Specialized release zones in chromaffin cells examined with pulsed-laser imaging

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INTRODUCTION

The release of chemical signaling molecules from one cell and their binding to a receptor on a target cell is perhaps the most common mode of intercellular communication. The signaling molecules can be released directly onto a neighboring cell (e.g. synaptic transmission) or into the blood stream for transport to their sites of action (e.g. hormones). Prior to their release, the chemical transmitter molecules are stored in the cytosol of the cell in specialized membrane-bound organelles (secretory vesicles). Exocytosis is the process of fusion of these secretory vesicles with the plasma membrane and the subsequent release of their luminal contents into the extracellular milieu.

Targeting of secretory vesicles to the plasma membrane

Various proteins that are proposed to have a role in membrane fusion have recently been isolated and characterized [1]. In vitro reconstitution of vectorial vesicular transport led to the identification of a cytosolic ATP-binding protein, N-ethylmaleimide-sensitive factor (NSF) required for the fusion reactions between intracellular membranes. NSF binds to target membranes by interacting with soluble NSF attachment proteins α, β and γ SNAP. In brain extracts, three previously characterized proteins – VAMP 2, SNAP-25 and syntaxins – were found to bind in an ATP-dependent manner to the NSF-SNAP complex [2]. The membrane protein receptors for the SNAP complex are known as SNAREs; v-SNAREs are on vesicles and t-SNAREs are located on the target membrane. The model postulates that each transport vesicle has its own v-SNARE and that this protein(s) can only interact with the t-SNARE on its target membrane. Formation of the complex between a v- and t-SNARE is thought to correspond to docking of synaptic vesicles, and NSF and SNAP have been postulated to initiate the fusion process [2,3].

Regulation of exocytotic fusion

The fusion of secretory vesicles with the plasma membrane is a highly regulated process. It has long been known that secretion can be stimulated by increases in the cytoplasmic free Ca2+ concentration [4]. In addition to the SNARE complex, other proteins have been proposed to play an important role in regulated exocytosis. For example, the synaptic vesicle protein synaptotagmin I, which binds phospholipids in a Ca2+ dependent manner, has been proposed to be a major Ca2+ sensor for transmitter release in central synapses [5]. Furthermore, synaptotagmin I binds the t-SNARE protein syntaxin 1A [6,7], suggesting that synaptotagmin may confer Ca2+ sensitivity to a SNARE based fusion machine. The t-SNARE
protein syntaxin IA interacts with an 87 amino acid region of the II-III cytoplasmic loop found in the α subunit of N-type calcium channel [8] which is directly involved in activating synaptic transmission [9,10]. When syntaxin IA was co-expressed with N-type calcium channels in the plasma membrane of Xenopus oocytes, the voltage dependency of inactivation of the calcium channels shifted to more negative potentials [11] suggesting that syntaxin IA inactivates N-type calcium channels. The authors of that study speculated that docking of a vesicle may remove syntaxin's inactivating influence, allowing calcium entry only at sites where docked vesicles are ready for fusion [11]. Thus, it is possible that the tight association between synaptotagmin, syntaxin and the N-type Ca channels may ensure that secretory vesicles dock near a Ca channel and in doing so channel inactivation is removed. Significantly, the C terminal domain of syntaxin that is responsible for inactivating the Ca channel is the target of the neurotoxin, botulinum C (BoNT/C), which cleaves syntaxin IA at the Lys253-Ala254 peptide bond [12]. A plausible mechanism of action for the toxin is to delocalize docked secretory vesicles from the N-type Ca channel, thus greatly reducing the efficacy of a Ca stimulus. The tight association between synaptotagmin, syntaxin and N-type calcium channels has the functional role of ensuring that secretory vesicles dock near a calcium channel. Synaptotagmin would be located in an ideal position to bind Ca following the opening of Ca channels and activate the fusion process.

Another significant finding is that of the cysteine-string proteins. These are secretory vesicle proteins that are thought to be regulators of plasma membrane Ca channels [13,14]. This led to the hypothesis that upon docking, the cysteine string proteins activate dormant Ca channels providing a tight spatial association between the synaptic vesicle and the site of Ca entry [14]. These observations, when taken as a whole, suggest that a SNARE complex, augmented by Ca sensitive proteins and proteins that regulate Ca channels, colocalizes with voltage-sensitive calcium channels in the plasma membrane to assemble specialized zones of exocytotic release.

What is the calcium stimulus for vesicle fusion?

Although the cytosolic concentration of Ca is well recognized as the major intracellular signal regulating exocytosis in excitable and non-excitatory cells, there is often poor quantitative correlation between changes in measured Ca concentration or Ca influx and the rate of exocytosis [15,16]. This poor correlation is likely a result of spatial components of the calcium signal that have become apparent only recently. It is now generally accepted that the trigger for vesicle fusion is a localized increase in Ca concentration under the plasma membrane. The existence of such Ca gradients is supported by modeling studies of Ca entry, diffusion, Ca buffering and Ca sequestering activities which show that localized Ca domains, at least in theory, exist [17-19]. The diffusion of cytosolic calcium is constrained by a large number of calcium binding proteins. These effectively reduce the calcium diffusion coefficient more than 20-fold [20]. Hence, entry of calcium through the plasma membrane creates gradients that are short ranged and maintain an elevated concentration for only tens of nanometers from the point of entry. Excessive stimulation can overcome the endogenous buffers and fill the cytosol with an elevated calcium concentration. Such an extreme condition is unlikely to arise physiologically. It is more likely that many cytosolic calcium signals arise only briefly and act locally.

Active zones of release and calcium channels

Electron microscopy studies have shown that neurons contain specialized regions in their presynaptic membranes, called active zones, where secretory vesicles accumulate and undergo exocytotic fusion [21]. It has been hypothesized that clusters of Ca channels are found in the plasma membrane at these active zones [22]. Thus, stimulation of the cell would lead to the formation of localized calcium gradients at the active zones. Hotspots consistent with this prediction have been observed in neuronal growth cones [23], in the squid giant synapse [24] and in the presynaptic active zones of hair cells [25]. These observations suggest that the spatial arrangement of secretory vesicles and calcium channels are crucial in orchestrating physiological secretory responses. However, there has been no adequate model system to study these spatial components in detail. The small size and fragility of central nervous system synapses pose severe technical difficulties that prevent a detailed study of the highly organized cellular architecture that regulates synaptic transmission. Ideally, direct and simultaneous measurements of Ca signaling, vesicle fusion and neurotransmitter release might reveal the molecular nature of the active zones where calcium channels and SNARE complexes are thought to colocalize to effect fast release of neurotransmitters. However, the current state of technology precludes studies such as these.

Our recent observations reveal that the bovine adrenal chromaffin cell is an ideal model cell in which to study the molecular architecture of discrete zones of exocytotic release. In this paper, we will describe a new set of tech-
Fig. 1 The standard configuration used to simultaneously record the fusion of secretory vesicles with the plasma membrane of a patch clamped chromaffin cell and the subsequent release of catecholamines from the fused vesicles is shown in Figure 1. A patch pipette in the whole cell configuration is depicted on the right of the figure and a carbon fiber electrode on the left. Fusion of secretory granules with the plasma membrane results in an increase in the plasma membrane area ($C_m$). Application of a constant potential to the carbon fiber electrode oxidizes catecholamines that are released from the secretory granules resulting in the generation of a small electrical current ($I_{amp}$). Individual spikes represent the release of the contents of a single vesicle. Exocytotic release was triggered by depolarizing the cell to +28 mV from a holding potential of -60 mV for 50 ms. The depolarization opens voltage-dependent Ca$^{2+}$ channels facilitating Ca$^{2+}$ influx into the cell and triggering the fusion of vesicles with the plasma membrane.

RESULTS AND DISCUSSION
High resolution measurements of exocytosis

There are two electrophysiological techniques which can be used to monitor secretion from single cells that have the resolution to follow fusion of a single vesicle with the plasma membrane. When a secretory vesicle fuses with the plasma membrane, its membrane becomes incorporated into the plasma membrane of the cell, leading to an increase in the surface area of the cell. It is this increase in the cell's surface area that is used to follow secretion by the first of the electrophysiological techniques, capacitance measurements [29,30,31]. Once a vesicle has fused with the plasma membrane its contents are released into the extracellular milieu. Some of the chemicals that are stored in secretory vesicles are readily oxidizable (e.g., catecholamines), and their release can be followed by using a carbon fiber electrode held at a constant poten-
The vesicular contents are secreted by the cell they will diffuse towards the carbon fiber. When the released transmitter molecules reach the surface of the carbon fiber they are oxidized and the electrons that are generated during this process cause a current to flow along the carbon fiber. This current is used as a measure of the cell's secretory response [32-34]. A schematic diagram illustrates the typical arrangement for these recordings (Fig. 1). A patch pipette is shown on the right-hand-side of a cell and a carbon fiber electrode is shown on the left-hand-side of the cell. Examples of the two types of measurements are also shown in the figure, i.e. a capacitance trace (C<sub>c</sub>) and an amperometric trace (I<sub>ampl</sub>). These were recorded from the patch pipette and the carbon fiber, respectively. The secretory response in this instance was elicited by transiently depolarizing a patch-clamped adrenal chromaffin cell from a holding potential of -60 mV to +20 mV for 50 ms. The voltage protocol used in this experiment is shown below the capacitance trace. This transient depolarization triggered the entry of Ca<sup>2+</sup> into the cell and triggered the fusion of secretory vesicles with the plasma membrane, which led to an increase in the cell surface area evident in the capacitance trace (C<sub>c</sub>, Fig. 1). An amperometric recording (I<sub>ampl</sub>) simultaneously made with the capacitance measurement is shown at the top of Figure 1. Catecholamines released from the cell were oxidized by the carbon fiber and detected as spike-like currents.

**The fusion pore**

The use of these two electrophysiological techniques has chronicled the fusion and release processes in exquisite detail and as such these are now very well characterized events. This is particularly true in studies of beige mouse mast cells. Their unusually large secretory vesicles make them especially useful in these studies. The exocytotic fusion pore connects the lumen of a secretory vesicle with the extracellular environment. This structure was first observed in degranulating mast cells using a combination of rapid freezing techniques and freeze-fracture electron microscopy [35]. The development of patch clamp-capacitance techniques permitted the direct observation of the activity and size of single fusion pores in isolated cells undergoing exocytosis [30,36,37]. These measurements revealed that the exocytotic fusion pore opens abruptly and is initially small (1-2 nm diameter). After opening, the pore expands rapidly. It then can either close again completely (transient fusion, sometimes called 'flicker') or expand irreversibly (see for example [34]). It was additionally discovered that a secretory vesicle could undergo multiple transient fusion events prior to irreversible fusion. The comparatively large size of the mast cell secretory granules makes them particularly amenable to these studies. Transient fusion events have been reported in only a few other cell types (e.g. eosinophils [38]).

**Release of secretory products through the fusion pore**

When a secretory granule fuses with the cell membrane, transiently or irreversibly, the fusion pore provides a path connecting the lumen of the vesicle with the extracellular medium. The exocytotic release of secretory products through the fusion pore has been oversimplified as the diffusional dumping of a 'soluble cocktail' into the extracellular environment. Direct observation of exocytotic events in mast cells using patch clamp and amperometric techniques, showed that far from a simple diffusional mechanism, secretory product release occurs by an ion exchange mechanism that is regulated by a polymer gel that fills the lumen of the secretory granules [34,39]. This regulation takes place after the fusion pore opens and determines the release of secretory products during transient and irreversible fusion events [39]. Ion exchange polymer gels have been shown to regulate release only in mast cell secretory granules. However, the mechanisms of storage and release of secretory products found in mast cells are likely to represent mechanisms that are common to most secretory cells including chromaffin cells [40,41]. Confirmation of this hypothesis has the potential for significantly changing our understanding of the regulation of secretory events.

**The fusion pore in chromaffin cells**

Figure 2a,b shows examples of the fusion of individual granules with the plasma membrane of patch-clamped chromaffin cells. These can be seen as step increases in membrane capacitance. Accompanying these fusion events are single amperometric spikes. Each individual spike represents the release, and oxidation by the carbon fiber, of the contents of a single vesicle [28,32,34]. In Figure 2a, the main amperometric spike is preceded by a smaller increase in current, or 'foot'. The foot is believed to be due to the leak of vesicular contents through a slowly expanding fusion pore [33,34]. It is also possible that the release of the granule's contents is governed by the kinetic properties of an ion exchange matrix found in the granule itself, in a similar fashion to that already described in mast cell granules [39]. Figure 2c,d shows two examples of transient fusion events recorded from a bovine adrenal chromaffin cell. Following the initial step increase in membrane capacitance there was a step-like decrease; this is an increase in membrane capacitance due to the transient opening and closing of the fusion pore. The fusion pore finally opened irreversibly and a sustained increase in capacitance was recorded. During the 'flickery' opening of the fusion pore the...
Capacitance and amperometry measurements of exocytosis have the resolution to discern the fusion of individual granules with the plasma membrane and the subsequent release of their contents. In (a and b), a single step increase in the membrane capacitance recording can be seen (lower trace), that is accompanied by a concomitant spike in a simultaneous amperometric recording (upper trace). Each step increase represents the fusion of a single granule with the plasma membrane, and each amperometric spike represents the release of a single vesicle's contents. Amperometric release spikes are often preceded by a smaller increase in current, a 'foot', that can be quite prolonged (a). (c and d) Show the transient fusion of secretory granules with the plasma membrane of an adrenal chromaffin cell stimulated by the uncaging of DM-nitrophen. Following the transient opening of the fusion pore seen as a step increase, followed by a step decrease in the capacitance trace (marked by asterisks), the fusion pore then opened irreversibly evidenced by the sustained increase in capacitance. This figure is modified from Robinson et al. [28].
Fig. 4 Performance of the combined application of pulsed laser photolysis of caged calcium and pulsed laser calcium imaging. (a) Shows a calibration curve made by adding rhod-2 (0.4 mM) to Ca\(^{2+}\)-EGTA buffered solutions (made in the same buffer that was used in patch clamp experiments) that contained known levels of free Ca\(^{2+}\). These buffers were placed in a glass-bottomed Petri dish on the stage of the imaging microscope, exposed to a pulse of light from the visible laser, and the level of the fluorescence was captured with the CCD camera. Changes in the fluorescence levels of rhod-2 in the different Ca\(^{2+}\) containing buffers were normalized with respect to 0.4 mM rhod-2 in a ‘zero’ Ca\(^{2+}\) (10 mM EGTA) containing solution. The maximum change in fluorescence obtained with Ca\(^{2+}\)-saturated rhod-2 was approximately 70 times that of rhod-2 in a Ca\(^{2+}\) free solution. The data in this figure were fit with the following equation:

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\frac{F_{\text{rat}}}{F_{\text{min}}} = \frac{K_s + F_{\text{rat}}}{K_s + [\text{Ca}\^{2+}]} \cdot [\text{Ca}\^{2+}] / ([K_s][\text{Ca}\^{2+}])
\]

where \(K_s = 1.3 \, \text{μM}\), \(F_{\text{rat}} / F_{\text{min}} = 73.01\) and \([\text{Ca}\^{2+}]\) was the free Ca\(^{2+}\) concentration calculated for each of the Ca\(^{2+}\) buffer solutions. (b) Shows a time course of release of Ca\(^{2+}\) from DM-nitrophen following its photolysis in vitro measured by rhod-2. This time course of the release of Ca\(^{2+}\) was captured by firing the pulsed dye laser with varied delays with respect to the uncaging stimulus. The change in fluorescence of rhod-2 versus time following the triggering of the UV laser is shown in (b). There was a delay of 860 μs following the triggering of the laser and the UV light pulse. The peak change in the Ca\(^{2+}\) concentration was seen at about 80 ps following the uncaging stimulus, i.e. the UV light pulse. This can be most easily seen in the inset in (b) which is an expanded time course of the first 2 ms of the recording. (c) Shows representative data from an experiment in which DM-nitrophen was photolyzed by the UV laser in vitro. The diaphragm of the epifluorescence microscope was stopped down so that only a small aperture remained open. The microscope was then focused such that the edge of a drop of solution containing DM-nitrophen and rhod-2 was over the aperture of the epifluorescence pathway. A time course of the change in fluorescence of rhod-2 following the uncaging of DM-nitrophen was constructed by varying the delay between the triggering of the visible laser with respect to the UV laser. The fluorescent image obtained 1 ms after the triggering of the laser (200 μs after the UV light pulse) has been deliberately saturated to enable the fluorescent signal from later time points to be seen more easily.

assembled structure of the fusion pore scaffold [40] is hypothesized to remain in place, and that following the closure of the fusion pore that the secretory granule remains docked with the plasma membrane.

Thus it would appear that the physics of fusion, the opening of the fusion pore and the release of granular contents from chromaffin cells are very similar to other cell types that have been studied with both capacitance and amperometric measurements. A possible exception are neuronal cells. Bruns and Jahn [42] recorded from the retzius cell of the leech two populations of amperometric spikes, differentiated by their magnitude. The larger spikes were often preceded by feet events. ‘Stand alone’ feet events were also observed, indicative of transient fusion events [42,43]. Feet events were not observed in the smaller population of amperometric spikes, attributed to the fusion of synaptic vesicles [42]. Bruns and Jahn [42] surmised that this was due to an abrupt opening of the fusion pore of synaptic vesicles to a relatively large conductance state. However, it is more likely that feet events were below the detection threshold of the carbon fiber and thus a complete characterization of the opening of the fusion pore of synaptic vesicles remains elusive. The frequency of transient fusion events in neurons and neuroendocrine cells is at present not known because most events are below the resolution of patch clamp-capacitance measurements. However, it is possible that transient fusion is a significant component in all secretory responses [30]. If confirmed, this concept will call for revision of ideas about the mechanisms involved in synaptic transmission and secretion in general.

Pulsed-laser fluorescence imaging

Excitable cells such as neurons and muscle cells are believed to be stimulated by the generation of short lived
and highly localized Ca\(^{2+}\) gradients near the sites of Ca\(^{2+}\) influx or the sites of Ca\(^{2+}\) release from intracellular stores in response to action potentials. The duration of the action potential in these cells is short, in the order of a few milliseconds. Visualization of these gradients requires an imaging system with high degrees of temporal and spatial resolution. The majority of systems used today acquire images as rapidly as possible following the triggering of an event. The temporal resolution of conventional imaging systems is ultimately limited by the speed of the cameras and other hardware employed. This limitation can be overcome by using photomultiplier tubes or the line scanning mode of confocal microscopes. However, these two techniques often compromise spatial components in the observed signal. Over the past few years, we have developed an imaging system that relies on a different principle. Our approach was pioneered by Kinosita and his colleagues and relies on the transient (350 ns) and intense illumination provided by a pulsed laser. A pulsed-laser imaging system captures 'snapshot' images of the fluorescence of indicator molecules that are transiently excited by the laser illumination. This allows images to be obtained with sub-microsecond temporal resolution [44,45]. The schematic diagram shown in Figure 3 shows the basic setup of the imaging system that we have used in this study. This system is a development of one that we have previously described and used successfully to image, in excitable cells, the generation and decay of Ca\(^{2+}\) gradients that can develop and decay in 10–15 ms [26,46]. The use of this imaging system is not restricted to documentation of Ca\(^{2+}\) gradients. It has also been used in a novel study of the flux of ions in a polymer gel matrix [47].

The organization of the pulsed-laser imaging setup is illustrated as a block diagram in Figure 3. The main elements of the imaging system are a cooled CCD camera, a pulsed dye laser (used to excite the fluorescence indicator molecules), a frequency doubled ruby laser (used to uncage photoactivatable molecules), and a host computer which contains a data acquisition and signal generation card. The computer serves two functions. The first is to precisely time the triggering of a stimulus event (e.g. the photolysis of a caged compound, or the depolarization of a patch clamped cell) and the firing of the pulsed dye laser with respect to this event. Time courses of changes in Ca\(^{2+}\) concentrations can be built up by taking a series of images at intervals following the stimulus. The second function of the computer is to acquire electrophysiological data and store fluorescence images. Data stored on the computer can be analyzed either on- or offline. Light from the two lasers was focused into the epi-fluorescence pathway of the microscope with a custom
Pulsed-laser imaging calibration of rhod-2 fluorescence

The images shown throughout this paper are pseudocolor images that represent fractional changes in rhod-2 fluorescence. These were obtained by dividing a stimulus image by a control image. The ratio corrects for differences in indicator concentration, indicator excluded volume and light path length. Thus the fractional change in fluorescence is a measure of the change in Ca2+ concentration. We calibrated the fluorescence changes by adding rhod-2 (0.4 mM) to Ca2+-EGTA buffered solutions (made in the same buffer that was used in patch clamp experiments) that contained a known level of free Ca2+ [15]. These buffers were placed in a glass bottomed Petri dish on the stage of the imaging microscope, exposed to a pulse of light from the visible laser, and the level of the fluorescence was captured with the CCD camera. Differences in the fluorescence levels of rhod-2 in the different Ca2+ containing buffers were normalized with respect to 0.4 mM rhod-2 in a Ca2+-free solution (containing 10 mM EGTA). The maximal fractional change in the fluorescence ratio of rhod-2 was about 70-fold for a saturating Ca2+ solution (Fig. 4a). The saturating Ca2+ solution was made by dissolving CaCl2 in water since EGTA cannot buffer Ca2+ at such high concentrations. The results of this calibration show that a sigmoidal change in the fluorescence of rhod-2 was obtained when the level of dye fluorescence was changed by adding different buffers containing known amounts of Ca2+. These data are consistent with data reported when the characteristics of this dye were first described [48]. These results suggest that the pulsed dye laser does not affect the chemistry or the fluorescent properties of rhod-2, and that it can be used in this system to capture changes in Ca2+ concentrations. The fractional fluorescence changes induced in this paper were typically in the range of 11-fold for in vitro experiments and from 1.25–3-fold for experiments involving chromaffin. These represent very small changes in Ca2+ concentration (a few tens to hundreds nM).

Pulsed-laser photolysis of caged compounds

The combined use of the flash photolysis of caged Ca2+ compounds and capacitance measurements of secretion has recently been applied to determine the Ca2+-sensitivity of the fusion machinery in neuroendocrine cells and nerve terminals [48, 50, 51]. The perceived advantage of caged Ca2+ chelators is the belief that their photolysis generates a uniform and global increase in the intracellular concentration of Ca2+. We have taken advantage of the temporal and spatial resolution of our imaging system to record the Ca2+ gradients generated by photolysis of caged Ca2+ chelators in patch clamped chromaffin cells. In order to fully exploit the temporal resolution of the system, we have added a frequency doubled ruby laser that emits light at 347 nm and has a pulse duration of 30 ns to photolyze caged compounds. Light from this laser was focused into the epifluorescence path of the microscope and followed the same pathway as light from the visible laser. Before using these compounds in patch clamped cells, we characterized the photolysis and change in Ca2+ concentrations in vitro (Fig. 4b,c). A small drop of solution (~2 μl) containing rhod-2 and DM-nitrophen (10 mM loaded with 3 mM Ca2+) made up in a solution with the same composition as that used to fill the pipettes in the patch clamp experiments was placed on a Petri dish on the microscope stage of the imaging system. This drop was covered with mineral oil (~100 μl) to prevent evaporation. The diaphragm of the epifluorescence microscope was stopped down so that only a small aperture remained open. The microscope was then focused to position the edge of the drop of solution, containing DM-nitrophen and rhod-2, over the aperture of the epifluorescence pathway. A time course of the release of Ca2+ was captured by varying the delay between firing the pulsed dye laser with respect to the firing of the UV laser. The peak change in Ca2+ concentration was seen 940 μs after triggering of the UV laser (Fig. 4c). It should be noted that firing of the pulsed laser is always delayed by exactly 860 μs with respect to its triggering, for convenience the times shown on the figures throughout this paper refer to the time from the triggering of the laser and not the UV light pulse itself. Thus, about 80 μs elapses from the uncaging stimulus to the peak change in Ca2+. Following the photolysis of DM-nitrophen it appears that there is a very rapid, almost instantaneous increase in Ca2+ concentration that decays to a basal level over a period of tens of milliseconds. Escobar et al. [52]
have reported a time course for the change in Ca²⁺ concentration following DM-nitrophen photolysis which is much faster than the one that we have observed. They showed that the binding and dissociation kinetics of different Ca²⁺-sensitive dyes have a marked effect on the apparent time course of changes in Ca²⁺ concentration that follow photolysis of DM-nitrophen. Thus it is likely that the actual free Ca²⁺ concentration in our system would return to basal levels at a much faster rate than that reported here by rhod-2 fluorescence (see [52]). An advantage of using rhod-2 in these studies, however, is that its excitation wavelength (525 nm; emission of the visible pulsed-dye laser) is very different from that used to photolyze the caged compounds (347 nm) thus there is little chance of uncaging a small amount of DM-nitrophen with the visible laser (see Fig. 4b,c). The time course and magnitude of the changes in Ca²⁺ concentration were reproducible in each experiment. This shows that the UV laser effectively uncages a fixed amount of DM-nitrophen each time the laser is fired. The result also demonstrates the very high temporal resolution of the imaging system. In this case the peak Ca²⁺ concentration peaked 80 μs after the UV light pulse and the generated gradients of Ca²⁺ decayed in tens of milliseconds. These gradients would be impossible to document with a video camera which would have acquired only one image over this entire time course.

Photolysis of DM-nitrophen as a Ca²⁺ stimulus of secretion

It is generally assumed that photolysis of caged Ca²⁺ chelators generates a uniform and global increase in the concentration of Ca²⁺ in cells. We have addressed this issue using the imaging system described above. Figure 5a shows the changes in intracellular Ca²⁺ concentration in two different patch clamped chromaffin cells following the photolysis of DM-nitrophen (10 mM, 30% loaded with Ca²⁺). The images were taken by firing the visible laser 5 ms after triggering the UV laser. The two images clearly show that following the uncaging of Ca²⁺ there was a uniform elevation in the Ca²⁺ concentration in the cytosol of the cell, but that the magnitude of this change was significantly smaller than the changes observed in the nucleus. The secretory vesicles in these cells are distributed throughout the cytosol. Thus, in terms of the secretory response, it is the change in the cytosolic concentration of Ca²⁺ and not in the nucleus that is important. The cause of these large changes in nuclear fluorescence is unclear at present. These imaging studies do, however, highlight one possible problem. If a photomultiplier tube is used to measure the changes in intracellular Ca²⁺ following the uncaging of Ca²⁺, it is clear that most of the light emanating from the cell will come from the nucleus. This would lead to a gross overestimate of the change in Ca²⁺ in the cytosol if it is assumed that the increase in Ca²⁺ is uniform throughout the cell (see for example [49,50]). Thus if the concentration of Ca²⁺ measured by the photomultiplier tube is used to assess the Ca²⁺ binding affinity of the secretory machinery, this value would be an underestimate of the true value. In our present study, the UV laser used to uncage DM-nitrophen elicited much smaller changes in the intracellular Ca²⁺ concentrations than was reported in previous studies in which flash lamps were used for the same purpose [49,50]. In the case of the UV laser, the light pulse lasts for 30 ns, whereas exposure times in the order of tens of milliseconds were more routine when flash lamps were used to photolyze caged compounds. It remains to be seen whether or not the fluorescence in the nucleus under these conditions is much larger than that of the cytosol.

One problem associated with imaging studies is that the finite numerical aperture of the objective results in distortion and reduced contrast of the image. Thus the image will be blurred and contaminated with out-of-focus light. Thus, if there is a localized high concentration of Ca²⁺ in a cell, out-of-focus light will make the apparent gradient much smaller and more spread out [53]. In chromaffin cells, the concentration of dye in the cytosolic compartment is lower as a result of dye exclusion by the densely packed secretory granules (see Fig. 3 of [53]). This may also account for some differences in the levels of fluorescence seen between the nucleus and the cytosol following the uncaging stimulus. Monck et al. [53] showed that unless raw images are corrected for out-of-focus light, ratioed images misrepresent Ca²⁺ levels in compartments from which the dye is excluded. Indeed the unprocessed images, shown in Figure 3b of Monck et al. [53], reveal a higher level of fluorescence in the nucleus of the cell than in the cytosolic compartment that is reminiscent of the images in Figure 5a. In contrast, when the out-of-focus light is removed, the Ca²⁺ levels in the cytosol are uniform.

The uncaging of DM-nitrophen by the UV laser in patch clamped adrenal chromaffin cells raised Ca²⁺ to a level that was sufficient to stimulate a secretory response. We chose to measure this response with an amperometric carbon fiber (Fig. 5b) because in recently comparing the secretory response measured by capacitance and amperometry following the flash photolysis of DM-nitrophen (induced by a flash lamp) we found striking differences between the responses measured by the two techniques. There was an immediate increase in capacitance of the chromaffin cell, but there was a considerable delay to the detection of the first amperometric spikes [54,55]. On average, this delay was 500 ms for the first uncaging stimulus given in a particular cell, and was...
considerably reduced for subsequent UV light flashes in the same cell. This change in delay between the uncaging stimulus and the first secretory response was observed more readily in mast cells [54,55]. We concluded from these studies that the capacitance changes induced by DM-nitrophen photolysis are complex and cannot readily be interpreted as the result of the fusion of secretory granules that contain oxidizable substances.

In the two traces shown in Figure 5b, it can be seen that there was again a delay to the detection of amperometric spikes following stimulation of the cell. These recordings (Fig. 5b) were picked at random from cells that had been stimulated to secrete several times, thus explaining the short delay times to the detection of the spikes (i.e. < 500 ms).

Figure 5c shows a typical time course of the changes in intracellular Ca^{2+} following the photolysis of DM-nitrophen in chromaffin cells. This time course was obtained by repeatedly uncaging Ca^{2+} in the same cell and varying the time of firing of the visible laser with respect to this pulse. The maximal increase in the intracellular Ca^{2+} concentration occurred 5 ms after the triggering of the UV light.

**Fig. 6** Pulsed laser calcium imaging of the photolysis of caged Ins 1,4,5P_3 in the cytosol of a chromaffin cell. This figure shows different patterns of Ca^{2+} elevations in patch clamped chromaffin cells loaded with 1 mM caged Ins 1,4,5P_3 and photolyzed by a 30 ns pulse of UV light delivered through the epifluorescence pathway of a microscope. It can clearly be seen that the increases in Ca^{2+} were non-homogeneous and were restricted one pole of the cell (a and b). (b) Shows the exocytotic response to the uncaging of Ins 1,4,5P_3 measured by an increase in cell membrane capacitance. The capacitance increase is due to the fusion of about 100 secretory granules with the plasma membrane (assuming each granule contributes 2 fF). Images of the Ca^{2+} distribution in the cell from which the capacitance recording was made are shown at the bottom of the figure. Arrows on the capacitance trace mark the times that images were obtained. It can be seen that the elevation of Ca^{2+} was polarized to one part of the cell. The maximal rate of secretion can be seen to be coincident with the maximal increase in the Ca^{2+} concentration (panel 2, b). About 5 s after the uncaging stimulus, Ca^{2+} had returned to basal levels and the secretory response ceased. The patch pipette was located at the 3 o'clock position in all cases.
laser. The first increases in Ca\(^{2+}\) were observed in the nucleus of the cell (apparent in the 2 ms image). Following the peak increase in the intracellular Ca\(^{2+}\) change at 5 ms, there was a slower decay of the Ca\(^{2+}\) concentration back to basal levels. (Note there was a delay of 860 μs between the triggering of the UV laser and the UV light pulse and that the times in the figures refer to the triggering of the UV laser.) This result is similar to that seen in the in vitro experiment (Fig. 4), however in the case of the cell, it took several milliseconds to reach the
peak change in intracellular Ca\(^{2+}\) concentration. The reasons for this are not known at present. Another point of interest is that the secretory response of the cell is very much prolonged compared to the time course of the change in the Ca\(^{2+}\) concentration in the cell. Rhod-2 indicated that the Ca\(^{2+}\) concentration had returned to basal levels 90 ms after the UV laser was triggered (though the return of Ca\(^{2+}\) to pre-stimulus levels was likely to have been faster than this, see above and Escobar et al. [52]). Thus the time course of the change in Ca\(^{2+}\) concentration and the secretory responses do not appear to be well correlated. The reason for this is not presently understood.

**Photolysis of caged Ins 1,4,5P\(_3\) as a stimulus of secretion**

In addition to neurotransmitters, the adrenal medulla is also stimulated in vivo by circulating hormones, such as angiotensin II and bradykinin. Both of these hormones have been shown to stimulate the production of Ins 1,4,5P\(_3\) in chromaffin cells and to generate an increase in intracellular Ca\(^{2+}\) concentration that is localized to the perinuclear region of the cell [56-58], in the vicinity of the endoplasmic reticulum [56]. In an elegant study involving the coculture of chromaffin cells with NIH-3T3 fibroblasts, it was indirectly shown that these localized elevations of Ca\(^{2+}\) elicited a polarized secretory response [57]. It is not clear from these studies, however, whether or not the localized elevations of Ca\(^{2+}\) were due to the clustering of receptors in one pole of the cell and the limited diffusion of Ins 1,4,5P\(_3\). In this present study we circumvented these problems by perfusing a patch clamped cell with caged Ins 1,4,5P\(_3\) (1 mM), and by use of the pulsed-laser imaging system to study the release of Ca\(^{2+}\) from intracellular stores following its photolysis. Figure 6a shows the pattern of elevation in intracellular Ca\(^{2+}\) in two different cells. In the examples shown, it can be seen that there was a diffuse elevation in intracellular Ca\(^{2+}\), however, it is clear that these increases were not homogeneous. Unlike the cells that were loaded with DM-nitrophen, no large increase in the fluorescence signal was seen in the nucleus of the cells loaded with caged Ins 1,4,5P\(_3\). Thus the very large increases in fluorescence observed in the nuclei of cells in which DM-nitrophen was uncaged is not due to the UV laser and is unlikely to be due to an effect of the uncaging process itself or the chemistry of rhod-2 in the nucleus of the cells.

Elevations of Ca\(^{2+}\) in these two examples were seen in the cytosol, i.e. in the area in which one would expect to see secretory granules. In both cases, the photolysis of caged Ins 1,4,5P\(_3\) induced a secretory response. Figure 6b shows the time course of both the secretory response and the increase in intracellular Ca\(^{2+}\) for the cell shown in the right of the panel of Figure 6a. The time course in this experiment was built up in a different way from the other experiments; a single control image was obtained before the UV pulse and this was used for each of the successive images obtained after the photolysis event. Thus, in this experiment, the images were obtained in a manner that more closely resembles that used in conventional imaging systems. The secretory response in this case was followed by monitoring membrane capacitance (top of Fig. 6b). A delay followed the UV light pulse used to photolyze caged Ins 1,4,5P\(_3\), and the onset of the secretory response. Images of the intracellular Ca\(^{2+}\) concentration in the cell were obtained at the times marked by the arrows, and are shown beneath the capacitance recording. The first image was taken at the time that an increase in membrane capacitance was first observed. It can be seen that Ca\(^{2+}\) was elevated in the left half of the cell. By the time the second image was obtained (at the time of the fastest rate of increase in membrane capacitance) there was an area near the plasma membrane in which the elevation in intracellular Ca\(^{2+}\) was very pronounced (from the 9 to 11 o'clock position in the cell). The Ca\(^{2+}\) level in the cell then declined to a basal level and the secretory response ceased. The capacitance technique does not yield spatial information about where the secretory response occurred, but, it would seem likely to have been localized to the 9 to 11 o'clock position in the cell (see [28,57] and Fig. 9).

Previous studies by O'Sullivan et al. [56] and Cheek et al. [57,58] showed that elevations in intracellular Ca\(^{2+}\) were restricted to the perinuclear region. In contrast, we observed Ca\(^{2+}\) changes throughout the cytosolic compartment of the cell. It has been proposed that the secretory vesicles of chromaffin cells contain the Ins 1,4,5P\(_3\) receptor [59]. It is possible that the secretory granules may be one source of Ca\(^{2+}\). In this study the caged Ins 1,4,5P\(_3\) would have equilibrated throughout out the cell before being photolyzed, and it is possible that Ca\(^{2+}\) released from the granules contributed to the responses we observed. In previous studies [56-58], this potential source of intracellular Ca\(^{2+}\) may not have been tapped due to the limited diffusion of Ins 1,4,5P\(_3\) generated in response to agonists. Direct investigation of this point would require the challenge of isolated granules with Ins 1,4,5P\(_3\) to see if it is possible to trigger the release of Ca\(^{2+}\) from them. Another possibility is that we simply stimulated release of Ca\(^{2+}\) from endoplasmic reticulum that is more distal from the nucleus.

**Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels as a stimulus of secretion**

The physiological stimulus for secretion in the adrenal medulla is the release of acetylcholine from the splanchinic nerve. This would trigger trains of action potentials in
chromaffin cells. It is possible to simulate this activation by transiently depolarizing a patch-clamped cell. This elicits Ca\(^{2+}\) entry into the cell through voltage-sensitive Ca\(^{2+}\) channels. We have already shown that the opening of Ca\(^{2+}\) channels in these cells can stimulate a secretory response (see Fig. 1). Capacitance measurements showed that on average 50 ms depolarizing pulses triggered the fusion of 2–24 granules [28]. Figure 7 shows the patterns of Ca\(^{2+}\) entry for different cells induced by a 50 ms depolarization from a holding potential of −60 mV to +20 mV. The pattern of Ca\(^{2+}\) entry into each individual cell is different. The increases in Ca\(^{2+}\) concentration are localized at the periphery of the cells near the plasma membrane. The entry of Ca\(^{2+}\) around the periphery of the cell is not uniform but is often highly localized in foci that are only 1–2 μm across. We have termed these localized Ca\(^{2+}\) entry sites ‘hotspots’ [26,28]. Although the pattern of Ca\(^{2+}\) entry was different in each cell, it was highly reproducible in the individual cells that were studied. We have already shown that the time course of the Ca\(^{2+}\) response to the photolysis of DM-nitrophen is longer than that predicted (compare Fig. 4b,c with Escobar et al. [52]) and that this is probably due to the slow dissociation rate of entry sites ‘hotspots’ [26,28]. Although the pattern of Ca\(^{2+}\) entry was different in each cell, it was highly reproducible in the individual cells that were studied. We have already shown that the time course of the Ca\(^{2+}\) response to the photolysis of DM-nitrophen is longer than that predicted (compare Fig. 4b,c with Escobar et al. [52]) and that this is probably due to the slow dissociation rate of Ca\(^{2+}\) from rhod-2. Similarly, the size of the hotspots of Ca\(^{2+}\) entry into the cell are likely to be overestimated in the images shown here due to a broadening effect that would result from the diffusion of rhod–2 bound to Ca\(^{2+}\) diffusing away from the initial site of entry.

By changing the timing of the firing of the visible laser with respect to the depolarizing pulse, we were able to obtain a time course of the development Ca\(^{2+}\) gradients generated in the cytosol following the depolarization induced Ca\(^{2+}\) entry. Although Ca\(^{2+}\) currents develop very rapidly during the depolarization of the cell, the Ca\(^{2+}\) gradients develop more slowly (Fig. 8). Very little change in intracellular Ca\(^{2+}\) concentrations were seen 10 ms after opening of the Ca\(^{2+}\) channels. After 20–30 ms, discrete elevations in Ca\(^{2+}\) levels were seen. At 55 ms after the opening of Ca\(^{2+}\) channels a broadening of the Ca\(^{2+}\) gradients was observed and they started to fill the center of the cell. After the depolarizing pulse, the gradients were observed to decrease over hundreds of milliseconds, leaving a spatially uniform increase in Ca\(^{2+}\) (Fig. 8 and see Monck et al. [26]). Thus, the pattern and the time course of the Ca\(^{2+}\) increases seen in chromaffin cells following the uncaging of Ca\(^{2+}\) from DM-nitrophen, photolysis of caged Ins 1,4,5P\(_3\), and depolarization induced Ca\(^{2+}\) entry are very different (compare Figs 5–8).

Colocalization of Ca\(^{2+}\) entry and release sites in chromaffin cells

We have shown that the sites of Ca\(^{2+}\) entry that follow a depolarization induced opening of voltage-sensitive Ca\(^{2+}\) channels are localized to discrete sites of the plasma membrane and induce secretion in these cells. Although capacitance measurements of secretion report the increase in the cell’s surface area, they do not convey spatial information about the sites of fusion events occurring at the plasma membrane. Schroeder et al. [27] used carbon fiber microelectrodes that had been etched in a Bunsen burner flame to yield tips which could detect the release of catecholamines from an area of only 1–2 μm to demonstrate that secretion occurred from restricted zones on the plasma membrane. We combined this technology with the pulsed-laser imaging system to determine whether or not the sites of Ca\(^{2+}\) entry and secretion colocalized. By placing the carbon fiber microelectrodes close (1.4 ± 0.2 μm) to a hotspot or away from a hotspot (3.6 ± 0.4 μm) and depolarizing the cell to induce the entry of Ca\(^{2+}\) and hence stimulate a secretory response we were able to show that the sites of Ca\(^{2+}\) entry and catecholamine release did colocalize. Figure 9 illustrates this point. The left hand panel (Fig. 9a) illustrates a Ca\(^{2+}\) image obtained from a chromaffin cell 5 ms after a 50 ms depolarization from −60 mV to +20 mV. There are three distinct Ca\(^{2+}\) hotspots in this cell, at the 2, 4 and 7 o’clock positions. To the right of this image two bright field images of the same cell are shown (Fig. 9b,c). In these two figures, the tip of a carbon fiber microelectrode can be seen. In Figure 9b the carbon fiber can be seen in the 9 o’clock position (6 μm away from the nearest hotspot; 1 μm away from the plasma membrane), in Figure 9c the carbon fiber was moved to the 7 o’clock position, 1.5 μm away from the nearest hotspot of Ca\(^{2+}\) entry. Beneath these bright field images are amperometric recordings made from the fibers shown in their respective positions above. It can be seen that when the carbon fiber was closely apposed to the hotspot that a secretory response was observed. When the carbon fiber was moved away from the hotspot secretory responses were observed less frequently. Pooled data showed a secretory response was recorded in 74% of the instances that the cell was depolarized when the carbon fiber was close to a hotspot, but when the carbon fiber was away from the hotspots, the frequency declined to 18% (see [28]). These results demonstrate that under conditions that lead to discrete elevations of Ca\(^{2+}\) in the cell, the sites of secretion and the sites of Ca\(^{2+}\) entry are colocalized. Thus there is an important spatial component of the Ca\(^{2+}\) signal underlying stimulus-secretion coupling in these cells that had not previously been reported.

Evidence for the clustering of voltage-sensitive Ca\(^{2+}\) channels in chromaffin cells

The imaging studies described above showed that depolarization of chromaffin cells led to the influx of Ca\(^{2+}\) at
Fig. 9 In chromaffin cells, the secretion of catecholamines colocalizes with the sites of Ca\(^{2+}\) entry. (a) Shows a snapshot image of the Ca\(^{2+}\) influx in a chromaffin cell taken 5 ms after a depolarizing pulse from -60 mV to +20 mV, duration 50 ms. A localized influx of Ca\(^{2+}\) led to the generation of hotspots of Ca\(^{2+}\) (in each case the patch pipette was in the 3 o'clock position). (b and c) Show the results of mapping release sites of secretion using flame-etched carbon fiber microelectrodes. The upper panels of (b and c) show bright field images for which the Ca\(^{2+}\) entry pattern is shown in (a). In each of the bright field images the position of the patch pipette can be seen in the 3 o'clock position, and the carbon fiber can be seen to be positioned firstly away from a hotspot (b) and then in close apposition (< 1 mm) to a hotspot in the 7 o'clock position (c). Amperometric recordings made from the positions shown in the bright field images are shown below (b and c). It can be seen that when the carbon fibers were away from the hotspots of Ca\(^{2+}\) entry that secretory events were not detected, but that when the fiber was moved to the hotspots that it was possible to see release events. This figure is modified from Robinson et al. [28].

Fig. 10
discrete sites in the plasma membrane of adrenal chromaffin cells. What these studies do not show, however, is whether or not the voltage-sensitive Ca\(^{2+}\) channels in these cells are clustered on the plasma membrane or whether there are large numbers of the channels that are in an inactive state. In order to address this question, we have used a monoclonal antibody that was raised against the class-specific cytoplasmic loop of N-type Ca\(^{2+}\) channels between the transmembrane spanning domains II and III. The antibody recognized in both brain and chromaffin cell homogenates a single polypeptide of \(\sim 200\) kDa (Fig. 10a), consistent with a neuronal Ca\(^{2+}\) channel \(\alpha_{1B}\) subunit [77]. In control experiments in which the primary antibody was omitted or replaced by serum from an adjuvant-immunized rat, no polypeptide bands were stained (data not shown). Thus the antibody used in this study was considered to recognize the \(\alpha_{1B}\) subunit of N-type Ca\(^{2+}\) channels with a high degree of specificity. It did not bind to a purified \(\alpha_{1B}\) subunit (provided by Dr K. Campbell). This antibody revealed a punctate pattern of staining in chromaffin cells. An example of the staining pattern is shown in Figure 10b. Using Nomarski optics it was possible to take optical sections through the cells that had been fluorescently labeled and count the numbers of fluorescent spots per cell. There were on average 4.9 \(\pm\) 0.2 (SEM; \(n = 100\)) spots of very bright fluorescence and 12.8 \(\pm\) 0.5 (SEM; \(n = 100\)) dimmer spots in each cell that were above the levels of background staining. Background fluorescence was determined from experiments in which the primary antibody was omitted or replaced by serum from a non-adjuvant rat. The cell shown in Figure 10b was chosen because three bright fluorescent spots could be seen in this focal plane. It was more usual that only a single spot was seen per optical section of each cell examined. The results of the antibody staining imply that at least the N-type Ca\(^{2+}\) channels are clustered in these cells. Should there be single channels, or clusters of smaller numbers of channels on the rest of the plasma membrane, these would be below the level of detection by immunofluorescence. It is also true that the imaging system would not have the resolution to visualize Ca\(^{2+}\) entry through a single channel.

Fig. 10 (opposite page bottom). Clustering of voltage-sensitive Ca\(^{2+}\) channels in chromaffin cells demonstrated by immunofluorescence of the \(\alpha_{1B}\) subunit of the N-type calcium channel. (a) shows a Western blot of chromaffin cell proteins with a monoclonal antibody raised against the cytoplasmic loop between the transmembrane spanning domains II and III of the N-type Ca\(^{2+}\) channel. The left-hand lane of the blot was loaded with mouse brain homogenate and the right-hand lane was loaded with cultured chromaffin cell homogenate. The antibody (dilution: 1/1000) recognized a single polypeptide band of estimated molecular weight about 200 kDa. The polypeptide recognized by the antibody in brain had a slightly higher electrophoretic mobility than that in chromaffin cells. The same antibody was also used in immunofluorescence studies in chromaffin cells and an example of the type of staining pattern seen is shown in (b) (antibody dilution, 1/10000). The staining pattern was punctate in nature, with the spots of fluorescence on the plasma membrane. Three brightly fluorescent spots are seen in this particular case. It was more usual for only a single spot to be seen per optical section of the cell examined. The dilution of antibody used in (b), 1/10000 was lower than that used in the analysis, 1/5000, in order to give higher background staining to facilitate visualization of the cell's outline. These images suggest that N-type channels are clustered in chromaffin cells. (c and d) Show images of the Ca\(^{2+}\) entry pattern seen in patch clamped chromaffin cells elicited by a depolarization of the cell before (c) and 7 min after the application of 1 \(\mu M\) \(\alpha\)-conotoxin GVIA to the cell (d). It can clearly be seen that the hotspots of Ca\(^{2+}\) entry at the 2 and 10 o'clock positions (marked by the arrows) disappeared after \(\alpha\)-conotoxin GVIA treatment.
Treatment of patch clamped chromaffin cells with the Ca\(^{2+}\) channel blocker, α-conotoxin GVIA, has provided further evidence for the clustering of channels in the plasma membrane. An image of the Ca\(^{2+}\) entry pattern into a cell that was depolarized from a holding potential of \(-60\) mV to \(+20\) mV for 50 ms is shown in Figure 10c. It can be seen that in this cell there were four hotspots of Ca\(^{2+}\) entry, at the 2, 7, 10 and 12 o'clock positions. The image in Figure 10d was acquired from the same cell shown in Figure 10c but 7 min after the application of 1 \(\mu\)M α-conotoxin GVIA. The hotspots at the 2 and 10 o'clock positions have disappeared but those at the 7 and 12 o'clock positions were little affected. This result suggests that in some cases there are clusters of channels that are α-conotoxin GVIA-sensitive and that there are clusters of channels in the same cell that are insensitive to this toxin. An effect of the α-conotoxin was observed in only 4 of 9 cells that were studied. The simplest explanation for this is suggested by the immunofluorescence staining of the N-type Ca\(^{2+}\) channels (Fig. 10b). It is clear that only a few clusters contain a large number of channels in these cells (about 5 per cell). If these clusters were out the plane of focus of the optical section that was used to obtain a Ca\(^{2+}\) image in a particular cell then one would not expect to observe a change in the pattern of Ca\(^{2+}\) influx. In order to confirm this it will be necessary to make a 3D reconstruction of the pattern of Ca\(^{2+}\) influx in each cell and to compare the imaging studies with immunofluorescence studies of the distribution of N-type Ca\(^{2+}\) channels in the same cell. It is known that chromaffin cells contain both L and P-type channels in addition to the N-type channel [60]. It seems plausible that these channels would also cluster in the plasma membrane. It will be of interest to determine if these channels are clustered such that only channels of a particular type are found together on the plasma membrane of the cells.

### SUMMARY AND PERSPECTIVES

#### A comparison of Ca\(^{2+}\) stimuli in chromaffin cells

The most striking features of this study were the differences observed in the patterns of elevation of intracellular Ca\(^{2+}\) concentration seen in patch clamped chromaffin cells following either the transient opening of voltage-sensitive Ca\(^{2+}\) channels or the photolysis of DM-nitrophen or caged Ins 1,4,5P\(_3\). The transient opening of voltage-sensitive Ca\(^{2+}\) channels often resulted in Ca\(^{2+}\) entry at very discrete regions beneath the plasma membrane (Figs 7–9). Gradients developed over a period of 10–50 ms following the opening of voltage-sensitive Ca\(^{2+}\) channels. By contrast, the flash photolysis of DM-nitrophen resulted in a fairly homogeneous increase in the cytosolic Ca\(^{2+}\) concentration. Accompanying this increase in the cytosolic Ca\(^{2+}\) concentration was a much larger increase in fluorescence in the uncaging of the cell's nucleus (see Fig. 5). Increases in intracellular Ca\(^{2+}\) peaked 4 ms after the DM-nitrophen, a much faster time for development of gradients by the opening of voltage-sensitive Ca\(^{2+}\) channels. Both of these patterns of Ca\(^{2+}\) elevation were markedly different from those observed following the photolysis of caged Ins 1,4,5P\(_3\) (see Fig. 6). In these experiments, there was a more diffuse elevation in intracellular Ca\(^{2+}\), but the increases were not homogeneous. The increases in intracellular Ca\(^{2+}\) concentrations that resulted from the photolysis of caged Ins 1,4,5P\(_3\) took several seconds to reach a peak. Thus the release of Ca\(^{2+}\) from intracellular stores occurs over a much longer time course than the changes in intracellular Ca\(^{2+}\) induced by the other two stimuli studied.

The physiological stimulus for secretion in the adrenal medulla is the release of acetylcholine from the splanchic nerve which triggers trains of action potentials in chromaffin cells. These would lead to an influx of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels. A localized elevation of Ca\(^{2+}\) generated by the first few action potentials would trigger the fusion of only a few secretory vesicles in a fashion analogous to that following the transient depolarization of a patch clamped cell (Figs 7–9, [26,28, 61,62]). A more prolonged train of action potentials would be expected to lead eventually to a more global elevation in Ca\(^{2+}\) concentration, such as is elicited by challenging chromaffin cells with nicotine [56] or by the uncaging of Ca\(^{2+}\) (Fig. 5). This would trigger a more robust and prolonged secretory response [49,54,55,62, 63]. One question remaining to be addressed in these cells is the role of the release of Ca\(^{2+}\) from intracellular stores in the generation of the Ca\(^{2+}\) gradients that follow depolarization stimuli. It is pertinent that chromaffin cells possess a Ca\(^{2+}\)-sensitive form of phospholipase C [64] and thus an elevation of Ca\(^{2+}\) in the cell would lead to generation of Ins 1,4,5P\(_3\) and release of Ca\(^{2+}\) from intracellular stores [56,65,66]. One role of these stores may be to augment Ca\(^{2+}\) increases in situations of prolonged stimulation.

#### Release sites in chromaffin cells

We propose that chromaffin cells have two functionally distinct arrangements of Ca\(^{2+}\) channels and secretory vesicles. Figure 11 illustrates our hypothesis. In regions of the plasma membrane that have hotspots of Ca\(^{2+}\) entry there exist clusters of Ca\(^{2+}\) channels. We have presented evidence for selective clusters of N-type channels in at least a subpopulation of cells (Fig. 10c,d). In the Introduction, we described how through a series of protein–protein interactions the synaptic vesicles are believed to be colocalized with N-type Ca\(^{2+}\) channels.
Thus it seems reasonable to postulate that at the sites of N-type Ca\(^{2+}\) channel clustering there is a tight colocalization between these channels, the secretory granules, and the fusion pore scaffold (Fig. 11a). Immunoprecipitation experiments have revealed the presence of a protein aggregate containing syntaxin, synaptotagmin and other members of the SNARE complex in chromaffin cells [67]. Furthermore, anti-syntaxin antibodies inhibit the Ca\(^{2+}\) triggered secretory response in permeabilized chromaffin cells [68]. Both of these observations support our hypothesis. Ca\(^{2+}\) signalling at clustered Ca\(^{2+}\) channels may be augmented by release of Ca\(^{2+}\) from intracellular stores. No molecular interactions between synaptic vesicle proteins or proteins of the SNAP/SNARE complex and other classes of Ca\(^{2+}\) channels have been reported. Thus, in contrast to the tight colocalization between secretory vesicles and N-type Ca\(^{2+}\) channels, we envisage that the remainder of the vesicles at the plasma membrane are not tightly associated with the L- or P-types of Ca\(^{2+}\) channels that are known to exist in chromaffin cells (see Fig. 11b).

The model that we have outlined allows some predictions of the time course of secretion expected from a chromaffin cell following different types of stimulus. The first is that short, transient openings of Ca\(^{2+}\) channels (in particular the N-type channel) would be expected to elicit the fusion of a small number of vesicles, those that are closely apposed to the sites of Ca\(^{2+}\) entry, and that these fusion events would occur with a short latency following the opening of the Ca\(^{2+}\) channels. This has been shown by several groups [28,61-63]. In order for vesicles that are more distal from the calcium channels to fuse with the plasma membrane, a more diffuse and global increase in the cytosolic Ca\(^{2+}\) concentration would be required. This could be achieved by repeated trains of action potentials or depolarizing stimuli [62,63], or by the flash photolysis of caged Ca\(^{2+}\) chelators [49,50,54,55]. A prolonged elevation of Ca\(^{2+}\) would lead to a build up of secretion during a train of action potentials and a delay in the cessation of the response thereafter due to the long time taken for Ca\(^{2+}\) to diffuse to the more distant vesicles [62]. A similar pattern of secretion is observed following the uncaging of Ca\(^{2+}\) [49,50,54,55].

**Future directions**

Some of the important issues raised during the course of these studies and amenable to answering include: (i) identifying which Ca\(^{2+}\) channels are found in the hotspots; (ii) determining if these channels are associated with secretory vesicles through the interaction of syntaxin 1A or Css; and (iii) determining the role of the release of Ca\(^{2+}\) from the intracellular stores in the propagation of the Ca\(^{2+}\) signal following the influx of Ca\(^{2+}\) through voltage-sensitive Ca\(^{2+}\) channels. The structural organization of secretory vesicles and Ca\(^{2+}\) channels proposed here for chromaffin cells may be similar to that thought to occur at the active zone of a synapse. Due to the inaccessibility and fragility of synapses, it has not yet been possible to test this hypothesis. The combination of pulsed laser imaging, patch clamp recording, amperometry and molecular biology should allow us to dissect the active release zones found in chromaffin cells.

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**APPENDIX**

**MATERIALS AND METHODS**

Cell preparation and culture

Chromaffin cells were prepared from bovine adrenal medullae by enzymatic digestion [69]. Isolated cells were suspended in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, 10% fetal calf serum, 8 mM 5-fluoro-2'-deoxyuridine, 50 µg/ml gentamycin, 10 µM cytosine arabinofuranose, 2.5 µg/ml fungizone, 25 µ/ml penicillin, 25 µg/ml streptomycin and plated at a density of 100,000–500,000 cells/ml on glass coverslips that formed the bottom of a 35 mm diameter Petri dish. Cells were cultured in a humidified atmosphere at 37°C in the presence of 5% CO₂ for 1–5 days prior to use.

**Solutions**

For experiments cells were washed in an extracellular medium comprising 120 mM NaCl, 20 mM HEPES, 4 mM MgCl₂, 2 mM CaCl₂, 2 mg/ml glucose and 1 µM tetrodotoxin at pH 7.2 (adjusted with NaOH). For depolarization experiments, the patch pipette solution contained 130 mM cesium-D-glutamate, 30 mM HEPES, 8 mM NaCl, 1 mM MgCl₂, 2 mM MgATP, 0.3 mM GTP and 0.4 mM rhod-2 (triammonium salt). Caged Ins 1,4,5P₃ (Calbiochem, San Diego, CA, USA) was added at a final concentration of 1 mM to this solution. Solutions used for the imaging of caged Ca²⁺ release contained 130 mM cesium-D-glutamate, 1.5 mM GTP, 0.2 mM MgATP, 3 mM CaCl₂, 10 mM DM-nitrophosphine, 0.4 mM rhod-2 and 10 mM HEPES (pH 7.2). This solution was used for both the in vitro experiments and for those involving patch clamped chromaffin cells. In the case of the in vitro experiments, small droplets of this solution were placed on glass coverslips (that formed the bottom of a 35 mm diameter Petri dish) and immersed in mineral oil. Experiments were carried out at room temperature.

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Capacitance measurements of secretion

Exocytosis was monitored by measuring the cell membrane capacitance using the whole cell mode of the patch clamp technique [70] in conjunction with a digital phase detector [71] implemented on a system comprising an AT-MIO16x interface (National Instruments) and an Axopatch 200A patch clamp amplifier (Axon, Rapid City, CA, USA). All the data acquisition and analysis programs were written in Labview (v3.01, National Instruments). A sinusoidal voltage (833 Hz, 54 mV peak to peak) on top of a holding potential of -60 mV was applied to the patch clamped cell and the current measured at two different phase angles, \(\Theta\) and \(\Theta - \pi/2\), relative to the stimulus. The phase was periodically adjusted using the phase tracking technique [72] so that the output at \(\Theta - \pi/2\) reflected changes in the real part of the cell admittance (Re [\(\Delta Y\)]), while the output at \(\Theta\) reflected changes in the imaginary part of the admittance, (Im [\(\Delta Y\)]). Capacitance and a.c. conductance data, along with voltage and d.c. conductance were captured with a temporal resolution of 9.6 ms per point.

Amperometric detection of secretory products

Two types of carbon fiber electrodes were used to monitor catecholamine release in this study. The first type had diameters of \(\sim 14 \mu m\) (approximating the diameter of the cell), the second type had tip diameters of \(1-2 \mu m\). In order to fabricate the electrodes, a carbon fiber was aspirated into a glass capillary pipette which was then pulled on a micropipette puller. In the case of the larger electrodes, the carbon fiber was sealed to the glass capillary tube by immersing the tips of the fibers into epoxy. The epoxy was cured overnight at room temperature, then at 100°C for 2 h and finally at 150°C for another 2 h [73]. Before use the tip of the fiber was bevelled at 45° with a micropipette beveler resulting in an elliptically shaped carbon fiber tip. The second type of carbon fiber electrodes used in these studies were pulled such that \(\sim 500 \mu m\) of the carbon fiber was exposed beyond the end of the glass capillary tube. The exposed carbon fiber was etched in a Bunsen burner flame which reduced the carbon to a cone shape with a submicron sized tip. The pipettes were filled with colloidal graphite and a chromel wire was inserted into the end of the pipette. The fibers were insulated with poly(oxyphenylene) which was electrochemically deposited on the fiber at +4 V for 8 min. [27,28]. The tip of the carbon fiber was bevelled for 5 s to remove the insulation at the very tip of the electrode. In either case, the carbon fiber was held at a potential of +650 mV and the redox current generated by the oxidation of the released catecholamines was monitored by a modified Axopatch 1B or a List EPC-7 amplifier.

The imaging system

The imaging system used in some of the experiments in this study has been previously described [26,28]. Most of the experiments described in this paper were carried out using a modified version of the original system. The modified system is shown schematically in Figure 1. The current system is comprised of an inverted epifluorescence microscope (Axiovert, Carl Zeiss, Oberkochen, Germany), a cooled charge couple device (CCD) camera (Photometrics, Tucson, AZ, USA) and a microcomputer (Gateway Pentium 75 MHz, Sioux City, ND, USA). Fluorescence images were transferred to the computer and analyzed using software from Photometrics. Patch clamp and amperometric data were acquired with either an Axopatch 200A or a List EPC-7 amplifier via an AT-MIO-16X interface board (National Instruments, Austin, TX, USA) and the acquisition program used was written in Labview (v3.01, National Instruments). Illumination in the imaging system was achieved by coupling a high intensity pulsed coaxial flash lamp dye laser (LumenX model LS-1400, Phase-R Corporation, New Durham, NH, USA) by a quartz fiber light guide and a custom made adapter to the epifluorescence path of the microscope. The lasing dye, Coumarin 525 (0.1 mM in methanol) was used to excite the Ca²⁺ fluorescent dye rhod-2. The custom made adapter also housed a dichroic mirror held at a 45° angle. This mirror reflected UV light from a frequency doubled ruby laser, output 347 nm (Lumonix, Rugby, UK) into the same epifluorescence light path as the visible laser (see Fig. 1). The use of this UV light emitting laser made it possible to uncage various photoactivatable compounds and image the resultant changes in Ca²⁺ concentrations. The epifluorescence block contained a 545 nm DCRX mirror and a 555 nm EFLP emission filter. A Zeiss 100× Neofluar oil immersion objective was used to both uncage photoactivatable compounds and obtain Ca²⁺ images. Image pairs of control and stimulated events were recorded.

Western blot procedure

Bovine chromaffin cells or whole mouse brain were lysed directly in Laemmli sample buffer [74]. About 20 \(\mu g\) of protein per lane were loaded in a 6% SDS-PAGE gel. The proteins were than transferred to nitrocellulose membranes (Hybond-ECL, Amersham, Little Chalfont, Bucks, UK). A rat monoclonal antibody raised against the II-III cytoplasmic loop of the \(\alpha_\text{II}\) subunit of the N-type calcium channels (CC20) [75] was used at a 1/1000 dilution to probe the blot [76]. The ECL Western blotting system was used to visualize the polypeptide bands and was used according to the manufacturer’s instructions (Amersham). Phosphate buffered saline, pH 7.2 (PBS; Gibco...
BRL, Gaithersburg, MD, USA) and 0.1% Tween 20 were used in all the blocking, incubation and wash steps. Anti-rat Ig, horseradish peroxidase-linked whole antibody was used at a 1/300 dilution. The autoradiograms were scanned in a ScanJet 4C scanner (Hewlett Packard, Palo Alto, CA, USA) at 600 dpi.

Immunofluorescence of chromaffin cells

Bovine chromaffin cells were cultured on glass coverslips coated with 0.1% poly-L-lysine for 2–12 days. Cells were washed 3 times with PBS before being fixed in 4% paraformaldehyde for 10 min. Permeabilization and blocking of nonspecific interactions of the antibodies were achieved by incubating the cells with 0.1% Triton X-100 and normal sheep serum (diluted 1/100 in PBS). This PBS buffer was used throughout the wash and incubation steps which were done following standard procedures [77]. Indirect immunofluorescence was detected using an anti-rat biotinylated secondary antibody from sheep (diluted 1/100; Amersham) and the fluorophore Cy2 conjugated to streptavidin (1/1000; Amersham). Micrographs were taken using a mercury lamp and a cooled CCD camera (Photometrics). Fluorescence images were transferred to a PC computer and analyzed using software from Photometrics.