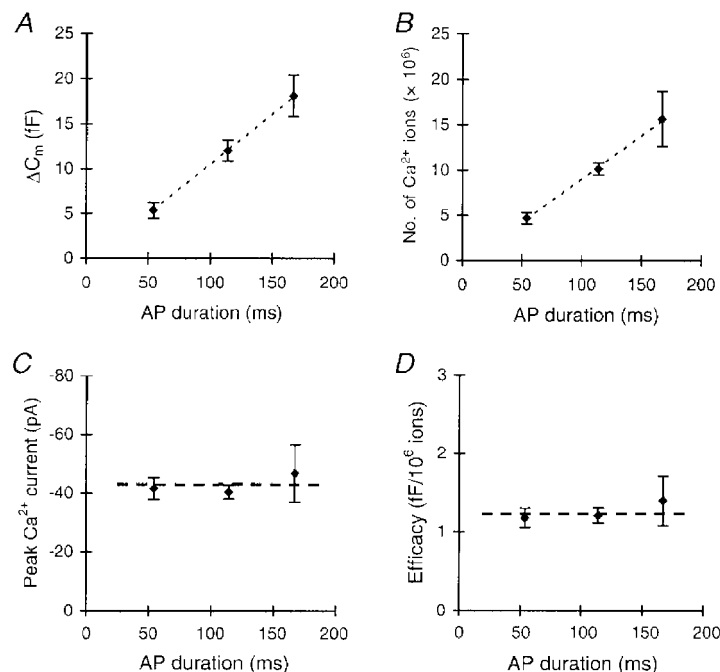
### The efficacy during different phases of the AP

Finally, we examined whether the linearity of the stimulus–secretion coupling also holds during an AP. Due to the different biophysical properties of the five different types of  $\text{Ca}^{2+}$  channel (Keja *et al.* 1992; Keja & Kits, 1994; Mansvelder *et al.* 1996), it is likely that the types of channel that are activated during the rising phase of the AP differ from the types of channel that are activated during the plateau phase. Furthermore, with the ongoing depolarization at the plateau, only the non-inactivating  $\text{Ca}^{2+}$  channels will remain activated. Moreover, the driving force for  $\text{Ca}^{2+}$  during the rising phase will be different from the driving force during the plateau phase. As a result, the different phases of the AP will most probably generate different  $\text{Ca}^{2+}$  concentration profiles under the cell membrane. This may affect the efficacy of  $\text{Ca}^{2+}$  in stimulating exocytosis during different parts of the long AP.

We stimulated melanotropes with increasing fractions of the long AP waveform (Fig. 7A1–4). The first fraction we used consisted of the first half of the rising phase, up to  $-45$  mV (Fig. 7A1). During this voltage trajectory, only LVA T-type channels are activated (see above). The LVA current stimulated exocytosis, albeit weakly (Fig. 7C1). On average, the LVA current had an amplitude of  $-23 \pm 1.9$  pA, which elicited a  $\Delta C_m$  of  $2.2 \pm 0.89$  fF ( $n = 7$ , Fig. 8A and C). We

then monitored exocytosis stimulated with the complete rising phase of the AP waveform (up to  $-17$  mV). The rising phase activated an extra component of the  $\text{Ca}^{2+}$  current, yielding a significantly larger peak current ( $-45 \pm 7.3$  pA;  $P < 0.01$ , Fig. 4C). The amount of exocytosis elicited was also increased to  $6.7 \pm 1.91$  fF (Fig. 7A2–C2). This trend continued when stimulus templates of longer duration were used (Fig. 7A3–C3 and A4–C4).

The amount of exocytosis and the number of entering  $\text{Ca}^{2+}$  ions evoked by the three longer waveforms showed a similar pattern of increase with increasing waveform duration (Fig. 8A and B). In both cases the shortest waveform did not conform to this trend. This most probably results from the significantly smaller amplitude of the LVA current during the shortest waveform (Fig. 8C). The number of entering ions was  $1.58 (\pm 0.13) \times 10^6$  when only the T-type current was activated and it increased to  $18.2 (\pm 2.98) \times 10^6$  upon activation of LVA and HVA currents (during the longest stimulus. Figure 8A and B thus clearly shows that over the entire duration of the waveform, the increasing number of entering  $\text{Ca}^{2+}$  ions correlates with a concomitant increase in exocytosis. As a result, the efficacy of  $\text{Ca}^{2+}$  in stimulating exocytosis was constant for all parts of the AP waveform. It amounted to  $\sim 1.4$  fF ( $10^6 \text{ Ca}^{2+}$  ions) $^{-1}$  for all stimulus durations (Fig. 8D). Thus, although different  $\text{Ca}^{2+}$



**Figure 6.** The efficacy of  $\text{Ca}^{2+}$  ions in stimulating exocytosis is the same for different APs

A, the mean increase in  $C_m$  per AP increases with increasing AP duration. Each cell was subjected once to stimulation with five successive APs at 1 Hz. The mean  $C_m$  response to these stimulations was calculated for each cell, and these means were averaged for a number of different cells. For each AP waveform different cells were taken. B, the number of  $\text{Ca}^{2+}$  ions that entered the cell during the AP increased with increasing AP duration. Dashed lines in A and B are linear regression lines. C, the peak  $\text{Ca}^{2+}$  currents during the APs were the same for the different AP waveforms. D, the efficacy of  $\text{Ca}^{2+}$  ions in stimulating exocytosis, expressed as the ratio of capacitance change per  $10^6 \text{ Ca}^{2+}$  ions, was constant for the different AP waveforms ( $n = 8$ –12). The duration of the idealized APs, as expressed on the horizontal axis in A–D, was measured at the half-maximal amplitude.



concentration profiles may be generated by the different phases of the AP waveform, exocytosis is always evoked with a constant efficacy. Furthermore, the results show that Ca<sup>2+</sup> ions flowing through T-type Ca<sup>2+</sup> channels are equally capable of stimulating exocytosis as Ca<sup>2+</sup> ions flowing through the HVA Ca<sup>2+</sup> channels. Taken together, the data presented in this paper suggest that, in melanotropes, the equal strength with which the Ca<sup>2+</sup> channels couple to exocytosis gives rise to a linear stimulus–secretion coupling during normal AP firing. This suggests that, in melanotropes, the duration of the AP effectively controls the amount of hormone secretion.

## DISCUSSION

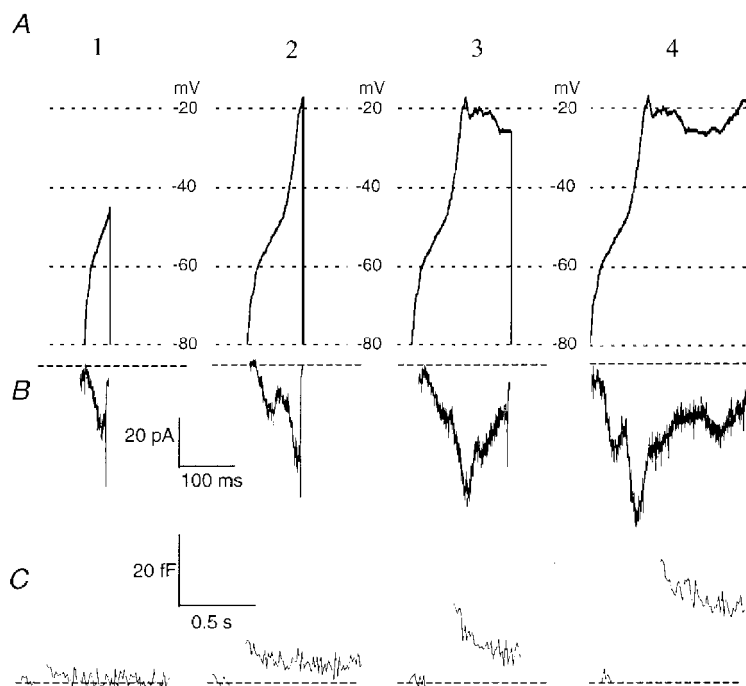
Melanotropes in the intermediate pituitary of the rat secrete hormones from LDCVs upon the influx of Ca<sup>2+</sup> through voltage-gated Ca<sup>2+</sup> channels. Five different types of Ca<sup>2+</sup> channel are expressed by these cells (Mansvelder *et al.* 1996; Ciranna *et al.* 1996). In the present study, we examined whether the different channels couple with different efficiencies to exocytosis. We found that blockers of L-, N-, P- and Q-type Ca<sup>2+</sup> channels all reduced exocytosis. However, none of them affected the efficacy of Ca<sup>2+</sup> ions in stimulating exocytosis, suggesting that HVA Ca<sup>2+</sup> channels couple equally efficiently to exocytosis. Also, when we

selectively activated the LVA T-type channels, exocytosis was evoked with the same efficacy as when the HVA components were also present. These data show that any influx of Ca<sup>2+</sup> ions, regardless of the Ca<sup>2+</sup> channel type through which this occurs, will contribute to exocytosis in an equal fashion.

### Ca<sup>2+</sup> channels and vesicles are not co-localized in melanotropes

In a previous study (Mansvelder & Kits, 1998), we showed that fast Ca<sup>2+</sup>-dependent exocytosis was affected by sub-millimolar concentrations of Ca<sup>2+</sup> buffers. This was taken as evidence that diffusion of Ca<sup>2+</sup> to the predocked vesicles causes a delay between channel activity and secretion that allows Ca<sup>2+</sup> chelators to interfere with exocytosis. Model studies (Kits *et al.* 1999) yielded an adequate description of these data when a distance between Ca<sup>2+</sup> channels and LDCVs of >100 nm was assumed. The present study demonstrates that all Ca<sup>2+</sup> channel classes couple with a similar strength to exocytosis. This reinforces the hypothesis that, in melanotropes, LDCVs do not co-localize with any specific type of Ca<sup>2+</sup> channel, nor with Ca<sup>2+</sup> channels in general.

In adult bovine chromaffin cells, secretion of catecholamines starts with a delay of 5–100 ms and persists for tens of milliseconds after Ca<sup>2+</sup> entry has stopped, which is mostly



**Figure 7. Different phases of the AP contribute equally to exocytosis**

*A*, stimulus templates of the different parts of the AP. Template 1 is the first half of the rising phase up to a voltage of  $-45$  mV. Template 2 comprises the complete rising phase. The last template (4) is the complete double AP without the repolarizing phase. All templates ended with an immediate step back to the rest holding potential of  $-80$  mV. *B*, examples of the Ca<sup>2+</sup> currents evoked by the templates shown in *A*. As in Fig. 2, different peaks can be distinguished in the traces. Trace 1 shows the current carried by LVA T-type channels, since the maximal voltage during stimulation was  $-45$  mV. In the other traces, HVA components were also present. *C*, changes in  $C_m$  evoked by the Ca<sup>2+</sup> currents in *B*. With increasing pulse duration,  $\Delta C_m$  also increased.

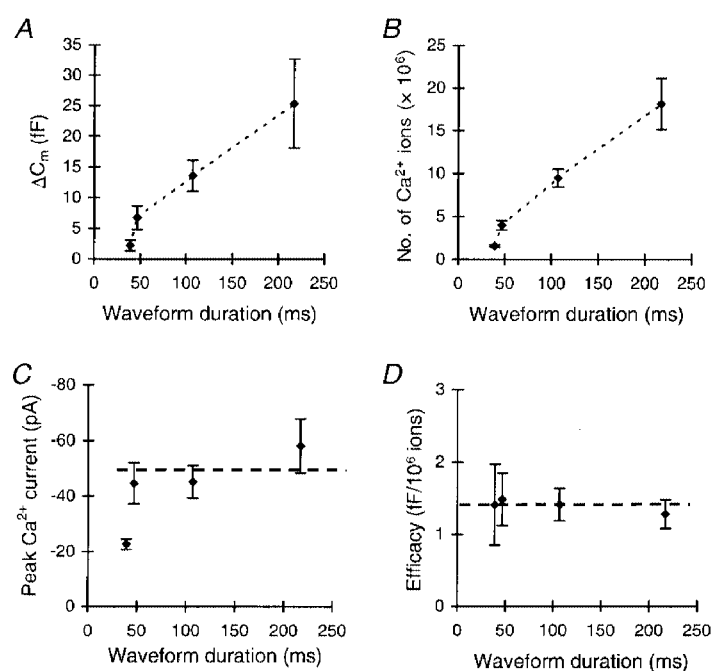
attributable to  $\text{Ca}^{2+}$  diffusion (Chow *et al.* 1992, 1994, 1996). Model studies by Klingauf & Neher (1997) showed that these processes were best accommodated by assuming a distance between  $\text{Ca}^{2+}$  channels and the majority of vesicles of  $\sim 200\text{--}300$  nm. Only a small fraction ( $\sim 8\%$ ) of the LDCVs had to be located at a distance of  $\sim 30$  nm to account for the short delays of  $\sim 5$  ms (see also Seward & Nowycky, 1996; Elhamdani *et al.* 1998). We cannot exclude the possibility that in melanotropes a small fraction of LDCVs are docked within  $\sim 30$  nm from a  $\text{Ca}^{2+}$  channel, since the ultrafast release of this fraction would probably not be detected with our approach. However, since only  $\sim 5\text{--}20$  vesicles (assuming 1 fF per LDCV) are released during an AP, this would pertain to only 1–2 vesicles, even if  $\sim 10\%$  of the vesicles were docked this close to the  $\text{Ca}^{2+}$  channels. We conclude that in melanotropes, as in chromaffin cells, the majority of the predocked, rapidly releasable LDCVs do not co-localize with  $\text{Ca}^{2+}$  channels.

The absence of co-localization of vesicles and  $\text{Ca}^{2+}$  channels indicates that none of the  $\text{Ca}^{2+}$  channel types expressed by melanotropes has a leading role in exocytosis. This distinguishes melanotropes from neurons and many other neuroendocrine cells. In different neuroendocrine cell types, preferential docking of release-ready LDCVs near specific types of  $\text{Ca}^{2+}$  channel does occur, thereby ensuring the coupling of secretion to these channels. Examples are vaso-

pressinergic neurohypophyseal nerve endings (Wang *et al.* 1997), mouse pancreatic  $\beta$ -cells (Bokvist *et al.* 1995), pancreatic A-cells (Gromada *et al.* 1997) and calf chromaffin cells (Artalejo *et al.* 1994; Elhamdani *et al.* 1998). In these preparations, the docking of LDCVs near  $\text{Ca}^{2+}$  channels appears to be precisely regulated and gives rise to distinct roles for specific  $\text{Ca}^{2+}$  channel types in exocytosis. This mechanism is most probably absent in melanotropes, rendering all  $\text{Ca}^{2+}$  channel types equally important in stimulating exocytosis.

#### A linear relationship between calcium entry and secretion characterizes stimulus–secretion coupling in melanotropes

One of the remarkable features of the release process of LDCVs in melanotropes is the linear relationship between the number of  $\text{Ca}^{2+}$  ions that enter the cell and the amount of exocytosis that is induced. This property holds during a train of step depolarizations, with different intracellular EGTA concentrations and with different pulse durations (Mansvelder & Kits, 1998; Kits *et al.* 1999; this study). Here, we found that exocytosis stimulated by AP waveforms with very different shapes and durations still displayed a constant relation to  $\text{Ca}^{2+}$  influx. Most probably, the equal strength with which  $\text{Ca}^{2+}$  channels couple to exocytosis results in a linear stimulus–secretion coupling when cells fire APs.



**Figure 8.** The efficacy in stimulating exocytosis is the same for different phases of the AP

*A*, mean capacitance change evoked by different phases of the AP, plotted as  $\Delta C_m$  against waveform duration, measured at the half-maximal amplitude. Each cell was subjected to one train of five stimuli. *B*, the number of  $\text{Ca}^{2+}$  ions entering the cell during a stimulus for the different stimulus templates. Note the very similar patterns of increase in the number of entering ions and in the  $\Delta C_m$  in *B* and *A*. *C*, maximal amplitude of the  $\text{Ca}^{2+}$  currents during the stimulations. Asterisks denote a significant difference from the peak current in the other groups at  $P < 0.05$ . *D*, the efficacy of  $\text{Ca}^{2+}$  ions in stimulating exocytosis for the different stimulus templates, expressed as the capacitance change per  $10^6$   $\text{Ca}^{2+}$  ions ( $n = 7$  for each template).

In general, the release of LDCVs from neuroendocrine cells has a third or fourth power relation to either the extracellular or intracellular Ca<sup>2+</sup> concentration:

$$\text{Release} \propto [\text{Ca}^{2+}]^n,$$

where  $n$  is 3 or 4 (Dodge & Rahamimoff, 1967; Augustine & Charlton, 1986; Stanley, 1986; Thomas *et al.* 1990; Heinemann *et al.* 1993; Borst & Sakmann, 1996). This power relation between concentration and secretion also exists in melanotropes (Thomas *et al.* 1990). However, little attention has been given to the question of how this power relation translates to the relation between the number of entering Ca<sup>2+</sup> ions and the amount of exocytosis that is stimulated by these ions. Our findings indicate that this relation can have a very stable linearity.

In adrenal chromaffin cells, exocytosis relates to the Ca<sup>2+</sup> concentration with a power of 3 (Heinemann *et al.* 1993), yet the relation between Ca<sup>2+</sup> entry and exocytosis is characterized by a power of  $\sim 1.9$  (Engisch & Nowycky, 1996). This relation is resistant to a number of different experimental conditions and protocols (Engisch & Nowycky, 1996). In nerve terminals in the posterior pituitary (Hsu & Jackson, 1996) and in the bullfrog sympathetic ganglia (Peng & Zucker, 1993) exocytosis of LDCVs increases linearly with Ca<sup>2+</sup> influx. Finally, in the giant axon of the squid, where the amplitude of the postsynaptic current also scales to the third or fourth power of the extracellular Ca<sup>2+</sup> concentration (Augustine & Charlton, 1986; Stanley, 1986), the postsynaptic current scales linearly to the duration of the AP and the ensuing presynaptic Ca<sup>2+</sup> entry (Augustine, 1990). Evidently, a linear relation between Ca<sup>2+</sup> entry and exocytosis in a system where a higher power relation between calcium concentration and exocytosis is found, is not limited to neuroendocrine systems, but also extends to neurons.

#### How the power relation might translate into linearity

How is it possible that a higher power relation between Ca<sup>2+</sup> concentration and exocytosis translates into a linear relation between Ca<sup>2+</sup> entry and exocytosis? To explain the linearity in the squid giant presynaptic terminal, Augustine (1990) suggested that Ca<sup>2+</sup> might trigger secretion from domains that do not overlap. Increasing the Ca<sup>2+</sup> current by increasing the duration of the AP would then trigger increasingly more domains to release. This most probably offers no explanation for the linearity of the relation between Ca<sup>2+</sup> entry and exocytosis in the melanotrope, since the distance between Ca<sup>2+</sup> channels and LDCVs is large (100–200 nm; Mansvelder & Kits, 1998; Kits *et al.* 1999). Domains from which secretion is triggered will most probably overlap.

For the melanotrope, we favour an alternative explanation which is based upon buffer saturation occurring locally under the plasma membrane (Kits *et al.* 1999). Multiple factors (e.g. size and duration of the single channel currents, diffusion and buffer conditions) influence the relation between influx and concentration. Using computer

simulations, we found earlier that the relation between entry and exocytosis can be linear, depending on the assumed conditions for diffusion (Kits *et al.* 1999). Under the assumptions of homogeneous calcium entry and free diffusion of Ca<sup>2+</sup> and intracellular calcium buffers in all directions, the efficacy of Ca<sup>2+</sup> ions in stimulating exocytosis is not constant, but increases with pulse duration (Kits *et al.* 1999). The constant relation emanates when an inhomogeneous cytoplasm is assumed, where diffusion of Ca<sup>2+</sup> ions and buffers is not free in all directions, for instance due to the presence of intracellular membranes. Mobile Ca<sup>2+</sup> buffers become saturated locally and the Ca<sup>2+</sup> concentration at the site of exocytosis is to a lesser extent determined by the buffer conditions. Under defined conditions, this results in a linear relation between Ca<sup>2+</sup> entry and exocytosis (Kits *et al.* 1999). Limited diffusion and buffer saturation thus obscure the third power relation between Ca<sup>2+</sup> concentration and exocytosis. The present finding that the linear relation bears relevance to the situation where APs stimulate exocytosis may indicate that also during an AP diffusion limited by barriers and buffer saturation primarily determines the relation between Ca<sup>2+</sup> entry and secretion. Extrapolating our results to the adrenal chromaffin cell, where the distance between Ca<sup>2+</sup> channels and the majority of the vesicles is also large (Klingauf & Neher, 1997), the deviation from the third power factor may suggest that buffer saturation plays a substantial role in stimulus–secretion coupling in these cells as well.

#### Physiological significance of a linear relationship between Ca<sup>2+</sup> entry and exocytosis

Earlier work on melanotropes revealed that APs contain a large Ca<sup>2+</sup> current-dependent component (Douglas & Taraskevich, 1980, 1982; Williams *et al.* 1989). This is confirmed by our findings: Ca<sup>2+</sup> currents were activated over the entire duration of the AP. In synaptic terminals, in squid as well as in mammals, where APs have a much shorter duration, the Ca<sup>2+</sup> current seems to be predominantly a tail current, reinforced by the increasing driving force during the falling phase of the AP (Augustine, 1990; Borst & Sakmann, 1996). At physiological temperatures, however, the peak of the Ca<sup>2+</sup> current can coincide with the peak of the AP (Sabatini & Regehr, 1997). In melanotropes, Ca<sup>2+</sup> currents are markedly activated during the rising phase of the AP. Most probably, LVA T-type Ca<sup>2+</sup> channels play an important role in initiating the rising phase of the AP, since activation of the Ca<sup>2+</sup> current had already started at  $-60$  mV and the first maximum was reached at around  $-50$  mV. By using only the initial phase of the AP to stimulate exocytosis, we found that the influx of Ca<sup>2+</sup> through the LVA T-type channels not only serves excitability (Llinás & Yarom, 1981), but also contributes to exocytosis. Interestingly, it stimulated exocytosis with the same efficacy as Ca<sup>2+</sup> entry during later phases of the AP.

Unlike in melanotropes, in other neuroendocrine cells such as mouse pancreatic  $\beta$ -cells and rat chromaffin cells, single

APs couple poorly to exocytosis (Ämmälä *et al.* 1993; Zhou & Mislser, 1995), while bursts of APs (four or more APs in  $\beta$ -cells) evoked exocytosis consistently. However, APs in the latter cell types only last a few milliseconds instead of 50–300 ms in melanotropes. In calf chromaffin cells (Elhamdani *et al.* 1998), single fast APs are able to elicit secretion reliably, because LDCVs are docked very close to  $\text{Ca}^{2+}$  channels. The slow rising phase of APs in melanotropes, with respect to the fast APs of chromaffin cells, may be well tuned to the large distance between  $\text{Ca}^{2+}$  channels and vesicles. With slow AP kinetics, a strict colocalization of channels and vesicles is simply not needed. The equal strength with which all  $\text{Ca}^{2+}$  channels couple to exocytosis guarantees that there is exocytosis over the entire duration of the AP.

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