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STIMULUS–SECRETION COUPLING: A PERSPECTIVE HIGHLIGHTING THE CONTRIBUTIONS OF PETER BAKER

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Summary

Many investigators are using numerous preparations for contributing to our present understanding of stimulus–secretion coupling, by which we mean stimulus-dependent exocytosis, sometimes known as the regulated pathway. However, a few model systems have been particularly illuminating and several of these were exploited by Peter Baker and his close associates: namely, the motor nerve terminal, the adrenal chromaffin cell, the sea urchin egg and the blood platelet. In fact, Peter's first real contribution in this area came from his seminal studies on calcium transport in his favourite preparation, the squid giant axon, where he investigated Ca^{2+}/Na^{+} exchange, Ca^{2+} distribution and voltage-gated Ca^{2+} entry. More direct investigations into stimulus–secretion coupling came from work on neurone transmitter release in collaboration with Andrew Crawford, and on catecholamine secretion from the adrenal medulla in collaboration (with TJR). His most important generic contribution to this field was in the development (with DEK), of the electropermeabilized cell, which allows control of the low molecular weight components of the cytosol while leaving the exocytotic apparatus and process intact. In the initial experiments on the cells it was finally proved that Ca^{2+}-dependent secretion of catecholamines is indeed from the granules and not from the cytosol. The quantification of the Ca^{2+} requirement of secretory exocytosis was an important step, as was the investigation of many factors purported to be important in the coupling mechanism or in the exocytotic process itself. Work with the human platelet, using this technique, has proved to be especially valuable in unravelling the complex interactions between different second messengers and has been neatly complemented by work in intact cells containing Ca^{2+}-indicator fluorescent dyes. Peter was also intrigued by post-secretory events both in the early seventies, and at the end of his career when he embarked on analysis of the membrane retrieval process and the associated uptake of extracellular medium.

Key words: secretion, calcium, exocytosis, stimulus–secretion coupling.

Introduction

We have two main objectives for this article: to outline some of the main steps on the road to our present understanding of stimulus–secretion coupling and the 'regulated' pathway of secretory exocytosis; and to highlight the main contributions made by our mentor and colleague, the late Peter Baker, and by some of the scientists he introduced to this field.

The term secretion is used to describe several rather different biological processes, ranging from neurotransmitter release to the production of milk. Here, we will be considering stimulus–dependent discharge of the contents of specific intracellular granules or vesicles by exocytosis. This is an exceedingly neat biological trick which we now recognize as having at least two major functions. It provides a way for the rapid and specific release of substantial amounts of carefully selected substances which can be of high molecular weight, very hydrophilic, and unlikely to pass readily through the cell membrane. It is also a way of rapidly inserting new membrane components into the surface membrane and increasing the surface area of specific parts of the membrane. Two important examples of this lesser known function of exocytosis are the insertion of water-permeable channels into the luminal membrane of distal nephron cells under the influence of antidiuretic hormone, and the insertion of proton-pumping vesicles into the apical membrane of the parietal cells of the gastric mucosa.

We can also note the diversity and versatility of this mechanism throughout an enormous range of biological function and evolutionary scale, and this was one of the aspects of secretion that appealed to Peter Baker. Secretory exocytosis underlies the millisecond events of neurotransmitter release, many forms of hormone secretion, fundamental processes of fertilization, the acrosome reaction of sperm and the cortical reaction of the egg and, more primitively yet, the discharge of the contractile vacuole of free-living protozoans such as amoeba.

It can be helpful for both conceptual purposes and to gain a historical perspective, to subdivide stimulus–secretion coupling into a number of steps or processes. First we will consider the storage of secreted products into specific granules or vesicles and the demonstration of their stimulated release by exocytosis. Next comes a consideration of the role of cytosolic calcium. More recently has come an investigation into other factors that can trigger and regulate secretory exocytosis. Then we will consider various theories and models for the mechanism and processes of the exocytotic event itself and, finally, briefly consider membrane retrieval.

As with many complex biological processes, our understanding has come through a concentration on particularly apt experimental systems. Several cell types and tissue preparations in hundreds of laboratories have made major contributions to our understanding of stimulus–secretion coupling, but we think all would agree that among the most important are the adrenal medullary chromaffin cell, cholinergic nerve terminals and in particular the frog neuromuscular junction, the sea urchin egg and the blood platelet.

Each of these was exploited by Peter Baker and/or his close colleagues. Experiments with these preparations will form the main focus of our account here, together with some of his seminal work on calcium transport in what was surely his favourite preparation, the squid giant axon.

Granules, vesicles and exocytosis

Following the discovery of hormones and the birth of endocrinology, it was recognized that many secretory tissues released their secreted product from a large prefabricated intracellular store. The granular nature of secretory cells, readily recognized in stained sections examined by light microsopy, hinted at the existence of specialized organelles for such storage. In the early 1950s, several investigators, including Blashchko & Welch (1953), showed that much of the catecholamine contained in the cells of the adrenal medulla was in a subcellular particle which was subsequently shown to be a distinct organelle, the chromaffin granule. However, in all fractionation studies a significant amount of the catecholamine was present in the supernatant and these studies could not, therefore, themselves resolve the issue of whether secretion was directly from the granular store or from the cytosolic pool. The first direct proposal of the mechanism of exocytosis as the basis of secretion came from electron microscopic analysis (De Robertis & Vaz Ferreria, 1957).

The next stage in building up evidence came from detailed analysis of the biochemical composition of the contents of the secretory granule and of the material released following stimulus of the chromaffin cell. The basic point is that the proportion of other substances to catecholamines was found to be similar within the granule and in the perfusate of stimulated adrenal glands (see, for example, Douglas, 1968). This was seen both with ATP, which is present in the chromaffin granule in a molar ratio of approximately 1:4 with catecholamines, and also with various proteins found in the chromaffin granule, such as a protein termed chromogranin and the soluble fraction of the enzyme dopamine β-hydroxylase. It was also demonstrated that the lipid components of the chromaffin granule were not released from the cell following stimulation (Schneider, Smith & Winkler, 1967; Douglas, 1968). These observations are easily fitted in with the model of secretion whereby the membrane of the secretory granule fuses with the inside of the plasma membrane to exteriorize the contents of the granule, but only with difficulty into any other model.

Another experiment very clearly demonstrating that stimulated secretion occurs from the granular pool came as a by-product of work by Peter Baker and one of the present authors (DEK) in the leaky, or the electropermeabilized, adrenal chromaffin cells (Baker & Knight, 1978). In this experimental preparation, disaggregated cells are exposed to intense high-voltage fields for very brief periods to induce the formation of pores in the plasma membrane which allow ready diffusion of small molecules, up to about 1000 Da. This allows one effectively to dialyse the cell interior yet leaves intact the cellular protein and macromolecular

apparatus including that required for stimulus–secretion coupling. Any cytosolic pool of catecholamines is, of course, immediately dispersed by this manoeuvre and yet, as will be discussed below, a very substantial pool of stimulus-dependent releasable catecholamine remains within the granules. Another recent contribution has been the on-line measurement of the capacitance of the surface membrane of single chromaffin cells by means of the patch-clamp technique (Neher & Marty, 1982). With this sophisticated approach it proved possible to demonstrate very small discrete step changes in the capacitance of the surface membrane when the cells were stimulated to secrete, which is the precise prediction of the exocytotic process whereby small, but significant, increments in membrane area must occur with every exocytotic event of granule fusion. Interestingly, one can go back to the 1930s to find what was actually the first clear evidence for exocytosis in experiments which measured the capacitance of *Hipponoe* eggs before and after fertilization by passing an a.c. signal through a suspension of the eggs. In doing this, Cole (1935) calculated a two- to three-fold increase in surface membrane area following fertilization; we now realize that this resulted from the exocytotic fusion of the cortical granules with the plasma membrane as a key part of the fertilization response in these eggs and the elevation of the fertilization membrane.

It was also in the 1950s that Katz and his colleagues discovered the quantal nature of neurotransmitter release at the frog neuromuscular junction, in which action potentials appeared to release discrete packets (or quanta) of acetylcholine (e.g. Del Castillo & Katz, 1956). Electron micrographs of the nerve terminals revealed numerous small vesicular structures which seemed likely to be the structural basis of these quanta. A few years later Whittaker and his co-workers fractionated the synaptic vesicles from mammalian brain and demonstrated that these contained acetylcholine (Whittaker, Michaelson & Kirkland, 1964). It has not proved possible to isolate the acetylcholine-containing vesicles from motor nerve terminals because these form such a minute fraction of the volume of tissue in the muscle; however, an evolutionarily homologous structure, the electroplax of the electric ray, contains massive accumulations of the vesicles in the cholinergic nerve terminals, and isolation of a pure fraction of these vesicles has been achieved (Whittaker, 1984). For many years it proved difficult to demonstrate convincingly that following nerve stimulation the expected 'omega figures' could be seen, to give a visual demonstration that exocytosis of a vesicle had, indeed, occurred. This turns out to be because the exocytotic process is very transient and occurs at somewhat dispersed localized spots.

However, later workers, including Heuser and Ceccarelli, found patterns of stimulation and other experimental manoeuvres which very clearly showed that the number of exocytotic figures seen in the electron micrograph fitted well with the electrophysiologically determined release of acetylcholine at the neuromuscular junction (e.g. Heuser, 1978; Torri-Telli, Grohavaz, Fesce & Ceccarelli, 1985). A recent experiment adds to the evidence in favour of exocytosis as the majo[means of neurotransmitter release; antigens which are normally oriented on the

inside of acetylcholine-containing synaptic vesicles appeared on the surface of the terminal following intense stimulation (Von Wedel, Carlsson & Kelly, 1981). We do not propose to discuss here the residual controversy over the precise role and type of exocytosis at the cholinergic nerve terminal, except to note that there is good evidence for a 'non-quantal' release process which occurs continuously, albeit at a very low level (Tauc, 1982). In our minds, a low level of release from the terminal across the plasma membrane fits quite well with expected physiology. Since choline acetyltransferase is a cytosolic enzyme, acetylcholine is presumably synthesized in the cytosol and then transported by secondary active transport and accumulated within the vesicle. For this to be effective there must be a cytosolic pool of acetylcholine and it seems entirely plausible that some of this should leak out across the plasma membrane on the choline transporter which is known to be present and is needed for the nerve terminal to obtain the choline substrate for the synthesis of acetylcholine. Another explanation of non-quantal release might be that pumps are inserted into the plasma membrane as a result of exocytosis. Acetylcholine is accumulated from the cytosol into intracellular vesicles by acetylcholine transporters in their membranes, these pumps operating to move acetylcholine from low cytosolic concentrations to high concentrations within the vesicle (Parsons & Koenigsberger, 1980). Immediately after exocytosis, the vesicular membrane including these pumps is incorporated into the plasma membrane. The continued operation of these pumps after insertion by exocytosis may give rise to the observed non-quantal component of acetylcholine release. Support for this comes from the finding that agents that block the transporter on the vesicle also block non-quantal release (Edwards *et al.* 1985). No doubt the debate will continue, especially with such findings as a Ca^{2+}-dependent acetylcholine transporter associated with the plasma membrane which is quite distinct from the pumps found in the vesicle membrane (Israel, Meunier, Morel & Lesbats, 1987). There has been increasing evidence that γ-aminobutyric acid (GABA) can be released from nerve terminals by depolarization but not by Ca^{2+}-triggered exocytosis and recent work from Schwartz (1987) seems to remove all doubt about this.

It was clear that if exocytosis were the mechanism of secretion, the cell needed a mechanism for membrane retrieval to maintain the steady state, and that this was probably some form of endocytosis, implying a post-stimulation uptake of extracellular fluid. In an early study of this type Peter Baker, with a number of continental colleagues, demonstrated a stimulus-dependent uptake of extracellular markers including sucrose and albumin into the neurohypophysis (Nordmann *et al.* 1974). The extent of this uptake was compatible with membrane retrieval in the microvesicles that were seen by electron microscopy in stimulated neurohypophyseal nerve terminals. There is a personal footnote to this section, in that the first project that Peter Baker suggested to one of us, (TJR) was a quantitative analysis of the stimulus-dependent changes in vesicle number and distribution in the frog motor nerve terminal by a combination of electrophysiology and electron microscopy. This was the type of ambitious project he liked to give to aspiring, and

naive, young scientists. A large selection of quite good electron micrographs, taken on a 1951 vintage Siemens electron microscope, still exists in the files. However, the publication, within 3 months of the start of this project, of a paper by Heuser & Reese (1973) achieving many of the objectives which Peter had set, allowed a relieved young scientist to turn his attention to the more feasible, if temporarily less exciting, territory of the adrenal chromaffin cell.

The role of calcium

It is now more than 100 years since Sidney Ringer first focused attention on the importance of calcium in the contractile activity of the heart. It is nearing 50 years since Harvey & MacIntosh (1940) came to the same conclusions about a role for calcium in neurotransmitter release. The influence of calcium on neurotransmitter release was studied in considerable detail by Katz and his colleagues in the 1950s (Katz, 1969). A striking dependence with an approximately fourth-power relationship was noted between the external concentration of calcium and the quantal content of the end-plate potential but, initially, it was not clear whether calcium was acting externally or at an internal site.

A more general role for calcium in the secretory process became evident from the pioneering work of Douglas and his colleagues, first in the adrenal medulla (Douglas & Rubin, 1961) and then in many other secretory systems. Noting that calcium was effective in eliciting secretion only under conditions when membrane permeability was expected to be increased, Douglas clearly expressed the proposition that calcium had to reach an intracellular site in order to trigger secretion (Douglas & Rubin, 1961; Douglas, 1968). Douglas and his group also demonstrated the increased uptake of tracer calcium during stimulation, supporting this conclusion. Various observations on nerve terminals have since supported this hypothesis for stimulus–secretion coupling. For instance, calcium-dependent action potential could be evoked in motor nerve terminals under special experimental conditions and lead to substantial acetylcholine release. In the voltage-clamped presynaptic nerve terminal of the squid giant synapse, Katz & Miledi (1977) showed that very strongly depolarizing pulses, reaching the expected reversal potential for Ca^{2+}, did not evoke transmitter release during the on-phase, but that on switching off the pulse there was postsynaptic activity consistent with transmitter release following a calcium 'tail current' carrying calcium ions to an intracellular site of action (see, for example, Katz, 1969; Katz & Miledi, 1967, 1969). Further evidence was obtained in the squid giant synapse when the photoprotein aequorin was injected into the presynaptic terminal and a calcium signal could be obtained concomitant with transmitter release (Llinas, Blinks & Nicholson, 1972).

During the period of these developments it had become clear that we badly needed experimental techniques to measure calcium levels in real time in intact cells undergoing secretion. We also needed some means of imposing defined changes in levels of calcium and in other cellular components of interest in a

preparation in which the secretory apparatus was directly accessible to the external medium, in a manner somewhat analogous to a skinned muscle fibre or an isolated myofibril preparation. One remembers meetings in the early 1970s at which it was clear that our understanding of stimulus–secretion coupling was one or two decades behind that of stimulus–contraction coupling, partly for want of these technologies. However, before moving on to the developments that have occurred in these areas over the last 10 years this seems an appropriate place to review some of the contributions made by Peter Baker to our understanding of cellular homeostasis of calcium and calcium transport. The key work was done in the late 1960s and early 1970s at the Marine Biological Association, Plymouth using squid giant axons. These studies complemented and extended those of Ashley & Ridgway (1970) in the even larger muscle fibres of the giant barnacle. Both these preparations allowed substantial control of the intracellular medium by axial injection or perfusion, many years before microinjection and 'whole-cell patch-clamping' were developed for small mammalian cells. The major achievements were: the discovery of a Na^+/Ca^{2+} exchange; the first measurements of cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, in nerve; and the analysis of a potential-dependent Ca^{2+}-entry, which served as an important conceptual model for ideas of voltage-operated calcium gating in many cell types.

Cytoplasmic viscosity and granule motion

The earliest observations on calcium and squid axons came from Peter Baker's Ph.D supervisor, Alan Hodgkin. In 1949, Hodgkin & Katz reported that exposing axoplasm extruded from giant axons to millimolar concentrations of Ca^{2+} caused the jelly-like axoplasm to liquify. This observation was somewhat influential over three decades for hypotheses in which physiologically elevated Ca^{2+} level was proposed to reduce cytoplasmic viscosity and, for instance, promote effective access of secretory granules or vesicles to release sites on the inner surface of the plasma membrane. Another mentor of Peter Baker, the late Trevor Shaw, was intrigued with this hypothesis and set up laser light-scattering experiments to investigate it in collaboration with one of the organizers of this symposium, David Sattelle. Peter Baker was similarly intrigued by this elegant hypothesis and also set up laser light-scattering measurements when he moved to King's College, London in the mid 1970s (Baker, Knight, Piddington & Ross, 1977). It was, in fact, these studies that led us to develop the isolated chromaffin cell preparation which was to prove so valuable in our later studies.

The idea was to measure the power spectrum of laser light scattered from the surface of medullary tissue of a perfused adrenal gland. Baker hoped to see a component of the power spectrum, that was associated with the granule, change during secretion. Unfortunately, however, the movement of the surface of the tissue gave such a large optical signal that it completely swamped any small one originating from granule movements. An attempt was made, without success, to reduce the noise by immobilizing thin strips of medullary tissue and by using optical fibres to carry the signals to cells deep in the medullary tissue. The

technique was therefore developed to isolate viable bovine chromaffin cells and immobilize them in a thin dialysis tube held in the path of a laser beam. Although this greatly reduced the background noise, it still proved difficult to distinguish between the different sources of the optical signals in secretory cells and thus to provide definitive support or rejection of the hypothesis. A more direct approach to factors regulating axoplasmic viscosity was used by Rubinson & Baker (1979), with a micro viscosity measurement on isolated axoplasm. Among the findings of this work were intriguing effects of different anionic components of the bathing medium. However, the basic Ca^{2+}-dependent liquefaction was subsequently shown to be mainly due to an irreversible hydrolysis of cytoskeletal proteins by Ca^{2+}-activated proteases (Gilbert, 1975), possibly not part of a physiological regulatory system. At about this time, one of the present authors (TJR) had been attempting to look for stimulated movements of secretory granules by direct Nomarski examination of disaggregated chromaffin cells. The granules could be clearly seen and were remarkable for their lack of any observable motion, even when the cells were stimulated with high K^+, or Ba^{2+}, a potent secretagogue. The lack of movement within cells was contrasted with the easily seen Brownian motion of isolated chromaffin granules resuspended at approximately the same density. Furthermore, even damaged cells that were clearly disrupted and stained with trypan blue had motionless granules despite the presence of 3·6 mmol l^{-1} Ca^{2+} in the medium. In these cells therefore, millimolar concentrations of Ca^{2+} did not appear to liquify the cytoplasm or to release secretory granules from the embrace of the cytoskeleton.

Calcium movement in squid axons

In 1957, Hodgkin & Keynes had examined the mobility and uptake of $^{45}Ca^{2+}$ in squid axons and calculated that no more than 2 % was freely diffusible, and that 10 μmol l^{-1} was the upper limit of $[Ca^{2+}]_i$. They also showed that action potentials were associated with a significant uptake of Ca^{2+}. In the late 1960s Peter Baker and his colleagues at Plymouth, including Hodgkin, Blaustein and Steinhardt, found a ouabain-insensitive Na^+ efflux that was dependent on external Ca^{2+}, a Ca^{2+} efflux that depended on external Na^+, and a Ca^{2+} uptake that was increased by high internal Na^+ and by replacement of external Na^+ (Baker, Blaustein, Hodgkin & Steinhardt, 1969; Blaustein & Hodgkin, 1969). Similar results on Ca^{2+} uptake were also found in crab nerve. These findings, which were explored in considerable detail and are summarized in an important review (Baker, 1972), strongly suggest the presence of a Na^+/Ca^{2+} exchanger with complex kinetics and a stoichiometry that could have been $3Na^+:1Ca^{2+}$, and which would operate in both directions depending on the conditions. In theory, this transporter could just have maintained the then estimated resting $[Ca^{2+}]_i$ of 0·3 μmol l^{-1}. But it was clear that axoplasmic ATP significantly influenced Ca^{2+} efflux. Subsequently, both Peter Baker (Baker & McNaughton, 1978) and other groups (e.g. DiPolo & Beauge, 1979) showed that the squid axon, like other cells, has a high-affinity Ca^{2+}-transport ATPase as well as the lower affinity, but higher capacity,

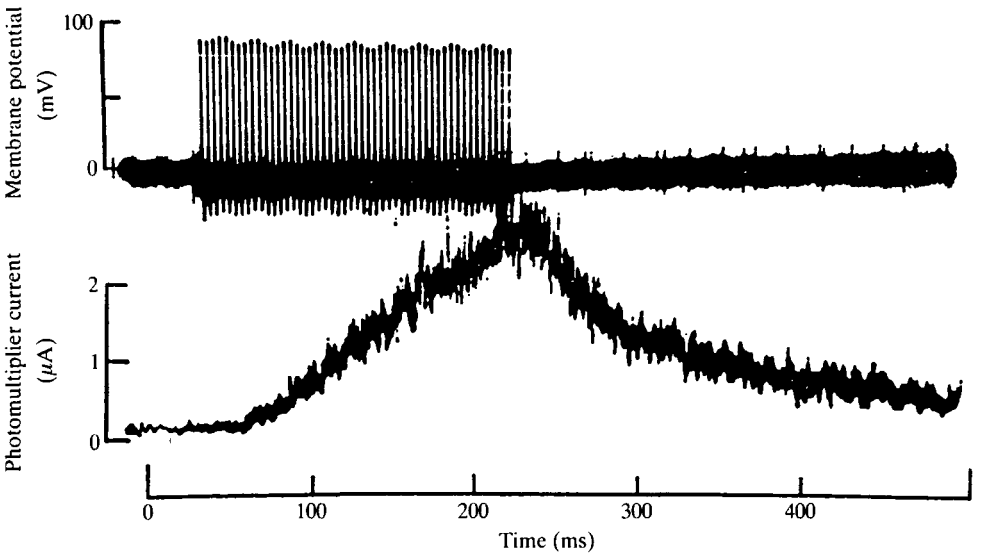

Fig. 1. Increase in light output from an aequorin-injected squid giant axon during a train of stimulated action potentials. Upper trace, membrane potential; lower trace, photomultiplier current. An increase in current denotes an increase in $[Ca^{2+}]_i$. This axon was bathed in artificial sea water containing 112 mmol l^{-1} $CaCl_2$.

Na^+/Ca^{2+} exchange. An excellent account of the later work from both sides of the Atlantic is provided in a review by Baker & DiPolo (1984).

Aequorin in squid axons

Perhaps the most memorable Ca^{2+} experiments with squid axons were those in which injected aequorin was used to monitor voltage-gated Ca^{2+} entry (Baker, Hodgkin & Ridgway, 1971). Legend has it that an eminent physiologist had demonstrated to Peter Baker, by meticulous calculation, that aequorin would be some orders of magnitude too insensitive to report Ca^{2+} transients in giant axons. It was characteristic of him to regard this as simply one more challenge and conduct the requisite experimental test. In the event, aequorin was able to provide an estimate of basal $[Ca^{2+}]_i$, to report effects of altering Na^+ gradients, to demonstrate $[Ca^{2+}]_i$ changes resulting from trains of action potentials (see Fig. 1), though not from single ones partly due to the response time, and to give usable signals during and after single voltage-clamp pulses. In the squid giant axon the signal could be calibrated *in situ* by finding the CaEGTA buffer which did not lower or raise the basal light output, an advantage with these giant cells. In voltage-clamp experiments the aequorin signal could be subdivided into two phases. Very short depolarizing pulses, up to 200 ms, led to a signal that was depressed by tetrodotoxin (TTX) and presumably represented Ca^{2+} entry through the Na^+ channel. Longer pulses gave a 'late-phase' entry which was insensitive to TTX but was blocked by Co^{2+} and Mn^{2+} and also by the organic calcium

antagonist D600 (Baker, Meves & Ridgway, 1973*a*). The similarity of these properties to those of Ca^{2+}-dependent neurotransmitter release was fully recognized. Another feature noted in these studies was the slow (taking many seconds) inactivation of Ca^{2+} entry with continued K^+ depolarization (Baker *et al.* 1973*b*). These studies, along with the increasing number of demonstrations of Ca^{2+}-dependent action potentials in various types of excitable cells, were important in focusing the attention of biologists on voltage-operated Ca^{2+} entry systems. Interestingly, it is still a matter of some debate as to whether the late-phase Ca^{2+} entry in squid axons goes through specific Ca^{2+} channels, or possibly through the delayed rectifier K^+ channel. Peter Baker and his colleagues went on to make several further studies on the mechanisms of Ca^{2+} buffering and transport in squid axons, but the main impact in thinking about stimulus–secretion coupling came from these pioneering early experiments.

Frog neuromuscular junction and adrenal medulla

The significance of these and other aspects of Ca^{2+} transport and handling of squid axons to stimulus–secretion coupling was discussed in perspective in a review (Baker, 1972) and various considerations from these studies stimulated work by Andrew Crawford on the frog neuromuscular junction and by T. J. Rink on the bovine adrenal medulla. Careful analysis of the influence of types of manipulation of the Na^+ gradient did not readily support a major and dominant role for Na^+/Ca^{2+} exchange in either the neuromuscular junction or the adrenal chromaffin cell. At the neuromuscular junction, the effects of reducing $[Na^+]_o$ or raising $[Na^+]_i$ mostly persisted in Ca^{2+}-free solutions, suggesting an effect on internal Ca^{2+} stores rather than on Na^+/Ca^{2+} exchange (Baker & Crawford, 1975). Interestingly, Li^+ entry had a marked stimulatory effect on neurotransmitter release (Crawford, 1975); one might now ask whether this reflects alteration of inositol phosphate metabolism. In one circumstance the evidence did suggest a role for Na^+/Ca^{2+} exchange. In the ouabain-poisoned junctions when the rate of miniature end-plate potentials was elevated, presumably reflecting elevated $[Ca^{2+}]_i$, removal of external Na^+ greatly increased the release rate and restoration of Na^+ reduced it again.

In adrenal medulla slices, secretory responses were seen with Na^+ replacement, but these depended critically on the nature of the Na^+ substitute (Rink, 1976). Measurement of $^{45}Ca^{2+}$ fluxes (both uptake and efflux) and of net Ca^{2+} movement by atomic absorption spectrophotometry did not provide ready support for a significant role for Na^+ exchange. However, later work by another of Peter Baker's younger colleagues, Gillian Pocock, suggested that Na^+/Ca^{2+} exchange might be more easily seen in disaggregated chromaffin cells, where it was found that not only did ouabain appear to increase the rate of secretion but it also inhibited Ca^{2+} efflux (Pocock, 1983). It is perhaps worth mentioning here, however, that the predicted increase in Ca^{2+} could not be detected by quin2 studies. Studies of Na^+/Ca^{2+} exchange in most tissues are still extremely complicated and the role of this transporter is far from clear in these cases. It also

seems that Na^+/Ca^{2+} exchange is prominent in only some cell types and lacking, or virtually so, in others.

In studies of the secretory response during prolonged K^+ depolarization, inactivation of Ca^{2+} entry into bovine adrenal cells, similar to that postulated in the squid giant axon, seemed to underlie the transient secretory response well known under these conditions (Baker & Rink, 1975). In those studies, a small-print section (an editorial technique much favoured by Peter Baker) reported the use of a fluorescent-sensitive dye to demonstrate that elevated K^+ did produce a prolonged depolarization in medullary slices; this was another example of his eagerness in early exploitation of a novel technique. Also in the mid 1970s came the introduction of the Ca^{2+}-transporting ionophore A23187. The ability of this antibiotic to stimulate secretion in many cell types, including chromaffin cells and platelets, was further strong support for a trigger role for Ca^{2+}. (Unusually, neither Peter Baker nor his associates were rapidly off the mark with this approach, though Ca^{2+} ionophores were to be of immense importance with the fluorescent Ca^{2+} indicators introduced by Roger Tsien and T. J. Rink in the early 1980s.) Although one can guess that A23187 works by moving Ca^{2+} into the cytosol, one cannot work out what level of $[Ca^{2+}]_i$ is achieved. As was mentioned above, we badly needed ways of imposing known, quantitated changes of $[Ca^{2+}]_i$ and of other factors on the secretory apparatus, and ways of measuring $[Ca^{2+}]_i$ in intact functioning cells. We spent much time in the mid 1970s trying to incorporate calcium-sensitive probes, such as arsenazo 3, into isolated chromaffin cells by various techniques such as the fusion of liposomes loaded with dye with the cells. Although we were able to prepare unilamellar liposomes of various lipid compositions we, like so many others at that time, were unsuccessful in getting them to fuse reliably and thus trap the Ca^{2+}-sensitive probes in the cells.

Electropermeabilization

At about this time we became aware of a technique to render membranes leaky by dielectric breakdown. Although one of the first demonstrations of this technique was to 'blow up' protozoa (Sale & Hamilton, 1968), we were introduced to it largely through the work of Zimmermann and his colleagues (Zimmermann, Pilwat & Riemann, 1974). The technique involved exposing isolated cells to an electric field of brief duration which caused a localized dielectric breakdown of the plasma membrane. We realized immediately the potential value of this technique in being able to permeabilize selectively the plasma membranes of secretory cells without altering the limiting membranes of the smaller secretory granules. This is because the effectiveness of the electric field to break down a membrane depends on the size of the particle or the cell exposed to the field. It was thus relatively easy to choose a field strength that breached the plasma membrane of the large cell but that did not perturb the structural integrity of the smaller osmotically sensitive intracellular organelles – or, for that matter, the protein matrix that formed part of the secretory machinery. Although we were able to diffuse extracellular solutes into the isolated chromaffin cell through the electrically formed pores, we were

unable to reseal them and thus trap the Ca^{2+}-sensitive probes inside. However, we did have a preparation, akin to the skinned muscle, with which we could gain access to the cytosol by the diffusion of solutes through these stable pores. By exposing the cells to a series of electric fields it proved possible to 'pepper' the plasma membrane of each cell with small pores. This preparation therefore allowed us to define and manipulate experimentally the chemical environment at the site of exocytosis and thus to investigate, quantitatively, the Ca^{2+} requirement for exocytosis (Baker & Knight, 1978). Other techniques designed to bypass the barrier set up by the plasma membrane were being and have since been developed and include the use of detergents, toxins and viruses (Dunn & Holz, 1983; Gomperts & Fernandez, 1985). However, as our electrical method was controlled, chemically clean, equally effective on a homogeneous population of cells in suspension and, perhaps most importantly, did not alter the ability of the cell to secrete, we always felt it offered distinct advantages over the other techniques. Shortly after we began these studies on catecholamine secretion from 'leaky' adrenal cells, Michael Whitaker and Michael Scrutton joined us to apply the technique to study the Ca^{2+}-dependence of secretion from other preparations, i.e. trychocyst discharge of *Paramecium*, cortical granule discharge from sea urchin eggs, and serotonin release from platelets (Baker & Whitaker, 1978; Baker, Knight & Whitaker, 1980; Knight & Scrutton, 1980). Another approach adopted at this time by Baker and Whitaker to control the site of exocytosis was to use the cortical plaque (lawn) in which a sea urchin egg was stuck to a glass slide with the unstuck portion of the egg hosed away (Vacquier, 1975; Baker & Whitaker, 1978). The inside of the cortex and its attached secretory vesicles were therefore left exposed and accessible to chemical control. The general conclusion from all these studies, together with many more later ones involving various neuronal, endocrine and exocrine tissues, was that exocytosis could be triggered by micromolar levels of Ca^{2+}. Another approach that has allowed the secretory response of a single cell to be measured, rather than the average response from a population, is to attach a single secretory cell to a patch pipette, perfuse the interior of the cell and monitor exocytosis by a change in capacitance (Neher & Marty, 1982; Fernandez, Neher & Gomperts, 1984).

A major result of the early experiments on electropermeabilized adrenal medullary cells was to show that although the plasma membrane was freely permeable to solutes of up to 1000 Da, less than 1 % of the total cellular catecholamine leaked out of the cell. This finding alone argued strongly against the involvement of a cytosolic pool in secretion, as central to such a model was the existence of appreciable amount of freely diffusible secretory product in the cytosol.

Catecholamine was released when micromolar levels of buffered Ca^{2+} were introduced into the cytosol and we provided evidence that this release mechanism was truly sensitive to micromolar levels of Ca^{2+}, and not to higher levels brought about by possible release of Ca^{2+} from intracellular stores. The mechanism underlying this release of catecholamine from leaky cells seemed to be exocytosis

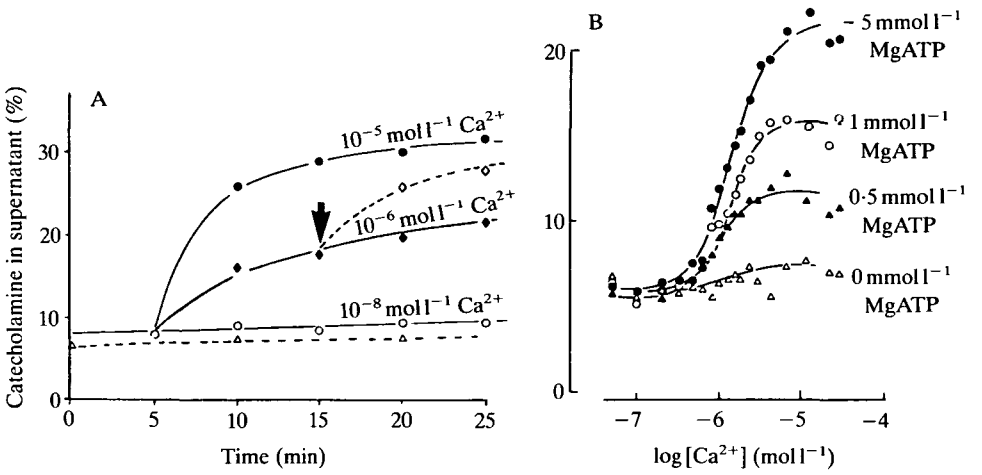

Fig. 2. Ca^{2+}- and MgATP-dependence of secretion from electropermeabilized chromaffin cells. (A) Submaximal levels of Ca^{2+}, i.e. 10^{-6} mol l^{-1}, affect mainly the extent of secretion rather than simply the rate. Further secretion is triggered when 10^{-5} mol l^{-1} Ca^{2+} is added after 10^{-6} mol l^{-1} Ca^{2+} (arrow). (B) Electropermeabilized chromaffin cells equilibrated with various concentrations of MgATP before being challenged with 10^{-5} mol l^{-1} Ca^{2+}.

as the same biochemical criteria used to determine exocytosis from intact cells were also satisfied by the leaky cells. Essentially this was that both catecholamine and dopamine-β-hydroxylase were released into the extracellular medium at the same rate and in the same proportions as found in the soluble component of the secretory granule, whereas the cytosolic enzyme lactate dehydrogenase was only released into the extracellular medium at a much slower rate that was quite independent of the Ca^{2+} level. Very little catecholamine was released from leaky cells held at Ca^{2+} levels close to 0·1 μmol l^{-1}, i.e. at resting levels of Ca^{2+}, whereas half-maximal release occurred at 1 μmol l^{-1} Ca^{2+} and maximal secretion at 10 μmol l^{-1} Ca^{2+} (Fig. 2A). The shape of the Ca^{2+} activation curve suggested that two calcium ions could be involved with each exocytotic reaction (Baker & Knight, 1981; Knight & Baker, 1982).

In the hope of shedding some light on the underlying mechanism of secretion, the effects of various agents introduced into the leaky cell were investigated. Although many agents had little effect, it was very clear that MgATP had to be present for Ca^{2+}-dependent secretion to proceed (Fig. 2B).

Electropermeabilized platelets

Although early experiments with electropermeabilized blood platelets yielded results that were very similar to those obtained from the chromaffin cell (Knight & Scrutton, 1980), later experiments that involved monitoring secretion of two different granule types within the same cell were intriguing and led to interpretations beyond those obtained from the adrenal cell. This was based on the finding

that in intact platelets, low levels of the natural agonist thrombin triggered the release of the contents of one type of secretory granule (serotonin from the amine-storage granules) but not the contents of another type of granule (acid hydrolases from lysosomes), whereas higher levels of thrombin triggered the release of both serotonin and acid hydrolase. This example of differential secretion from the same cell could have been explained in terms of the exocytotic machinery of the first type of granule having a higher sensitivity to Ca^{2+} than that of the other type of secretory granule. When platelets were rendered leaky, however, and the intracellular Ca^{2+} levels clamped, it was found that their sensitivities to Ca^{2+} were the same. The differential secretion triggered by thrombin in intact cells therefore provided evidence for a transduction pathway that was more effective on one granule type than on the other and led us to consider the involvement of a 'non-Ca^{2+}' factor involved in the triggering and control of exocytosis (Knight, Hallam & Scrutton, 1982) – a suggestion that was entirely compatible with the quin2 results outlined below.

Fluorescent calcium indicators

Just after these developments came Roger Tsien's invention of the fluorescent Ca^{2+} indicator, quin2, and a way of introducing it into intact cells by means of ester permeation and cytosolic hydrolysis and trapping of the tetracarboxylic acid dye (Tsien, Pozzan & Rink, 1982*a*). The technique was developed first in lymphocytes and then rapidly applied to many other cells starting with the blood platelet (Rink, Smith & Tsien, 1982). Resting $[Ca^{2+}]_i$ measured by this method was close to 100 nmol l^{-1} in nearly all cell types, including platelets (Rink *et al.* 1982) and chromaffin cells (Knight & Kesteven, 1983). In quin2-loaded platelets, step changes in $[Ca^{2+}]_i$ could be imposed by application of increasing concentrations of Ca^{2+} ionophore. Secretion could be measured simultaneously, by measuring release of radioactive 5-hydroxytryptamine (5-HT) or, in parallel, by following secretion of the ATP also contained in the amine-storage granules by luciferin–luciferase (Rink *et al.* 1982; Rink & Hallam, 1984). These experiments showed an apparent threshold for secretion near 1 μmol l^{-1} $[Ca^{2+}]_i$, with maximal effects at several micromolar beyond the range of resolution of quin2. These results were consistent with the data from permeabilized platelets. Interestingly, shape change, another important functional response, was much more sensitive to Ca^{2+}, being activated between 300 and 800 nmol l^{-1}. These studies were the first in which secretion was measured along with quantitation of $[Ca^{2+}]_i$ in an intact cell, and provided the data to support what had been supposed from other more indirect measurements.

The role of protein kinase C

The surprise, and thus the real interest and excitement, came when experimental conditions were found in which secretion occurred in response to thrombin or collagen while $[Ca^{2+}]_i$ remained at or near basal levels, and well below the $[Ca^{2+}]_i$

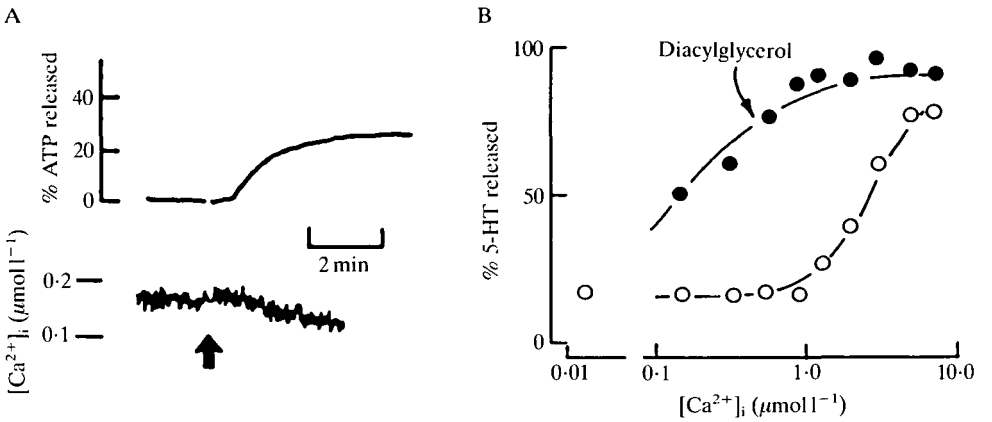

Fig. 3. Experiments showing apparent Ca^{2+}-independent secretion and its possible explanation. (A) Collagen, 10 mg ml^{-1} added at the arrow, promotes secretion of ATP from intact platelets without a rise in Ca^{2+} level as measured with quin2. (B) In electrically permeabilized platelets, diacylglycerol (which is produced following exposure to collagen) brings about a marked increase in the Ca^{2+}-sensitivity of the secretory process.

threshold needed when Ca^{2+} ionophore translocated Ca^{2+} into the cytosol bypassing receptor-mediated events (Rink, Sanchez & Hallam, 1983; Rink & Hallam, 1984) (see Fig. 3A). Just at this time we began to appreciate the importance of diacylglycerol production from receptor-mediated phosphoinositide hydrolysis and its role in activation of protein kinase C. Nishizuka, with Castagna and others (Castagna *et al.* 1982; Nishizuka, 1984), had also just shown that a particular phorbol ester, phorbol myristate acetate (PMA), could substitute for diacylglycerol in activating protein kinase C. We had previously observed that PMA caused platelet aggregation with no rise in quin2 fluorescence (Tsien *et al.* 1982*b*) but could not work out what this meant. Now all fell into place and we showed that PMA, and an exogenous diacylglycerol which also activated kinase C in platelets, could stimulate extensive, if slow, secretion from both classes of granules (Rink *et al.* 1983). In these experiments the retention of cytosolic quin2 served as an in-built control for selective secretion from the granular store. We proposed therefore that, in the particular conditions we used, thrombin and collagen were stimulating secretion at basal $[Ca^{2+}]_i$ by their ability to promote diacylglycerol formation and activate kinase C. These findings, incidentally, also strongly suggested that receptor-mediated phosphoinositide secretion preceded and was thus independent of, any elevation of $[Ca^{2+}]_i$, at least in this system. We also confirmed and extended the previous observations of synergy between elevated $[Ca^{2+}]_i$ and diacylglycerol in accelerating the exocytotic process.

The ability of surface ligands to stimulate exocytosis without promoting any elevation of $[Ca^{2+}]_i$ came as a considerable surprise, but has subsequently been seen in many cell types. In retrospect, the assumption that $[Ca^{2+}]_i$ was a mandatory part of the exocytotic process was not sensible. Many models of the

process did not call for a specific role for this divalent cation and, in any case, many other examples of membrane fusion, including that of constitutive secretory exocytosis, clearly did not need elevated $[Ca^{2+}]_i$ (Judah & Quinn, 1978; Shoback, Thatcher, Leombruno & Brow, 1984; DiVirgilio, Lew & Pozzan, 1984; Pozzan *et al.* 1984). Nonetheless, it is clear that in many secretory processes, particularly those in which speed is important, elevated $[Ca^{2+}]_i$ under the plasma membrane, just where it is needed, is the major and probably requisite trigger in stimulus–secretion coupling.

Support for a role of protein kinase C in secretion came from the finding that in electropermeabilized chromaffin cells PMA could increase the Ca^{2+}-sensitivity (albeit modestly) of the exocytotic machinery (Knight & Baker, 1983*a*). This experiment paved the way for the crucial experiments a year later which showed that PMA or diacylglycerol increased the Ca^{2+}-sensitivity of serotonin secretion from electropermeabilized platelets to such an extent that PMA could elicit appreciable secretion at resting $[Ca^{2+}]_i$ levels, i.e. 100 nmol l^{-1} (Fig. 3B; Knight & Scrutton, 1984*a*,*b*). We had earlier shown that the signal transduction process involving the thrombin receptor remained functional in electropermeabilized platelets (Knight & Scrutton, 1983, 1984*a*; Haslam & Davidson, 1984*a*), and also shown that it operated by increasing the Ca^{2+}-sensitivity of serotonin secretion. These two events were firmly linked when it was shown that activation of the thrombin receptor led to phospholipase C activity in the electropermeabilized cells and hence an elevated diacylglycerol level (Haslam & Davidson, 1984*a*,*b*).

Another interesting finding was that although PMA, diacylglycerol or activation of the thrombin receptor increased the Ca^{2+}-sensitivity of serotonin release, their effect on secretion from another type of granule (i.e. secretion of acid hydrolase) was quite different. Here the extent of secretion was increased without the Ca^{2+}-sensitivity being altered. This demonstration that two distinct populations of secretory granules in the platelet (i.e. serotonin-containing amine-storage granules and enzymes in lysosomes) could exhibit quite different responses to activators of kinase C provided a mechanism for effecting differential release of secretory products. Thus, at low cytosolic Ca^{2+} levels, a rise in diacylglycerol level induced by either thrombin or collagen would trigger the release of serotonin but have little effect on lysosomal release, whereas higher levels of thrombin, which elevated the cytosolic Ca^{2+} still further, would trigger secretion of both (Knight, Niggli & Scrutton, 1984).

Are different classes of protein kinase C responsible for different secretory responses?

The secretory responses seen in the presence of activators of protein kinase C seem to fall into three categories. First, there is the type of response typified by serotonin release from platelets where activators of the kinase cause a very large increase in the sensitivity to Ca^{2+} of the secretory process (Fig. 3B; Knight & Scrutton, 1984*a*,*b*). At high concentrations of these activators, the sensitivity to Ca^{2+} increases to such an extent that the response can appear to be

Ca^{2+}-independent. The second type of response is characterized by a fairly modest increase in the sensitivity to Ca^{2+} and this is typified by the secretion of catecholamine from chromaffin cells (Knight & Baker, 1983*a*). The third type of response is where activators of the kinase enhance the extent of secretion without altering the Ca^{2+}-sensitivity. This is shown in the case of lysosomal enzyme secretion from platelets (Knight *et al.* 1984). The different types of response may result from different forms of kinase C (Knopf *et al.* 1986; Kariya & Takai, 1987; Ido, Kazuo, Kikkawa & Nishizuka, 1987). Alternatively, if the enzyme has a preferred order of binding Ca^{2+} and diacylglycerol (Baker, 1986), then the amount of enzyme associated with Ca^{2+} and diacylglycerol as a function of $[Ca^{2+}]$ will depend on the order of binding (i.e. diacylglycerol followed by Ca^{2+}, or Ca^{2+} followed by diacylglycerol, or when there is no preferred order of binding). The relationship between the amounts of enzyme associated with Ca^{2+} and diacylglycerol for the three orders of binding is strikingly similar to Ca^{2+} activation curves of the three types of secretory response. Therefore, the three different secretory responses may result from similar enzymes with different preferred orders of substrate binding (Baker, 1986).

Theories and models of exocytosis

Sub-maximal calcium levels

A rather interesting finding from experiments with electropermeabilized cells was that suboptimal levels of Ca^{2+} affected mainly the extent of secretion rather than the rate (Fig. 2A). If the kinetics of release from these intact cells resembled that from intact cells then the significance of this finding was that a maintained elevated Ca^{2+} level would trigger a transient secretory response rather than a maintained one. Although secretion from leaky cells ceased after a few minutes in response to a maintained Ca^{2+} challenge (say $1\,\mu mol\,l^{-1}$), further secretion could be triggered by this same Ca^{2+} concentration if the Ca^{2+} level was briefly lowered to resting levels (i.e. $0{\cdot}1\,\mu mol\,l^{-1}$) before being raised again (Knight & Baker, 1982). These results suggest that oscillating Ca^{2+} levels would lead to a greater secretory response than that due to a maintained elevated Ca^{2+} challenge. If brief bursts of nervous impulses, interspersed by quiet periods, result in an oscillation of $[Ca^{2+}]_i$, whereas a maintained firing pattern gives rise to a maintained elevated $[Ca^{2+}]_i$, then these observations with leaky cells provide an explanation of why bursts of nervous activity give rise to more secretion than do tonic firing patterns.

Sites of action of calcium

Protein kinase C is one likely receptor for Ca^{2+} in its control over secretion (Nishizuka, 1984; Shapira, Silberberg, Ginsburg & Rahamimoff, 1987; Hu *et al.* 1987) and this is supported from experiments on the chromaffin cell and platelet,

where it has been shown that the Ca^{2+}-sensitivity of secretion is altered by activators of the kinase, and there is an absolute requirement for MgATP.

However, other possible receptors should not be discounted and include synexin (Creutz, Pazoles & Pollard, 1978), chromobindins (Creutz *et al.* 1983) and calelectrins (Sudhof, Walker & Fritsche, 1985). Just how these Ca^{2+}-binding proteins could trigger membrane fusion leading to exocytosis is not clear, but a clue could come from the method by which certain enveloped viruses are able to fuse with membranes. The method seems to involve a special pH-sensitive spike protein that the viral membrane has projecting from its surface (White, Kielan & Helenius, 1983). Viruses attach to the cell surface, become internalized into endosomes and, as the H^+ concentration inside the endosomes rises to about $10\,\mu mol\,l^{-1}$, the spike protein undergoes a conformational change revealing a hydrophobic sequence that buries itself into the neighbouring wall of the endosome. It is this step that seems to lead to fusion of the viral and vesicle membranes, permitting the escape of the viral contents into the cytosol. Although it seems most improbable that such a pH-sensitive process is involved in exocytosis, the underlying principle may well be the same, i.e. that in association with either the secretory vesicle or plasma membrane there is a protein specialized for effecting fusion, Ca^{2+} being the trigger that reveals some hydrophobic sequence.

Calmodulin has been suggested as another receptor for Ca^{2+} in its control over secretion (Steinhardt & Alderton, 1982; Llinas *et al.* 1985). Support for an involvement of calmodulin also comes from experiments which show that calmodulin antibodies introduced into chromaffin cells block Ca^{2+}-dependent secretion (Trifaro & Konigsberg, 1983).

Calmodulin- and protein kinase-C-dependent processes could both play a part in the control of exocytosis. For example, rather than protein kinase C being the integral part of the machinery controlling exocytosis, the enzyme could be involved as a modulator of an underlying calmodulin-dependent process. If this were the case, it should be possible to separate the two processes pharmacologically. Unfortunately, we have been unsuccessful in showing this as, using a range of putative inhibitors, we have been unable selectively to remove the PMA-induced shift of the Ca^{2+} activation curve without also removing the underlying (PMA-insensitive) Ca^{2+} activation curve. Either the agents we have used are not specific enough to disentangle the different pathways, or protein kinase C may well operate as the integral part of the exocytotic machinery.

Role of MgATP

What was very clear from the experiments on electropermeabilized chromaffin cells was that for them to respond to a Ca^{2+} challenge, MgATP had to be present (Fig. 2B). The rather specific requirement for ATP, and the ineffectiveness of non-hydrolysable analogues, suggested a possible involvement of phosphorylation and/or dephosphorylation in secretion. This would be in agreement with a role f[illegible] protein kinase C in secretion. However, protein phosphorylation studies in leaky

chromaffin cells or platelets failed to identify a single phosphoprotein uniquely associated with Ca^{2+}-dependent secretion, a very large number being phosphorylated (Niggli *et al.* 1984; Knight *et al.* 1984). There is good evidence that in some tissues the phosphoprotein, synapsin 1, may be involved in exocytosis, although this protein is apparently absent from chromaffin cells (Llinas *et al.* 1985). The MgATP dose–response curve obtained from electropermeabilized chromaffin cells was best fitted by a model in which a chemical reaction closely associated with exocytosis involved one molecule of MgATP per round of exocytosis. The data therefore provided no evidence for cooperative sites of action of MgATP involved in secretion. The kinetics of catecholamine release studied under conditions of clamped intracellular Ca^{2+} and MgATP levels suggested that if phosphorylation or dephosphorylation was a key step in exocytosis, then the rate of phosphorylation/dephosphorylation would be the controlling factor in secretion rather than the absolute levels of protein phosphorylated (Knight & Baker, 1982). Some recent studies favour phosphatase activity to be the regulatory step (Zeiseniss & Plattner, 1985).

Although MgATP is certainly required for secretion from bovine chromaffin cells, its role is still somewhat unclear. For example, two sets of data lent some support to the idea that one role of MgATP is simply to protect or prime the secretory system. First, the millimor concentrations of nucleotide needed for a secretory response could be reduced by over an order of magnitude if the leaky cells were incubated and challenged in a medium of low ionic strength. Although this can be explained in terms of the affinity for MgATP being dependent on the ionic strength, another interpretation is that the nucleotide protects a system otherwise destabilized by anions, the order of potency of the anions following the lyotopic series. Second, the cells respond better if incubated continuously in a medium containing ATP, rather than one in which the nucleotide is added just prior to the Ca^{2+} challenge.

An involvement of MgATP in secretion is less clear in other preparations. In the case of secretion from electropermeabilized platelets, for example, the ability of the cell to secrete in response to a Ca^{2+} challenge decreases over minutes when the cells are suspended in a solution lacking ATP, even though the endogenous nucleotide is lost from the leaky cell within seconds. In the case of the cortical granule discharge seen in sea urchin egg plaques, the secretory response is not lost immediately MgATP is removed from the bathing solution, but instead decreases slowly – sometimes over hours. In other preparations, e.g. transformed cells, it seems clear that exocytosis can also be triggered over a long period in the absence of added ATP (Ahnert-Hilger & Gratzl, 1987). Although it may be possible that there is a bound pool of ATP, another explanation is that MgATP could be used mainly to prime (or protect) the secretory apparatus (Vilmart-Seuwen, Kersken, Sutzi & Plattner, 1986). In the case of skeletal muscle contraction, for example, ATP is used (cleaved) to prime the myosin head group. The actual power stroke that causes the filaments to move relative to each other does not involve hydrolysis of ATP. One could imagine, therefore, that ATP is required for secretion simply to

prime a component involved in the granule/plasma membrane interaction process rather than in the exocytotic mechanism itself. Similarly Ca^{2+} may exert its control of secretion by a troponin-type molecule rather than by a phosphorylation/dephosphorylation step as seen in smooth muscle.

Osmotic forces

One possible role for MgATP in secretion is to serve the ATP-dependent proton pump found in the membrane of many secretory granules. This pump maintains not only an acid interior of the granules but also a potential difference across the granule membrane. The idea arose that these potential energy sources within the secretory granule were in some way involved in the exocytotic mechanism, and this formed the basis of the chemiosmotic hypothesis (Pollard, Pazoles & Creutz, 1977). The mechanism proposed that neutralization of the intragranular H^+ with extragranular anions, or exchange of H^+ with extragranular cations, would lead to an accumulation of osmotically active particles within the secretory granule. This, in turn, would cause the granule to swell and thus facilitate membrane fusion (Finkelstein, Zimmerberg & Cohen, 1986). The finding that an increase in the osmotic pressure outside the granule inhibited secretion from electropermeabilized chromaffin cells (if not from digitonin-treated cells; Holz & Senter, 1986) supported the idea that osmotic forces were involved in exocytosis (Knight & Baker, 1982). In spite of this we were unable to support the model as it seemed exocytosis could proceed in the virtual absence of extragranular monovalent cations and was inhibited, rather than stimulated, by cations that were predicted to lead to the greatest osmotic instability, e.g. chloride. More significantly, we were able to estimate, and experimentally manipulate, the pH and voltage gradients across the secretory granule wall in electropermeabilized chromaffin cells, and test if this affected exocytosis. These data showed a complete lack of correlation between either granular pH or potential and the ability of the cell to participate in Ca^{2+}-dependent exocytosis (Knight & Baker, 1982, 1985*a*; Holz, Senter & Sharp, 1983). Further evidence against the model has come from the electrical measurements of voltage in exocytosing secretory granules (Breckenridge & Almers, 1987). The role of osmotic instability in the membrane fusion step of exocytosis was also studied in detail using the beige mouse mast cell (Zimmerberg, Curran, Cohen & Brodwick, 1987). Here the fusion step was clearly shown to precede granule swelling – rather than the other way round as would be predicted by the chemiosmotic theory. These data therefore argued strongly against a role of osmotic forces in membrane fusion. Osmotic forces may come into play, however, after the fusion step has taken place by regulating the release of the contents of the secretory granule into the extracellular fluid through the fusion pore (Zimmerberg *et al.* 1987; Whitaker & Zimmerberg, 1987). Increasing the osmotic pressure seems to inhibit the appearance of secretory product outside the cell although the fusion step between the vesicle and inner surface of the plasma membrane has already occurred.

The cytoskeleton

Baker & Whitaker gave evidence that the cytoskeleton was not intimately associated with exocytosis of cortical granules in sea urchin egg plaques (Whitaker & Baker, 1983). In this case the cortical granules were fixed in position and not expected to move. In electropermeabilized chromaffin cells, where secretory granules can move towards the periphery of the cell, agents that might be expected to perturb the cytoskeleton also had no effect on Ca^{2+}-dependent secretion. Recent work on leaky (Perrin, Langley & Aunis, 1987) and intact (Cheek & Burgoyne, 1986) chromaffin cells, however, suggests a role for the cytoskeleton in secretion. In the latter studies it was shown that actin filaments were disassembled around the periphery of the cell in response to nicotinic stimulation and over a time course that closely followed secretion. A potassium challenge was reported to cause a much smaller amount of secretion and, as it did not lead to actin disassembly, the higher secretion with nicotine was linked with the breakdown of the filament network. [In our early experiments we also observed that nicotinic stimulation led to a larger amount of catecholamine release than a potassium challenge, but we only observed this at room temperature when the secretory response due to nicotine was more maintained (Knight & Baker, 1983*b*). At 37°C we saw very little difference in the amounts secreted in response to these two stimuli, whether from freshly isolated cells, cultured cells or a perfused gland. We interpreted our data in terms of the acetylcholine receptor not desensitizing as quickly at the lower temperature, rather than the result of another second messenger possibly affecting the cytoskeleton.]

The finding that forskolin, an agent that elevates cyclic AMP level, not only reduces the nicotine-evoked release but also blocks the disassembly of the actin filaments suggested that cyclic AMP could modulate secretion (Burgoyne & Cheek, 1987). The earlier results from electropermeabilized cell preparations did not support this idea, as the Ca^{2+}-dependent secretion observed from the electropermeabilized cell was unaffected by cyclic nucleotides or by agents that should have either disrupted or prevented disassembly of the cytoskeleton. One of the ways of reconciling these data is if the act of rendering the cell leaky by electric fields causes the dissassembly of the actin filaments. Another possibility is that an ingredient essential for cyclic nucleotide effects was lost from the cytosol of the leaky cell.

Guanine-nucleotide-binding proteins

Although the data from platelets offered strong evidence for a role of protein kinase C in stimulus–secretion coupling, our earlier experiments with chromaffin cells were less convincing. These showed that although the phorbol ester PMA shifted the Ca^{2+} activation curve in the expected way, the diacylglycerol 1-oleyl-2,acetylglycerol had no effect (Knight & Baker, 1983*a*). This diacylglycerol had been shown to activate protein kinase C isolated from brain in much the same way as did PMA. Several explanations for the lack of effect came to mind including the

possibility that kinase from the medulla was not activated by this particular diacylglycerol, that the lipid could not get into the cell or that it was being rapidly metabolized. (We later found that other diacylglycerols, e.g. 1,2-dioctanoylglycerol and other activators of the kinase, e.g. mezerein, did increase the Ca^{2+} sensitivity in the same way as did PMA.) Shortly after these early experiments Richard Haslam and Monica Davidson showed that the thrombin-induced production of diacylglycerol in the permeabilized platelet was enhanced by GTP or GTPγS and they suggested that phospholipase C was under guanine-nucleotide-binding-protein control (Haslam & Davidson, 1984*c*). A similar control over phospholipase C activity was later shown in other preparations (Cockcroft & Gomperts, 1985; Merritt, Taylor, Rubin & Putney, 1986).

As phospholipase C in platelets could be stimulated by GTPγS in the absence of an agonist, we looked for an effect of this nucleotide on Ca^{2+}-dependent secretion from chromaffin cells. We hoped that endogenous diacylglycerol would be produced in the cell by this nucleotide, and that this would increase the Ca^{2+}-sensitivity of the secretory process in a way similar to PMA. As predicted, we found that GTPγS stimulated Ca^{2+}-dependent catecholamine release from chicken chromaffin cells. It was a surprise, however, to find that GTPγS inhibited the extent of secretion from leaky bovine adrenal medullary cells (Knight & Baker, 1985*b*). We could not interpret these data in terms of GTPγS inhibiting endogenous diacylglycerol production as the effect could not be overcome by direct activation of protein kinase C with PMA. We suggested, therefore, that the site of action of the GTPγS in the bovine chromaffin cell was downstream of the sites of action of phospholipase C and protein kinase C, and perhaps was at the site of exocytosis itself (Knight & Baker, 1985*b*). Quite independently, this hypothesis was also proposed by Bastien Gomperts and his colleagues (Barrowman, Cockcroft & Gomperts, 1986) who showed in neutrophils that the site of exocytosis might be under the control of a GTP-binding protein. However, in their systems GTP was stimulatory. A stimulatory effect of GTPγS on leaky bovine adrenal medullary cells was found by Ronald Holz and his colleagues (Bittner, Holz & Neubig, 1986). One explanation for this difference might lie in the different method of making the cells leaky, i.e. electropermeabilization *versus* digitonin treatment.

With the evidence mounting for a possible role of a guanine-nucleotide-binding-protein at or near the site of exocytosis, the question arose whether the stimulatory effect of GTPγS on serotonin release from platelets could in part be due to action at such a site rather than exclusively at the receptor/phospholipase C site. In support of the idea of two sites was the finding that under some conditions the GTPγS-evoked release was simply additive to the thrombin-evoked release, and that the pharmacologies of the two pathways were somewhat different. However, measurements of the kinetics of secretion gave strong support to the idea that GTPγS acted only at the level of agonist-mediated phospholipase C activity, as the pronounced time lag between applying GTPγS and the onset of secretion wa completely abolished in the presence of the agonist thrombin (Knight & Scrutton, 1987). (One rather surprising result, that may turn out to be significant, is that not

only do micromolar levels of GTPγS and GTP enhance the thrombin effect on Ca^{2+}-dependent secretion from electropermeabilized platelets, but the same enhancement is also seen by micromolar levels of GDT, GMP or cyclic GMP.)

Other second messengers

The evidence so far is that the main messengers acting on exocytosis are Ca^{2+} and diacylglycerol. Cyclic AMP has also been postulated to act as a second messenger for secretion (Gardner & Jensen, 1981). The experiments that have been directed towards clarifying this, however, show that where there is an effect it appears to be expressed through a Ca^{2+}-dependent process. This may result either as a modulation of the Ca^{2+}-sensitivity of the secretory process (Jones, Fyles & Howells, 1986) or by modulating the production of another second messenger, e.g. Ca^{2+} or diacylglycerol (Knight & Scrutton, 1984*a*,*b*). Secretion can be triggered in some systems not only by an increase in Ca^{2+} level, but also by GTγS acting in a seemingly Ca^{2+}-independent manner. Whether this nucleotide is operating on a pathway that is truly separate from the Ca^{2+}-dependent pathway, or whether it operates downstream of the site of action of Ca^{2+} is not yet clear (Barrowman *et al.* 1986; Fernandez *et al.* 1984; Oetting *et al.* 1986; Wollheim, Ullrich, Meda & Vallar, 1987; Penner, Pusch & Neher, 1987).

Identifying the proteins associated with exocytosis

One method of identifying some of the proteins essential for secretion is to allow the cytosolic proteins to diffuse out of the leaky cell and see at what point the cell becomes refractory. This approach has recently had some success when it was shown that associated with the onset of refractoriness was the appearance of proteins in the extracellular fluid, and that these proteins, when concentrated and added back to the cells, could restore Ca^{2+}-dependent secretion (Sarafian, Aunis & Bader, 1987).

Toxins, such as bungarotoxin, act on specific proteins, and the finding that botulinum toxins specifically blocked both basal and evoked secretion by acting at or near the site of exocytosis suggested that it could be used to target a protein specifically associated with exocytosis (Knight, Tonge & Baker, 1985; Penner, Neher & Dreyer, 1986). The mechanism by which botulinum toxin blocks secretion is not clear, but the finding that the toxin could ADP-ribosylate a protein in secretory tissue (Ohashi & Narumiya, 1987) raised the possibility that it could operate in the same way as do cholera or pertussis toxins, i.e. by ribosylation of some key protein. However, detailed measurements of both the extent of botulinum toxin-induced ADP-ribosylation and the inhibition of secretion reveal that the two are not necessarily linked. It is possible, for example, to trigger toxin-induced ADP-ribosylation without inducing inhibition of secretion, or to inhibit secretion by botulinum toxin without any measurable ADP-ribosylation (Adam-Vizi, Aktories, Knight & Rosener, 1988). It would seem that the ribosylation factor in botulinum toxin types C and D is not that which causes botulism (Aktories, Weller & Chatwal, 1987). It has been generally accepted that

botulinum toxins are, like cholera toxin, internalized by the cell before they can express their inhibitory effect. Another possibility, however, is that the toxin acts on a protein positioned on the outside of the cell surface, and which is revealed to the toxin when the cell is depolarized.

Post-secretory events – endocytosis

With the exception of perhaps cortical granule discharge from the sea urchin egg and trychocyst discharge from *Paramecium*, the constancy of cell size means that any increase in the cell surface resulting from exocytosis must in the long term be balanced by endocytosis of an equivalent area of membrane (Linng, Fischer-Colbrie, Schmidt & Winkler, 1983; Phillips, Burridge, Wilson & Kirshner, 1983). We showed that endocytosis, as measured by horseradish peroxidase uptake, occurred in electropermeabilized chromaffin cells alongside exocytosis (Baker & Knight, 1981). Therefore, if secretion included a cycle of events involving both exocytosis and endocytosis, we reasoned that the intracellular conditions controlling secretion might not necessarily reflect those exclusively for exocytosis but could also include those for endocytosis. Experiments designed to differentiate the intracellular conditions controlling these two pathways have only recently begun. Peter Baker and his colleagues showed that horseradish peroxidase could be taken up into intact chromaffin cells after catecholamine release had been stopped (von Grafenstein, Roberts & Baker, 1986). In this experiment, cells were stimulated with carbamylcholine and then the secretory stimulus was blocked by the addition of hexamethonium; at the same time, the extracellular marker was added. Uptake of this marker continued for several minutes even though exocytosis had stopped. They showed that after exocytosis had been stopped, the subsequent endocytosis could be interrupted by lowering the temperature, only to resume as normal when the temperature was raised again. Removal of extracellular calcium during this interrupted period did not alter the subsequent uptake of horseradish peroxidase, strongly suggesting that endocytosis was $[Ca^{2+}]_i$-independent. This result has recently been supported, and the study extended, using electropermeabilized cells. In these experiments in which exocytosis is blocked by lowering the Ca^{2+} level after a few minutes, horseradish peroxidase uptake continues independently of Ca^{2+} level and, as in the intact cells, can be interrupted if the temperature is lowered. Endocytosis seems to continue normally when the temperature is raised only if micromolar levels of MgATP are present, and completely ceases in the presence of nonhydrolysable analogues, e.g. ATPγS (von Grafenstein, 1988).

Ways forward

The experiments described in this paper revealed some of the intracellular factors that control secretion. We still know very little about the actual proteins involved in exocytosis or about the basic mechanism underlying membrane fusion. A better understanding may come from using toxins to target proteins, adding back different proteins as described above, and from the development of cell-free systems in which exocytosis and endocytosis can be followed under precisely

defined conditions (Davey, 1987; Crabb, Morden & Jackson, 1987). Other advances may come from techniques that allow the synthesis of foreign secretory proteins within a model cell or from the use of secretory mutants. It is a great loss to us all that Peter Baker is not here to help pursue and unravel these challenging problems.

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