ION CHANNELS FOR COMMUNICATION BETWEEN AND WITHIN CELLS

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by

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Around 1970, the fundamental signal mechanisms for communication between cells of the nervous system were known. Hodgkin & Huxley (1952) had provided the basis for understanding the nerve action potential. The concept of chemical transmission at synapses had received its experimental verification by detailed studies on excitatory and inhibitory postsynaptic potentials (see B. Katz, 1966, for a concise description of the electrical signals in nerve and muscle). The question of the molecular mechanisms underlying these signals was still open, however. Hodgkin & Huxley (1952) used the concept of voltage-operated gates for a formal description of conductance changes, and by 1970 the terms Na-channel and K-channel were used frequently (see review by Hille, 1970), although no direct evidence for the existence of channels was available from biological preparations. This was different for the case of artificial membranes. Müller & Rudin (1963) introduced 'black-lipid membranes' as experimental model systems, which in many respects resemble the bimolecular lipid membrane of living cells. These membranes are rather good insulators. However, when they are doped with certain antibiotics or proteins they become electrically conductive. R.C. Bean et al. (1969) and Hladky & Haydon (1970) showed that some of these dopants induce discrete, steplike changes in conductance when they are added in trace amounts . All the evidence suggested that the conductance changes observed represented the insertion of single pore-like structures into the membranes.

In biological membranes similar measurements were not possible at the time, since the methods available for recording currents in living cells typically had background noise levels higher, by about a factor of a hundred, than the 'single-channel currents' observed in bilayers (see Fig. 1). Indirect methods, however, provided strong evidence that channels similar in conductance to those in artificial membranes should be operative in nerve and muscle cells. Early attempts to count the number of Na channels by Tetrodotoxin binding indicated that the contribution of a single channel to Na conductance might be as much as 500 pS. Later, the technique of noise analysis (Katz & Miledi, 1972; Neher & Stevens, 1977) provided more
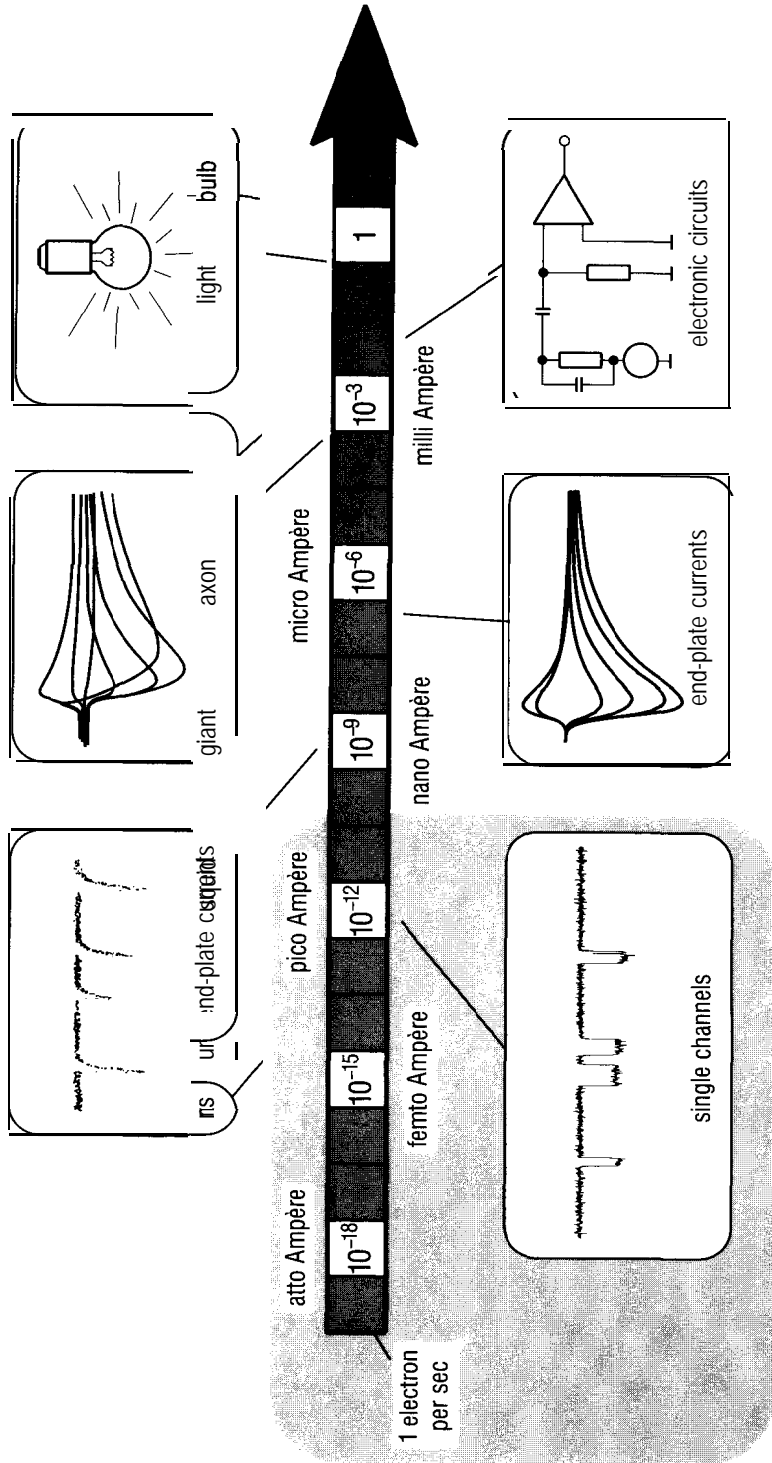
Figure 1. A graphical representation of the quantity 'current' in logarithmic scale with representative examples of current signals or current-carrying elements from electronics and biology. The shaded region is that which was dominated by background noise before the development of the patch clamp technique.

accurate numbers. Anderson & Stevens (1973) estimated the conductance contribution of single Acetylcholine-activated channels (ACh-channels) at the frog neuromuscular junction to be 32 pS. This is close to the conductance of single gramicidin channels as measured by Hladky and Haydon. Thus, it was very tempting to think about better methods for recording currents from biological preparations. There was good reason to hope that an improved technology would reveal a whole 'microcosmos' of electrical signals in a multitude of electrically and chemically excitable cell types. In this lecture I will give a short account of our joint effort to solve this problem, and then focus on further developments that the solution of the problem led to. Bert Sakmann, in the second lecture, will present some of the detailed knowledge that high-resolution current recording provides access to.

RATIONALE FOR USING 'PATCH PIPEITES'

A basic limitation for any current measurement, disregarding instrumentation noise, is the 'Johnson' or thermal noise of the signal source, which for a simple resistor is given by

$$\sigma_n = \sqrt{4kT\Delta f/R}$$

where σ_n is the root-mean-square deviation of the current, k is the Boltzmann constant, T the absolute temperature, Af the measurement bandwidth, and R the resistance. From this it is clear, that the internal resistance of a signal source (or, more generally, the complex impedance) should be very high for low-noise current recording. Specifically, to record a current of 1 pA at a bandwidth of 1 kHz with 10 % accuracy, the internal resistance of the signal source should be about $2\,G\Omega$ or higher. We now know that the input resistances of small cells can be as high as that. But early in the 1970s the conventional microelectrode techniques required large cells for reliable current measurements, and these typically had input resistances in the range $100\,k\Omega$ to 50 MΩ. Thus, it seemed impossible to reach the required resolution with standard techniques and standard preparations. What was required, was a smaller signal source.

   With these considerations in mind, we directed our efforts to isolate a small patch of membrane for the purpose of the electrical measurement. I had gained experience with suction pipettes being placed onto the surface of cells for local current measurement in the laboratory of H.D. Lux in Munich, where I did my doctoral thesis. Such pipettes had been used before in various contexts for either stimulation of cells or for current measurements (Pratt & Eisenberger 1919; Strickholm 1961; Frank & Tauc 1963; Neher & Lux 1969; Fishman 1973). It was clear to us, that they should be good tools for single-channel measurements, if only the 'pipette-to-membrane seal' could be made good enough. The impedance of the patch itself should be higher than required, even for a patch as large as 10 µm in

diameter. An incomplete seal, however, is 'seen' by the measuring amplifier in parallel to the patch, and its noise is superimposed onto the patch signal.

EARLY SINGLE-CHANNEL MEASUREMENTS

When Bert Sakmann and I started measurements by placing pipettes onto the surface of denervated muscle fibres, we soon realized that it was not so easy to obtain a satisfactory 'seal'. Although Bert Sakmann was very experienced in enzymatically treating cell surfaces through his work in B. Katz's laboratory, and although the work of Katz & Miledi (1972) and our own voltage-clamp measurements had shown that denervated muscle should have an appropriate density of diffusely dispersed ACh-channels, our initial attempts failed. Our seal resistances were just about 10-20 MΩ, two orders of magnitude lower than desired. However, by reducing the pipette size, and by optimizing its shape we slowly arrived at a point where signals emerged from the background - first some characteristic noise, later on blips which resembled square pulses, as expected. In 1976, we published records (Neher & Sakmann, 1976) which, with good confidence, could be interpreted as single-channel currents (see Fig. 2). The fact that similar records could be obtained both in our Göttingen laboratory and in the laboratory of Charles F. Stevens at Yale, (where I spent parts of 1975 and 1976), gave us confidence that they were not the result of some local demon, but rather signals of biological significance. The square-wave nature of the signals was proof of the hypothesis that channels in biological membranes open and close stochastically in an all-or-none manner. For the first time one could watch conformational changes of biological macromolecules in situ and in real time. However, the measurement was far from perfect. There still was excessive background noise, concealing small and more short-lived contributions of other channel types. Besides, the ampli-
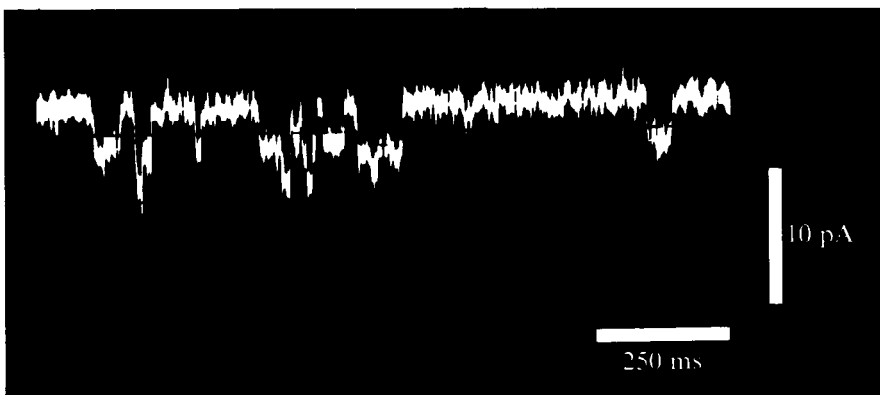
Figure 2. **Early single-channel currents from denervated frog** *(Ram pipiens)* **cutaneous pectoris muscle. The pipette contained 0.2 μM suberyldicholine, an analogue of acetylcholine which induces very long-lived channel openings. Membrane potential – 120 mV; temperature 8°C. Reproduced from Neher & Sakmann 1976.**
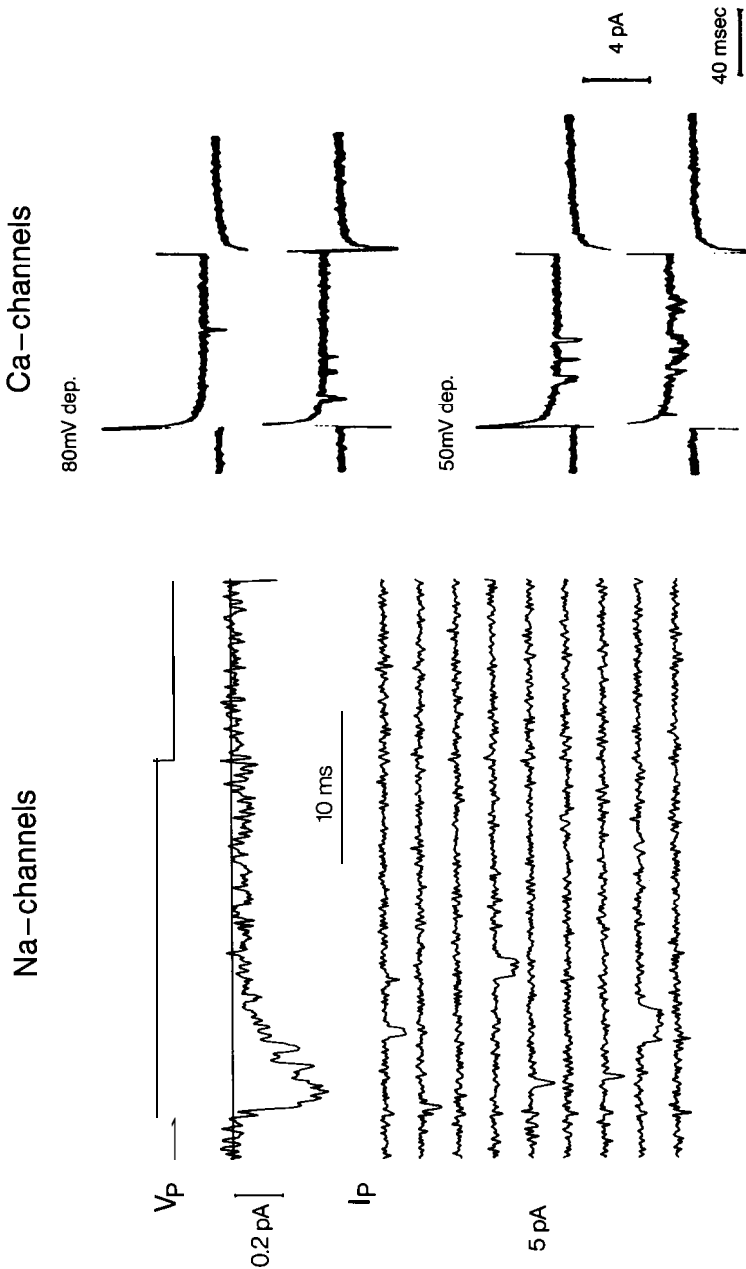
Figure 3. Early records of voltage-activated single channels. The left side shows Na channels (adapted from Sigworth & Neher, 1980). The top trace is the voltage protocol. The second trace shows the average response from 300 voltage pulse depolarizations, and the following traces give examples of individual responses. It is seen that in some-but not in all-traces there are individual openings (downward deflection) of Na channels. The patch was hyperpolarized by 30 mV, and 40 mV depolarizing pulses are given.

   The right side shows individual Ca-channel currents (adapted from Fenwick, Marty & Neher, 1981). Depolarizing pulses as indicated were given from normal resting potential. The pipette contained isotonic Ba solution. Single channel responses are seen superimposed onto a residual capacitive and leak artifact. In the left panel these artefacts were digitally subtracted.

tudes of single-channel currents had a wide distribution, since the majority of channels were located under the rim of the pipette, such that their current contributions were recorded only partially.

We made many systematic attempts to overcome the seal problem (manipulating and cleaning cell surfaces, coating pipette surfaces, reversing charges on the glass surface, etc.) with little success. Nevertheless, some important properties of single channels could be elucidated in the years 1975 to 1980 (Neher & Steinbach 1978; Sakmann 1978; Sakmann & Boheim 1978; Conti & Neher 1980; Sakmann, Patlak & Neher 1980).

By about 1980, we had almost given up on attempts to improve the seal, when we noticed by chance, that the seal suddenly increased by more than two orders of magnitude when slight suction was applied to the pipette. The resulting seal was in the gigaohm range, the so-called 'Gigaseal'. It turned out that a gigaseal could be obtained reproducibly when suction was combined with some simple measures to provide for clean surfaces, such as using a fresh pipette for each approach and using filtered solutions. The improved seal resulted in much improved background noise (Hamill et al. 1981). Fortunately, Fred Sigworth had just joined the laboratory. With his experience in engineering he improved the electronic amplifiers to match the advances in recording conditions. Thus, several types of ion channels could rapidly be characterized at good amplitude and time resolution (Fig. 3).

UNEXPECTED BENEFITS

Solving the seal problem turned out not only to improve the electrical recording, but also to provide useful tools for manipulating patches and small cells. Although the physical nature of the 'Gigaseal' is still unknown, we soon realized that it provides not only electrical stability but also a tight mechanical connection between the measuring glass pipette and the membrane. Owen Hamill and Bert Sakmann (1981), simultaneously with Horn & Patlak (1980), found that patches could be removed from cells, by simply withdrawing the pipette. This results in 'excised patches', which are accessible for solution changes from both sides. Alternatively, a patch can be ruptured by a short pulse of suction or voltage without loss of the glass-to-membrane seal. Thus, an electrical connection is established between measuring pipette and cell, with the pipette-cell assembly well insulated against the outside bath. This configuration was termed 'whole-cell recording'. Fig. 4 gives a schematic representation of the different procedures and resulting configurations.

Whole-cell recording is very similar to conventional microelectrode impalement, with some important differences, however:

1. The leak between cell interior and bath is extremely small, such that this form of penetration is tolerated by cells as small as red blood cells (Hamill 1983).

2. The electrical access resistance is low (l-10 MΩ) as compared to electrode resistances of impalement electrodes (20-100 MΩ typically, for small cells). Thus, voltage-clamp conditions are achieved easily without
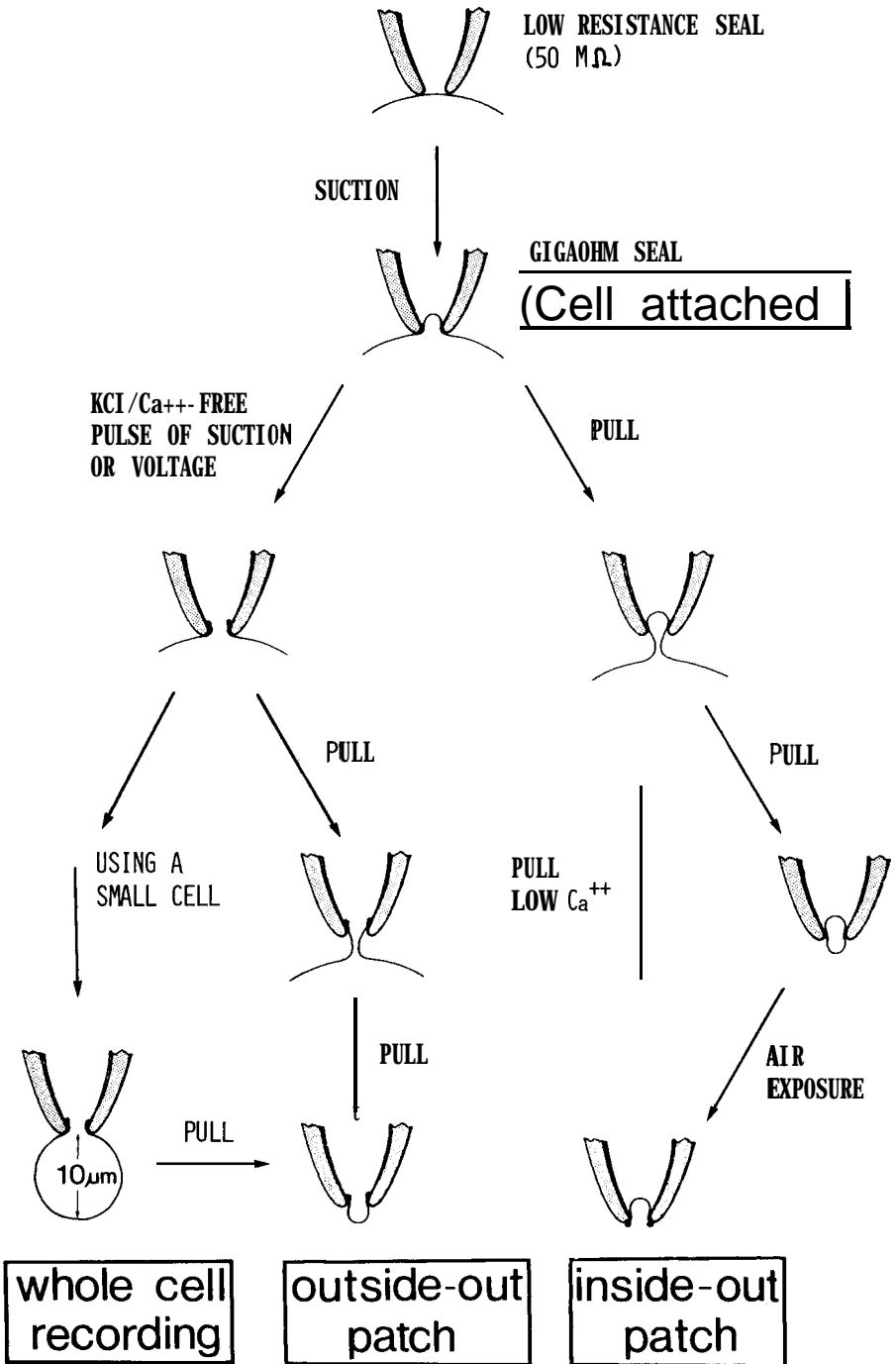

Figure 4. Schematic representation of the procedures that lead to the different patch clamp configurations. From Hamill et al. 1981.

feedback circuits and additional electrodes, if small cells are used (membrane resistance 100 MW-10 GW).

3. There is rapid diffusional exchange and equilibration between patch pipette and cell (Marty & Neher 1983; Pusch & Neher 1988). This provides control over the composition of the medium inside the cell. A cell can easily be loaded with ions, chelators, second messengers, fluorescent probes, etc., simply by including these substances in the measuring pipette. However, this exchange also implies that the internal milieu is disturbed, and that signaling cascades may be disrupted (see below).

With these properties, 'whole-cell recording' evolved to be the method of choice for recording from most cell-culture preparations and from acutely dissociated tissues. Many cell types, particularly small cells of mammalian origin, became accessible to biophysical analysis for the first time through whole-cell recording, since they would not tolerate multiple conventional impalements. Individual current types could be separated through control of solution composition on both sides of the membrane (Fenwick et al. 1982b; see Fig. 5 for an example of whole-cell Ca-channel currents). This development shifted the emphasis of electrophysiological studies away from large-celled   preparations, which usually were of invertebrate origin, towards mammalian and human cell types. In the first half of 1981, just before we first published a whole-cell characterization of a small mammalian cell (bovine adrenal chromaffin cells) only 5 out of 14 voltage-clamp studies in the Journal of Physiology were performed on cells of mammalian origin. The first 199 1 issue of the same journal alone contained 10 voltage-clamp studies on mammalian cells, none on invertebrates, and all using either the whole-cell or single-channel recording techniques

DISTURBING SECOND MESSENGER EQUILIBRIA

All measurement techniques have to deal with a conflict with respect to their objectives. In some instances, it is desired to observe a process, disturbing it as little as possible; in other instances one would like to obtain quantitative data under as much experimental control as possible. The two aims are, of course, mutually exclusive. The cell-attached measurement comes close to the first ideal, since it leaves the cell largely intact, and allows one to observe channels open and close, or to record action potentials extracellularly (Fenwick et al. 1982a). Excised patches constitute the other extreme, where membrane patches are removed from their natural environment for optimal control of solution composition on both sides of the membrane. The whole-cell recording method is at an intermediate position in this respect. It does provide excellent control over membrane potential, if cells smaller than 20 mm in diameter are used. However, the chemical composition of the internal medium is neither undisturbed, nor is it under good control. We found that small mobile ions typically exchange by diffusion between pipette and cell in a few seconds (for cells of approximately 15 mm diameter and pipettes of 2-5 MW resistance). Molecules of intermedi-
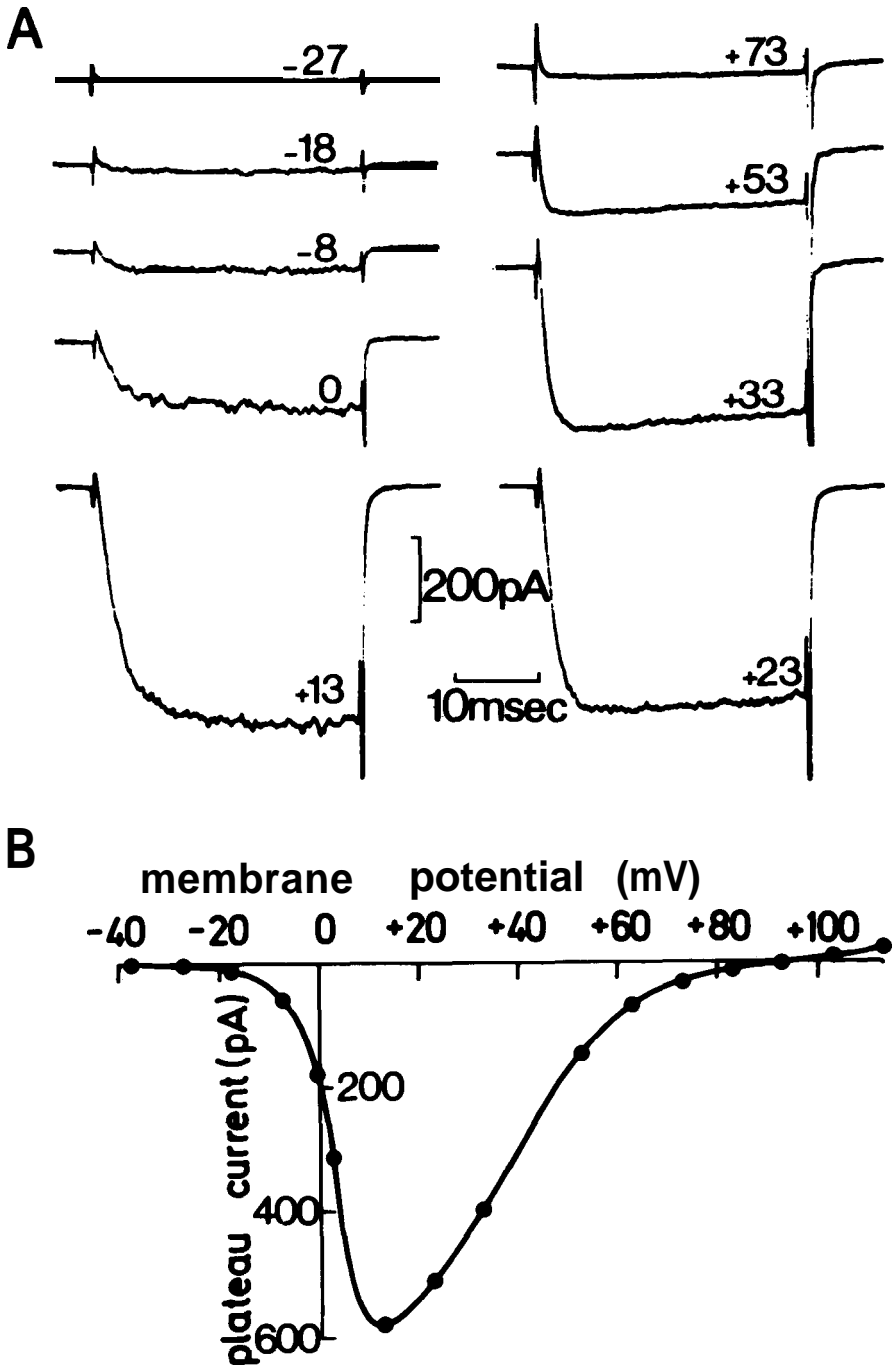
Figure 5. Whole-cell membrane currents in chromaffm cells bathed in isotonic Ba solution plus 20 μg/ml TTX. The membrane potential was stepped to values as indicated from a holding potential of -67 mV. The pipette solution contained mainly CsCl and TEA. With this solution composition the currents flowing are predominantly carried by Ca channels. Part B shows the current-voltage relationship. Reproduced from Fenwick et al. 1982b.

ate size, like second messengers, typically 'wash-out' or 'load' into cells within 10 seconds to a minute, and small regulatory proteins may take several minutes and longer for complete equilibrium (Pusch & Neher 1988).

In retrospect, it seems fortunate, that we started our measurements with ACh channels and Na channels, which happen to be relatively robust with regard to diffusible regulatory components. Thus, we initially avoided complications of 'channel modulation'. However, when switching to channels which now are known to be subject to modulation by second messengers, G-proteins and phosphorylation (such as Ca-channels), we soon realized that channel activity would disappear rapidly as a result of the perturbation imposed by the measurement, both in 'whole-cell' and, more so, in excised-patch measurements (Fenwick et al. 1982b). Such 'washout' had been observed earlier in studies on dialyzed giant neurones (Kostyuk 1980). The prototype of a channel modulated by an intracellular second messenger, the Ca-activated K^+-channel was characterized by Alain Marty (1981). These early studies already showed the ambivalent nature of the new tools: On the one hand there was the advantage of control over intracellular calcium to elucidate the mechanism of Ca-modulation. On the other hand there was the loss of cellular function due to the loss of regulators, which at that time were unknown. Subsequently, ingenious use of these tools by many laboratories has revealed a whole network of interactions between channels, second messengers, G-proteins, and other regulatory proteins (see Hille 1989, for review). In order to uncover this network it was necessary not only to record electrically from cells, but also to control or change systematically the concentrations of second messengers (Marty 1981; Kameyama et al. 1986; Penner, Matthews & Neher 1988; Fesenko et al. 1985). Later on it became possible to impose step-like changes in regulators using caged compounds (Morad et al. 1988), or to load cells with fluorescent indicator dyes (Almers & Neher 1985) and regulatory proteins (Hescheler et al. 1986).

All this was made possible by utilizing diffusional exchange between patch pipette and cell or by exposing the cytoplasmic surface of excised patches. Later, ways were found to avoid the adverse effects of 'washout' by making the patch selectively permeable to small ions (Lindau & Fernandez 1986; Horn & Marty 1988). This technique, at present, seems to be the least invasive method to study the functioning of small cells.

AN ELECTROPHYSIOLOGICAL APPROACH TO STUDY SECRETION

An outstanding property of an electrical measurement with giga-sealed pipettes is its high sensitivity. This can be used not only to record currents, but also to study the membrane electrical capacitance, which is a measure of cell surface area. It had been observed before, that membrane capacitance increases under conditions where massive exocytosis of secretory vesicles is expected to occur. Presumably, this is due to the incorporation of vesicular membrane into the plasma membrane (Jaffe et al. 1978; Gillespie 1979).

The low background noise of the giga-seal measurement made it possible to resolve area changes which result from the exocytotic fusion of single vesicles. This was shown by Neher & Marty (1982) for exocytosis from chromaffin cells of the adrenal medulla and by Fernandez, Neher & Gomperts (1984) for secretion of histamine from rat peritoneal mast cells. In the latter case, the granules are somewhat larger, leading to well-resolved step-like increases in capacitance (see Fig. 6). These records show that capacitance measurement is a *high-resolution* technique. The figure also shows, however, that capacitance is not a very *specific* measure for secretion. This is evident from the fact that there is a continuous, smooth decrease in capacitance before exocytotic events show up. We found (Almers & Neher 1987) that the rate of this decrease depends on the concentration of free intracellular calcium $[Ca]_i$, and that it has many properties expected for pinocytosis.

We used capacitance measurement together with current recording and microfluorimetry (fura-2) to simultaneously study, in a single cell, changes in $[Ca]_i$ and secretion at subsecond time resolution. We were surprised to find quite different effectiveness of regulators of secretion in different cell types (Fig. 7). For chromaffin cells, which in many respects resemble neurones, the classical role of calcium as prime regulator of secretion was fully confirmed (Augustine & Neher 1992). In mast cells, however, which are not electrically excitable, changes in calcium concentration (in the physiological range) had little effect. Ca-independent secretion had been described in a number of inexcitable cell types (Haslam & Davidson 1984; Sha'afi et al. 1983; Di Virgilio et al. 1984). But for us, who were used to work with electrically excitable cells, it was a shock not to be able to elicit secretion
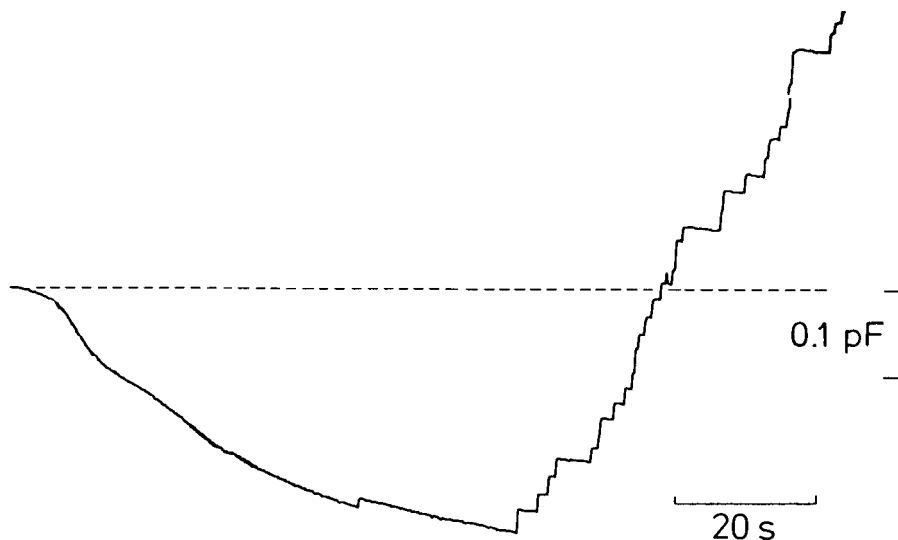
Figure 6. High resolution capacitance recording during the onset of a mast cell degranulation. Whole-cell recording from a rat peritoneal mast cell with a pipette containing 20 μM GTP-γ-S. Initially the capacitance slowly decreased, probably due to retrieval of very small pinocytotic vesicles. After some delay degranulation started, leading to a staircase-like increase in capacitance, each step representing fusion of a single granule (adapted from Almers & Neher 1987).

with an intracellular solution buffered to about 1 μm free calcium. Initially, we hypothesized, that the whole-cell configuration resulted in a loss of Ca-dependent regulators by washout - in analogy to early work on muscle contraction in skinned fibers. Later, we learned that cells in the whole-cell recording configuration were still able to secrete, in response to GTP-γ-S, a nonhydrolyzable analogue of GTP (Fernandez, Neher & Gomperts 1984).
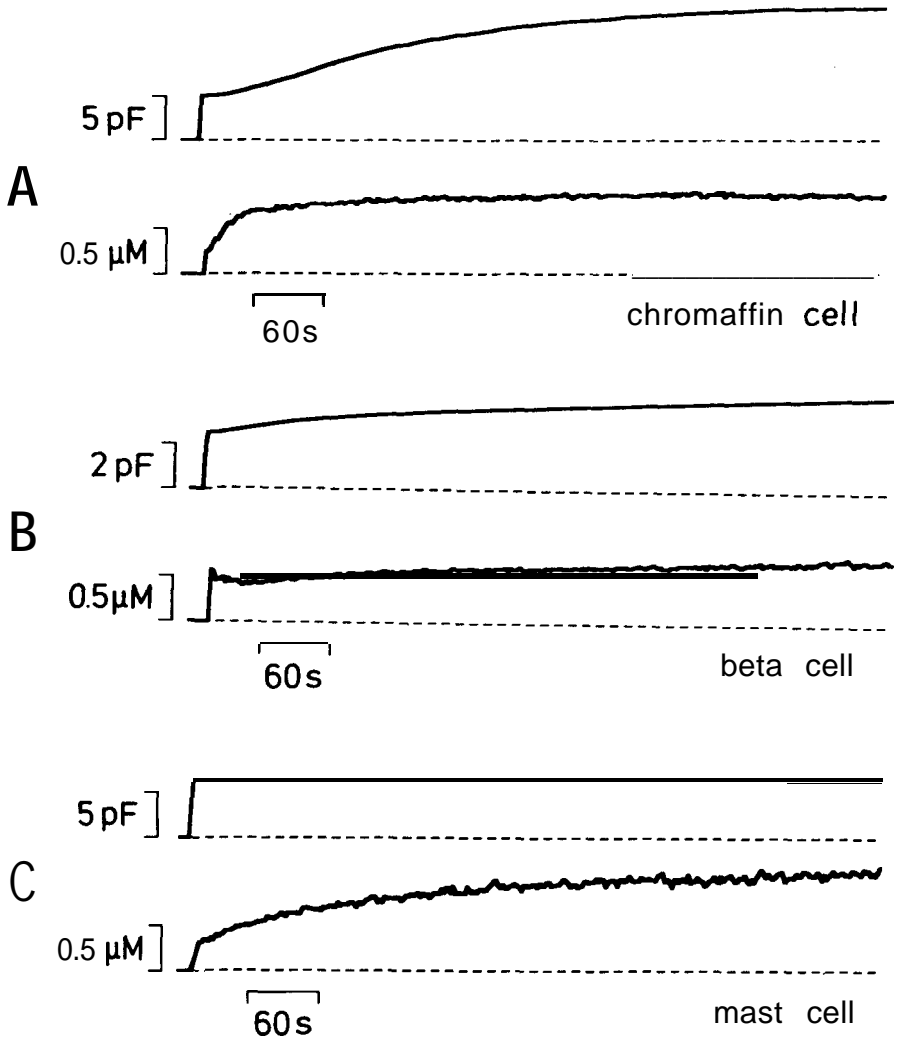
Figure 7. Different effectiveness of intracellular free calcium in inducing a capacitance increase. Part A represents a measurement from a bovine chromaffin cell. The traces represent the time courses of capacitance (top) and free calcium concentration (bottom; measured by fura-2 fluorescence) following a whole-cell penetration. Calcium rapidly rises, since the pipette was filled with a Ca-EGTA mixture adjusted for free calcium of approximately 1 μM. On the capacitance trace the step at the beginning ($\equiv 6$ pF) represents the initial capacitance of the cell, which becomes 'visible' at the moment of 'break in'. Capacitance then rises about two to threefold due to exocytosis. Part B shows a similar measurement on a pancreatic beta cell, with only little capacitance increase, and part C shows the complete lack of response in a rat peritoneal mast cell. Reproduced from Penner & Neher 1988.

With this response in hand, it was possible to show that calcium, although not able to elicit secretion by itself, was still effective in accelerating an ongoing secretory response. There was no indication of a loss of a Ca-regulator; rather it appeared that the GTP-γ-S stimulus primed the cell to render it more Ca-sensitive (Neher 1988).

GTP-γ-S nonspecifically activates intracellular signal pathways, most prominently the dual-signal pathway (Cockcroft & Gomperts 1985; Berridge & Irvine 1989). Using the repertoire of patch clamp methods, Penner (1988) was able to show that various external secretagogues, which are known to also activate the dual signal pathway, lead to a characteristic pattern of secretion. This is accompanied by IP_3-induced Ca release from intracellular stores. Combining the fura-2 technique with patch clamping one can study the temporal relationship between this prominent transient Ca signal and secretion. In spite of the modulatory effect of calcium described above it was found that there is no strict correlation. The secretory response very often starts well after the peak of the Ca signal. Also, the Ca peak can be abrogated by including EGTA in the patch pipette without drastic effects on secretion (Fig. 8). Phenomenologically, this can be explained by the fact that the Ca peak occurs very early at a time when the above-mentioned priming effect of a chemical stimulus has not occurred yet. A sustained phase of increased calcium, which very often follows the Ca-release peak, is more efficient in accelerating secretion, however, since it is more appropriately timed. In terms of molecular mechanisms, the priming is likely to represent protein kinase C activation (Heiman & Crews 1985; Penner 1988). Additionally it has been shown that there is another G-protein-mediated pathway, which links a hormonal stimulus to secretion (Gomperts, Barrowman & Cockcroft 1986; Penner 1988). This link is sensitive to pertussis toxin, and to intracellular application of cAMP (Penner 1988).

Our studies on mast cells (see Penner & Neher 1989 for review) have taught us that secretory control is not necessarily dominated by calcium, but that rather it involves a meshwork of interacting second messenger pathways. In neurones, it appears that changes in calcium (Augustine et al. 1987) or calcium plus voltage (Parnas et al. 1986) largely determine the kinetics of fast secretory events. However, there is increasing evidence that other second messengers are responsible for plastic changes in synaptic signals, possibly by regulating the availability of vesicles for secretion (Llinas et al. 1991). Unfortunately, nerve terminals are usually not accessible to the kind of biophysical investigations described here. However, recent studies on neurosecretory cells reveal new details on the kinetics of Ca-induced secretion (Thomas, Suprenant & Almers 1990; Augustine & Neher 1992; Lim, Nowycky & Bookman 1990; Lindau et al. 1992). They promise to allow a differentiation between the exocytotic event per se and some of the other steps in the life cycle of a secretory vesicle. Together with the ability to control second messengers such studies may soon lead to a better under-

standing of exocytosis and of the molecular processes that direct a vesicle to its site of action.
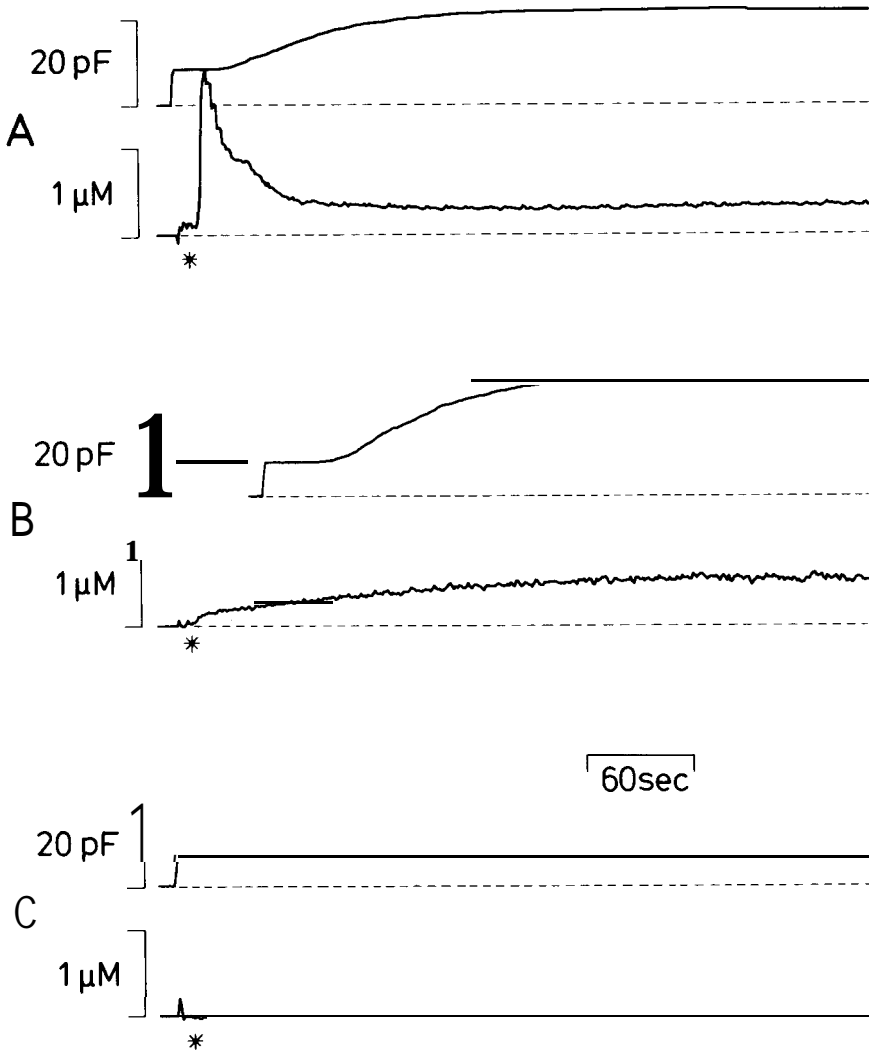
Figure 8. Responses of mast cells to stimulation by the secretagogue compound 48/80 under different Ca-buffering conditions. The individual panels show combined capacitance-calcium (fura-2) measurements similar to those of Fig. 7. Panel A shows the 'unbuffered' case. No Ca-buffer was added to the pipette (except for 100 μM fura-2). A Ca transient developed in response to stimulation (asterisk). Secretion typically proceeded mainly during the falling phase of the Ca-transient or following it. In panel B an EGTA/Ca mixture (10 mM) was added to the pipette which suppressed the transient, and fixed the Ca concentration to the range 200-500 μm. Nevertheless, secretion proceeded with a time course similar to case A. In panel C 10 mM EGTA was added to clamp calcium to low values. This suppressed both the Ca signal and the secretory response (from Neher & Penner 1988).

nels, and taught me to use microscopic tools. Superb working facilities in Gottingen were provided by H. Kuhn, O. D. Creutzfeldt and T. Jovin, who established a Young Investigators Laboratory for Bert Sakmann, F. Barrantes and myself where we could independently pursue our goals. In more recent years my work was generously supported by a Leibniz Award of the Deutsche Forschungsgemeinschaft.

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