AN EXOCYTOTIC RELEASE PATHWAY TRIGGERED BY Ca^{2+} INFLUX VIA Ca^{2+} CHANNELS IN CHROMAFFIN CELLS FROM RATTUS NORVEGICUS.

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Secretion of catecholamine in bovine chromaffin cells is thought to occur primarily when exocytosis of docked secretory vesicles is triggered by μ-domains of Ca^{2+} which develop to a concentration of several μM near the inner opening of Ca^{2+} channels (Augustine and Neher, J. Physiol. 450:247-271, 1992). It is also recognized that the global, as well as the local, intracellular concentration of Ca^{2+} may cause exocytosis or determine the size of the pool of release-ready, docked vesicles. Furthermore, it is thought that intracellular Ca^{2+} stores contribute to the control of secretion, that different types of Ca^{2+} channels (L,P,Q etc.) may not trigger secretion equally, and that both the internal stores and the diversity of Ca^{2+} channels vary from species to species.

In the present study we have examined the control of secretion in single rat chromaffin cells under stringent experimental conditions where the time course and magnitude of the secretory response was compared directly to both Ca^{2+} current and global intracellular Ca^{2+} concentration. For this purpose we used simultaneously the whole-cell voltage clamp technique to measure Ca^{2+} current (I_{Ca}), fluorescent dyes to detect intracellular Ca^{2+} transients (Ca_i-transients), and mono-filament carbon fiber electrodes to record the current resulting from oxidation of released catecholamines.

Primary cultures of rat chromaffin cells were prepared by enzymatic digestion followed by separation on a sucrose gradient (Fan, Cleemann, Lara, Gandia and Morad, Bull. MDIBL 34:12-13, 1995). The cells were suspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin and were plated onto glass cover slips which had been coated with 50 μg/ml collagen and then dried and baked. Experiments were performed at room temperature after culturing from 2 to 6 days. The standard external solution contained (in mM): 125 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES at pH 7.4. K^+ was omitted from this solution in order to suppress Ca-activated K^+ current. The whole cell patch-clamp electrodes had a resistance of 4-5 MΩ and were filled with a dialyzing solution containing (in mM): 80 Cs-aspartate, 30 CsCl, 5 Mg-ATP, 0.05-0.2 Ca-indicator dye and 10 HEPES at pH 7.2. The Ca-indicator dyes (Calcium-green or Fluo-3) were excited with epi-illumination using 488 nm light from an argon laser and emitted light was recorded with photo-multiplier detector placed behind a 500 nm glass barrier filter. This method allows measurements of relative rather than absolute increases in cytosolic calcium. The carbon fiber electrodes used for the recording of exocytosis of single catecholamine containing vesicles were insulated with polyethylene tubing, were biased with 600 mV, and had an exposed 8 μm tip which was pressed gently against one side of the voltage-clamped cell (diameter 10-20 μm, capacitance 8-25 pF). The secretion was measured as the average oxidation current recorded typically during 3 to 10 voltage clamp pulses.

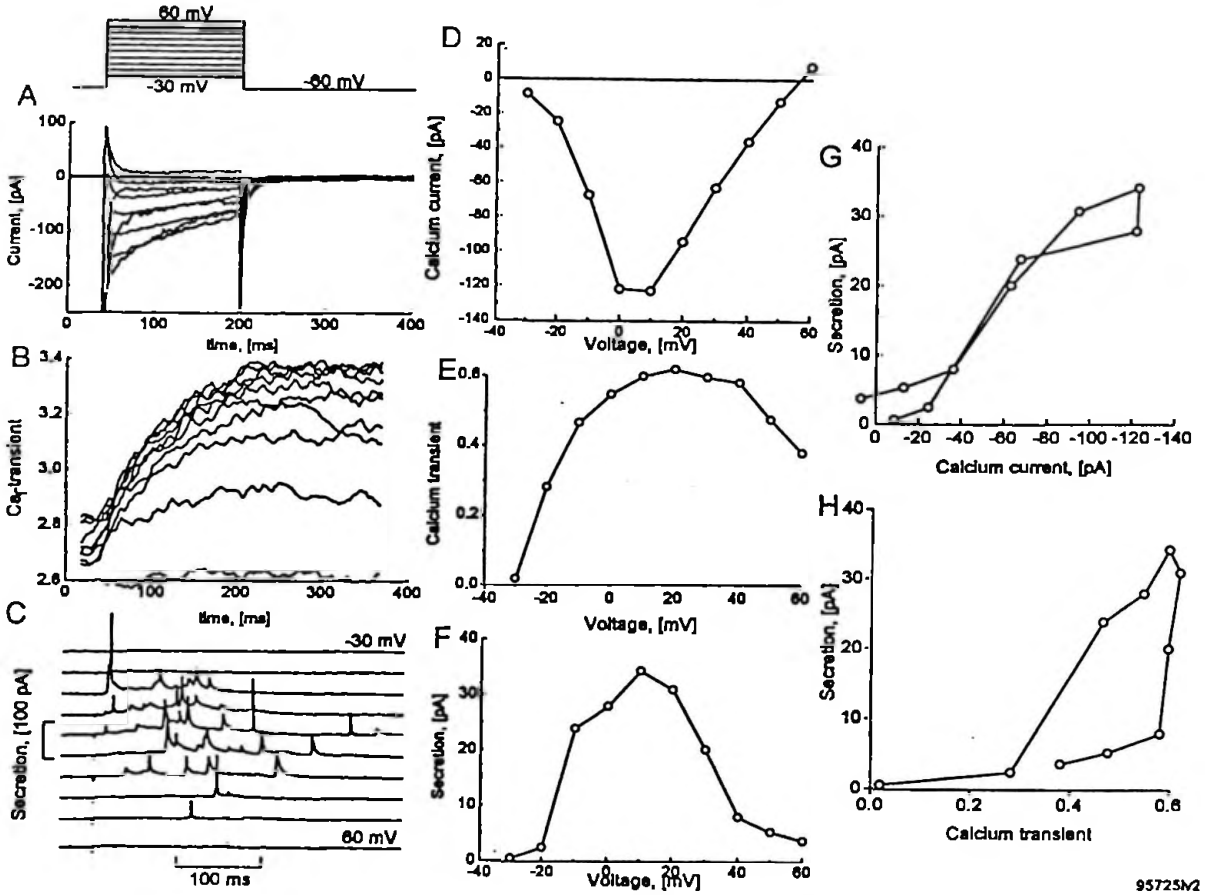

Figure 1. Voltage dependence of I_{Ca} (panels A, D and G), Ca_i-transients (panels B, E, and H), and secretion (panels C, G, F and H). Original records are shown on the left, voltage relations in the middle and correlations on the right. The Ca^{2+} current (panel D) was measured 40 ms after depolarization. The Ca_i-transients (panel E) are uncalibrated and were measured as the change in fluorescence from the start to the finish of the clamp pulse. Secretion (panel F) is the average oxidation current calculated as the integral charge of the secretory spikes divided by the clamp duration (160 ms).

The voltage dependencies of I_{Ca}, the Ca_i-transient and the secretory response are illustrated in Figure 1. The left side of the figure shows original records from a series of 160 ms voltage clamp pulses where the membrane was clamped, in 10 mV increments, to potentials in the range from -30 to +60 mV. I_{Ca} is seen as the slowly inactivating component which follows the initial capacitive current and Na^+ current (panel A). During the clamp pulse the Ca_i-transients rose, first rapidly and then more slowly (panel B). Notice that the decay of the Ca^{2+} transients is barely noticeable during the first 200 ms following repolarization. In fact, more than 5 sec was generally required for Ca^{2+} transients to decay 90%, and there was often, as in the present case, some carry-over from one depolarization to the next, even when they were separated by 30 sec. The recorded oxidation currents clearly show the current spikes associated with individual secretory vesicles (panel C). Unlike the Ca^{2+} transients, the secretion rapidly fell to zero when I_{Ca}

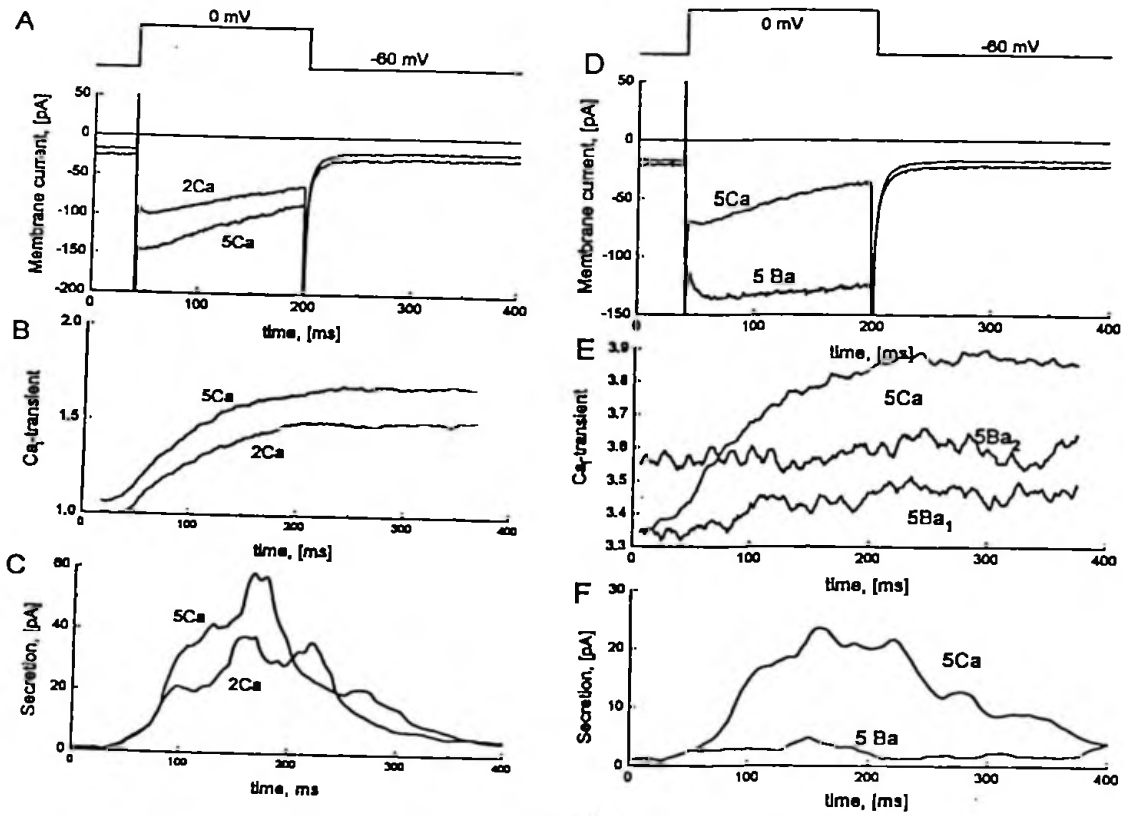

Figure 2. Effect of increased and blocked Ca^{2+} current on Ca_i-transient and secretion. From top to bottom the panels show the membrane current (panels A and D), the fluorescence signal (panels B and E) and the secretion (panels C and F). The left side of the figure shows the results of increasing the extracellular Ca^{2+} from 2 to 5 mM. The right side shows the effect of substitution of 5 mM Ba^{2+} for 5 mM Ca^{2+}. Panel E shows the average Ca_i-transient in the presence of Ca^{2+} and the two first recordings ($5Ba_1$ and $5Ba_2$) after the change to Ba^{2+} containing solution. The oxidation currents are averages from a number of depolarizations and over a 40 ms window.

was terminated by repolarization. The middle panels of Fig. 1 show the voltage dependencies of the three parameters (panels D,E and F). In all cases we found a bell shaped relationship with a maximum near 10 mV and smaller values at both higher and lower potentials. Some significant differences are revealed, however, when the three parameters are in turn compared (panels G and H). The secretion was proportional to the Ca^{2+} current (panel G), but showed significant non-linearity and hysteresis when compared to the Ca^{2+} transient (panel H). We conclude that both the time course and voltage-dependence of the secretory response followed the Ca^{2+} current much more closely than the Ca^{2+} transient.

A causal relationship between Ca^{2+} current and secretion was also found in experiments with double pulses and clamps of different duration. For instance, clamp pulses of 50, 250 and 450 ms duration , produced secretory responses of similar durations. This showed that the secretion lasts

as long as the Ca^{2+} current, and dispels the possibility that the termination of secretion (as in fig. 1C) might be due to depletion of a pool of release-ready vesicles.

It might be argued that saturation of the Ca^{2+} indicator dye could account for its broader bell shaped voltage-dependence (Fig. 1E). Against this idea, however, we found that the indicator dye registered a higher Ca^{2+} concentration than during previous depolarizations when the seal of the patch electrode was lost. Furthermore, the Ca^{2+} transients at 0 mV increased in amplitude when the Ca^{2+} current (Fig. 2, panel A) increased following elevation of the extracellular Ca^{2+} concentration from 2 to 5 mM (panel B). Notice that this intervention increased I_{Ca} and secretion proportionally.

The obligatory role of Ca^{2+} influx in the secretory response was demonstrated in experiments where secretion was completely blocked (Fig. 2, panel F) when extracellular Ba^{2+} was substituted for extracellular Ca^{2+} as charge carrier through the Ca^{2+} channel (Fig. 2, panel D). Panel E of Fig. 2 suggests that entry of Ba^{2+} into the cell caused an increase in the fluorescence of the indicator dye.

The present study of rat chromaffin cells support and extend results from bovine chromaffin cells. The extensive use of simultaneous measurements of I_{Ca}, Ca_i-transients and single secretory events is a novel feature which makes it possible to guard against a number of artifacts (run-down of I_{Ca} and Ca^{2+} overload) and yields highly significant results with high sensitivity and excellent time resolution. The different voltage-dependencies of I_{Ca} and Ca_i-transient is a new finding. The major result is that both magnitude and duration of secretion in rat chromaffin cells is controlled directly by the Ca^{2+} current and is influenced very little, if at all, by the global intracellular Ca^{2+} concentration. On the other hand, it is not known why the voltage dependence of Ca_i-transients differs from that of the Ca^{2+} current. A likely explanation is that the Ca_i-transients, in part, reflect other Ca^{2+} pathways such as release of Ca^{2+} from internal stores, or entry via a Na-Ca exchanger. Our results are consistent with the idea that secretion is controlled by μ-domains of Ca^{2+}

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