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REVIEW ARTICLE

Gaining access to the cytosol: the technique and some applications of electropermeabilization

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Introduction

Three general experimental approaches have been used to analyse the mechanisms (cellular signal transduction mechanisms) which permit extracellular signals to influence cellular responses. First, the intracellular chemical changes resulting from stimulation by such a signal may be determined in an intact cell either by chemical content or radiochemical analysis, or by introducing probes which monitor chemical changes. Such probes include ion-sensitive microelectrodes (in larger cells) (Thomas, 1981, and references therein); or chemical-sensitive dyes which may be inserted by microinjection (Blinks, 1981; Scarpa, 1981, and references therein) or by presentation to the cell in a membrane-permeant form (Tsien, 1981), Second, the response to the signal may be reconstituted *in vitro* using isolated cellular components and the chemicals proposed as constituents of the signal transduction mechanism (see, for example, Bikle *et al.* 1976). And third, recognizing that the presence of the plasma membrane is the major barrier to successful manipulation of signal transduction mechanisms, localized lesions may be introduced selectively in this limiting membrane. This modification then allows controlled variation of a chemical component of the cytosol and thus determination of the effect of such a variation on the response.

Initially most studies on cellular signal transduction mechanisms were based on the use of intact cells and/or isolated cellular components. However, disadvantages exist with both these approaches. For secretion, for example, where the cellular response involves participation of the plasma membrane, reconstitution experiments have not, with a few notable exceptions (Vacquier, 1975; Davis & Lazarus, 1976; Gratzl *et al.* 1977; Konings & de Potter, 1981), been successful. Even when such reconstitutions have succeeded in other systems (e.g. Bikle *et al.* 1976) it is often difficult to relate the conditions used to those which may prevail in the intact cells. Hence the relevance of the data obtained to the state *in vivo* is uncertain. Similarly, it is difficult to establish in an intact cell that a given intracellular chemical participates in the signal transduction mechanism from measurements of changes in its concentration as a consequence of stimulation by the extracellular signal. Attempts can be made to modify the changes in the concentration of this chemical induced by the signal, or to induce such changes independently of the signal, and then determine the effect of such maneouvres on the response. However, the agents used are rarely wholly selective, and in any case interaction between different signal transduction pathways in an intact cell usually ensures that any effects are not restricted to a single pathway. It is also possible to introduce chemicals directly into the cytosol of cells by perfusion (Kostyuk & Krishtal 1984), dialysis (Brinley, 1981), microinjection (Meech, 1981) or iontophoresis (Simonneau & Tauch, 1981) but such methods are limited in most instances to experiments in which responses from single cells can be studied.

More recently there has therefore been a marked increase in the use of cell preparations having lesions in the plasma membrane (permeabilized cells) for studies on signal transduction mechanisms, since these preparations offer the opportunity to manipulate the chemical composition of the cytosol without gross distortion of the architecture of this compartment or of small membrane-bound organelles. Furthermore, provided that large areas of the plasma membrane are unaffected by the method used to induce the localized lesions, responses involving this membrane can be studied. These disruptive techniques however invariably dissipate the plasma membrane potential and therefore any dependence of the singal transduction mechanisms on this potential will be lost. In this article we will examine such methods with especial reference to one procedure (electropermeabilization) which appears most fully to meet the required criteria.

Methods used for cellular permeabilization

A number of methods have been used in an attempt to obtain selective breakdown of the plasma membrane of cells (permeabilization). Some of these methods, e.g. treatment with organic solvents or use of osmotic shock, are probably not selective to this membrane and are inappropriate for analysis of signal transduction mechanisms. For other methods which do apparently cause selective permeabilization of the plasma membrane, e.g. incubation with Sendai virus (Impraim *et al.* 1980) or with EGTA + ATP^{4-} (McClellan & Winegrad, 1978) we have at present no clear understanding of the mechanism(s) by which membrane breakdown is achieved. Hence it is difficult to comment on the advantages and limitations of these methods. However it is notable that for some cells permeabilization can be achieved by incubation with EGTA, e.g. pancreatic acinar cells (Schulz *et al.* 1981), or ATP^{4-}, e.g. mast cells (Gomperts, 1983), when added alone and that such effects are often reversible by removal of EGTA and/or addition of excess Mg^{2+}.

A more satisfactory level of insight exists for detergent-induced- and electro-permeabilization, which

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PAF, platelet-activating factor.

Table 1. Comparison of permeabilization methods: electropermeabilization and detergent-induced permeabilization

	Permeabilization using a detergent, e.g. digitonin, saponin	Electropermeabilization
Special apparatus required	None	High voltage power supply, capacitor (2–5 μF), permeabilization cell, discharge switch (Knight, 1981)
Basis for membrane breakdown	Selective removal of cholesterol (Gogelein & Huby, 1984)	Induction of potential (approx. 1.5 V) across membrane by applied high voltage field (Knight, 1981, and references therein)
Basis for selective breakdown of plasma membrane	Higher cholesterol content of plasma membrane as compared with that of intracellular membranes (Menashi *et al.*, 1981)	Radius of cell relative to that of intracellular organelles (Knight, 1981, and references therein)
Is membrane breakdown localized?	Probably not, since cholesterol unlikely to be localized within particular regions of membrane	Yes – maximal potential develops for given applied field at points on cell 0° and 180° to direction of field (Knight, 1981, and references therein)
Establishment of conditions for selective effect on plasma membrane	Not predictable and must be obtained empirically (see for example Zuurendonk & Tager, 1974)	Predictable if dimensions of cell and intracellular organelles known (Knight, 1981)
Stability of permeabilized preparation	Unstable, since cholesterol removed from intracellular membranes once these are accessible to the detergent	Reasonably stable, although some cells, e.g. erythrocytes, tend to repair the lesions and so reseal (Kinoshita & Tsong, 1977)
Properties of permeabilized preparation	Often rather heterogeneous. Evidence of extensive damage to plasma membrane in electron micrographs and marked leakage of cytosolic macromolecules (see for example Purdon *et al.*, 1984)	Apparently homogeneous. Little evidence of plasma membrane damage in electron micrographs. Minimal leakage of cytosolic macromolecules, especially from smaller cells (Knight, 1981; Scrutton *et al.*, 1985)

are two procedures now used more widely for studies on cellular signal transduction. Table 1 presents a summary of some aspects of these two methods to facilitate their comparison. Permeabilization using detergents is cheap, but its apparent technical simplicity is deceptive due to the narrow range of detergent concentration, incubation time, incubation temperature, etc., over which the desired selective effect can be obtained. An example of the narrow working range with respect to detergent concentration is shown in Fig. 1 for Ca^{2+}-induced 5-hydroxytryptamine (5HT) secretion from platelets. An optimal secretory response induced by Ca^{2+} is obtained in this system on addition of approximately 5 μg of digitonin/ml. The decreased secretion seen at lower [digitonin] probably reflects incomplete permeability of the cells to Ca^{2+}, while that observed at higher [digitonin] represents inhibition of the secretory response, possibly due to loss of essential cytosolic macromolecules or to gross modification of membrane composition. Such inhibition of the secretory response is seen with other detergents and in other cells (Baker *et al.* 1985) and hence is not a specific effect of digitonin. It also occurs at detergent concentrations lower than those required to induce granule lysis, which is seen here as Ca^{2+}-independent 5-hydroxytryptamine release (Fig. 1). Digitonin concentrations which partially inhibit a secretory response induced by Ca^{2+} do not however impair thrombin-induced transmembrane signalling, since the sensitivity of the secretory response to Ca^{2+} is enhanced by the addition of this agonist even though the overall response decreases (Fig. 1). A similar enhancement of the Ca^{2+}-sensitivity of the secretory response by thrombin is observed in the electropermeabilized platelet (Knight *et al.* 1984; Haslam & Davidson, 1984*a*).

As indicated in Table 1, electropermeabilization is less susceptible to many of the problems inherent in the use of detergents. Although special apparatus is required, the switch and permeabilisation cell can be constructed by any competent workshop and only the capacitor is unlikely to be available in many biochemical laboratories. The basic principles of electropermeabilization have been discussed extensively elsewhere (Knight, 1981, and references therein) and hence the treatment here is restricted to the brief outline necessary to understand Table 1 and the following discussion.

A spherical membrane-bound particle of radius r (e.g. a cell or intracellular organelle) when placed in a plane electrical field of strength E will experience a voltage difference V across the membrane at point P (Fig. 2*a*) which is given by:

$$V = C\,r\,E\cos\theta \qquad (1)$$

where C is a constant and θ is the angle made by point P relative to the direction of the field. The maximum voltage difference, $V_{max.}$, will therefore develop across the membrane when $\cos\theta = \pm 1$, i.e. at points A and B

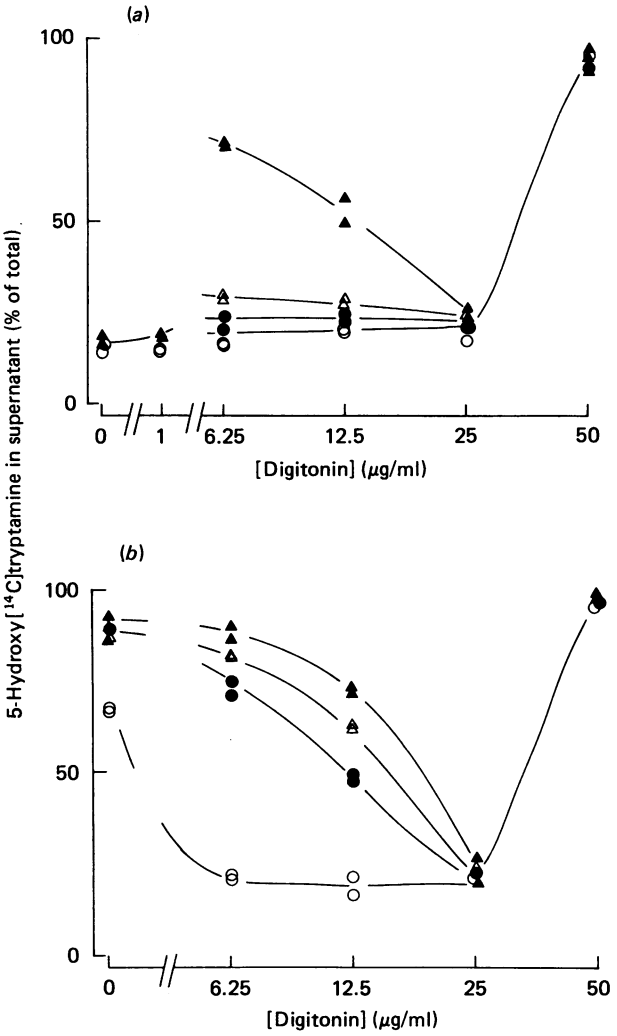

Fig. 1. Permeabilization induced by a detergent: permeabilization of human platelets by digitonin in the absence (*a*) and presence (*b*) of thrombin

Platelets previously loaded with 5-hydroxy[^{14}C]tryptamine and suspended in a glycine/potassium glutamate medium containing $MgATP^{2-}$ and EGTA (Knight *et al.*, 1984) were incubated for 5 min at 20 °C with the concentrations of digitonin as indicated. The cell suspension was then added to a Ca–EGTA buffer in the absence (*a*) and presence (*b*) of 6 nM-thrombin + 20 μM-GTP such that the final calculated Ca^{2+} concentration was approx. 0.01 μM (○), 0.4 μM (●), 1 μM (△) or 10 μM (▲) and incubated for a further 10 min at 20 °C. The 5-hydroxy[^{14}C]tryptamine content of the supernatant fraction was determined as described by Knight *et al.* (1984).

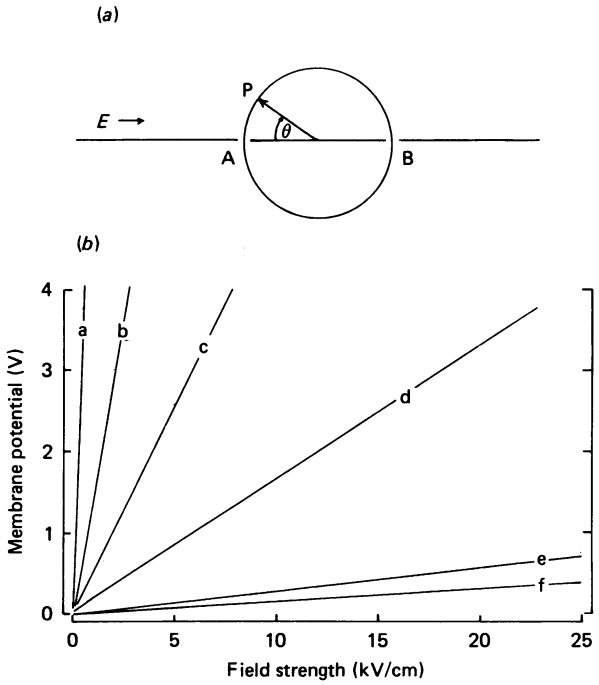

Fig. 2. Principles of electropermeabilization

(*a*) Relationships relevant to eqn. (1). (*b*) The relationship between membrane potential (V) and field strength (E) for spherical membrane-bound particles having radii of 50 μm (a), 10 μm (b), 3.5 μm (c), 1 μm (d), 0.2 μm (e) and 0.1 μm (f) assuming a value for C of 1.5. These particle sizes correspond approximately to the dimensions of a sea urchin egg (a), an adrenal medullary cell (b), an erythrocyte (c), a platelet (d), a mitochondrion (e) and a secretory granule (f), although erythrocytes, platelets and mitochondria show marked deviation from spherical geometry.

in Fig. 2(*a*) in line with the field direction. The value of C is determined by the radius of the cell, the thickness of the membrane and the relative electrical conductances of the membrane and of the fluids within and outside the particle. If, as might be expected, the conductance of the membrane is much smaller than that of the internal and external fluid, and the thickness of the membrane is much less than the radius of the particle, then the value of C approaches a limit of 1.5 and:

$$V_{max.} = 1.5\, r\, E \qquad (2)$$

Hence under such conditions the value of $V_{max.}$ for a given applied field is determined by the radius of the particle. Fig. 2(*b*) shows this relationship in another way by defining the dependence of $V_{max.}$ on E for spherical particles of different radii which approximate the size of various cells and organelles. For example, exposure to a field of 2000 $V \cdot cm^{-1}$ will place a $V_{max.}$ of 3 V across the plasma membrane of a cell ($r = 10$ μm) but of only 30 mV across the membrane of a typical intracellular organelle ($r = 0.1$ μm). Since membrane breakdown only occurs when $V_{max.}$ reaches approx. 1.5 V (Knight, 1981, and reference therein) selectivity for localized breakdown of the plasma membrane can clearly be obtained even when much smaller cells are used. [Unless very unusual conditions prevail the time required for the applied field to impose the voltage difference across the membrane is less than 1 μs (Jeltsch & Zimmerman, 1979) and if the imposed voltage difference is of a sufficient magnitude membrane collapse occurs in nanoseconds (Riemann *et al.* 1975). Hence permeabilization can be achieved by exposure of cells to an appropriate electric field having a duration of 1–2 μs.] Thus in many studies (see for example Baker & Knight, 1978; Knight *et al.* 1982; Moos & Goldberg, 1985) secretory cells have been rendered permeable to CaEGTA ($Ca^{2+} \approx 0.1$ μM) by this method without causing release of constituents stored in intracellular organelles and without impairing the

functions of such organelles. The localized nature of membrane breakdown caused by electropermeabilization has been most convincingly established using the sea urchin egg in which cortical granule discharge is initiated only at opposite ends of the cell when it is exposed to an electric field of appropriate strength in the presence of micromolar Ca^{2+} (Baker *et al.* 1980; Knight, 1981).

However, few cells are truly spherical and size heterogeneity is not uncommon. Furthermore it is often difficult to obtain single-cell preparations which retain the functional responses found in small cellular aggregates and many cells respond normally only when attached to a substratum. We should therefore consider the impact of these complications on the application of electropermeabilization. Heterogeneity of cellular size is clearly important (eqn. 2; Fig. 2*b*). If all cells in a heterogeneous suspension are to be permeabilized, the applied field must be adjusted to achieve the critical voltage potential across the plasma membrane of the smallest cell in the population with consequent risk of damage to the larger cells. Alternatively, in a mixed cell population advantage can be taken of the relationship between $V_{max.}$ and r (eqn. 2) to achieve selective permeabilization of the larger cells while leaving the smaller cells intact. However such selectivity may be compromised by deviations from spherical geometry, which introduce additional significant terms into eqn. (1) and so may invalidate the simple treatment given above. The method is applicable to immobilized cells, e.g. attached to electrically conducting cell culture beads (D. E. Knight, unpublished work) or embedded in agar (Whitaker, 1985), although immobilization may restrict the ability to make multiple plasma membrane lesions as a consequence of repeated exposures to the applied electric field. This option is available for cells in suspension due to changes in orientation with respect to the field which occur as a consequence of cellular tumbling.

Cellular aggregates may be recognized by the applied field as a single large membrane-bound structure, rather than as a collection of individual cells, due to intercellular communication within the aggregate. As a result plasma membrane breakdown may occur in only a few of the cells leaving the bulk unaffected. Unless the cells can be uncoupled. e.g. by the use of media containing a low concentration of Ca^{2+} or possibly of low ionic strength, it may be difficult to apply electropermeabilization to such a system.

Some practical considerations in electropermeabilization

Cells can be exposed briefly to an intense electric field either by rapid passage through a steady field or by pulsing the field through a static cell suspension. In the former case a Coulter Counter system is used. Cells are drawn through a narrow orifice across which a current flows, with the time of exposure to the field being determined by the time of passage through the orifice (Zimmermann *et al.* 1980). The second strategy, which has more generally been used in electropermeabilization studies, can be achieved either by using a switched system which exposes the cells to a low output impedance, high voltage source for several μs (Jeltsch & Zimmerman, 1979) or by discharging a capacitor through the system with the time of exposure to the field being determined by the time required for capacitor discharge (Zimmerman *et al.* 1975; Knight, 1981). Table 1 presumes this latter version which, although less elegant, is more practical since it does not require a specialized power supply or sophisticated switching equipment. In this system the capacitor is charged to the required voltage over several seconds using a relatively high output impedance current source which allows the use of relatively cheap and safe high voltage power supplies. The switch, which must be designed to discharge the capacitor in a single event, can be simply two metal contacts pushed together manually. The voltage used is a function both of the radius of the cell (eqn. 2) and the spacing of the electrodes in the permeabilization cell. If capacitor life is to be prolonged and heating minimized the applied voltage should be kept as low as possible by decreasing the electrode spacing. For example, an applied voltage of 20 $kV \cdot cm^{-1}$, which is necessary to achieve the requisite voltage gradient across the plasma membrane of a small cell such as a platelet ($r = 1$ μM) (Fig. 2*b*) can best be obtained by discharging a capacitor charged to 2 kV across a cell in which the electrodes are placed 1 mm apart.

When the capacitor is discharged through the cell suspension the applied field decays exponentially with a time constant that is a product of the capacitance of the capacitor and the resistance of the suspending medium. Since the cells should be exposed to the full field for several μs (see above), the system must be arranged so that the time constant for decay of the field is greater than 2–3 μs. For example, in a system in which 1 ml of a cell suspension in a medium of physiological ionic strength is placed between electrodes spaced 1 cm apart, the resistance will be approx. 100 ohms and hence discharge of a 2 μF capacitor will yield an applied field that decays with a time constant of 200 μs. If the electrode spacing is decreased (and the size of the electrodes increased to obtain a reasonable working volume of cell suspension) then the resistance will decrease. Hence to obtain the desired minimal value for the time constant either the size of the capacitors must be increased or the ionic strength of the medium reduced (hence increasing its resistance). The latter option is usually preferable, although the modified medium must still possess a conductivity which is considerable greater than that of the cellular membranes (see above).

The actual composition of the suspending medium is also of importance since on permeabilization it will enter the cytosol and dilute the low-M_r constituents present in this compartment. Hence this medium should either mimic the composition of the cytosol so far as this is known, or at least should not contain substances which are detrimental to the responsiveness of the preparation. Hence the media used typically contain a high concentration of K^+, low concentrations of Na^+ and Cl^-, millimolar Mg^{2+} and approx. 0.1 μM-Ca^{2+}, with $[Ca^{2+}]$ being defined by the use of a Ca–EGTA or Ca–BAPTA buffer (Martell & Sillen, 1964; Tsien,, 1980). Millimolar concentrations of $MgATP^{2-}$ are often included since many processes depend on the availability of this component of the cytosol.

Some knowledge of the effective size, and of the stability, of the membrane lesions created by exposure to the electric field is crucial to the effective use of electropermeabilized cell preparations. In larger cells the size of the lesions can be estimated by determination of the rate at which substances enter or leave the permeabilized cell. For example, the rate of leakage of several markers from adrenal medullary cells which have been permeabilized by exposure to a 2 $kV \cdot cm^{-1}$ field

indicates an effective pore diameter of approx. 4 nm (Knight, 1981). This approach cannot easily be used for small cells, since the rates of marker entry or leakage are too rapid (Knight & Scrutton, 1980; Knight *et al.*, 1982), and since changes in cellular volume tend to occur on permeabilization due for example to swelling caused by the colloid osmotic pressure (Haslam & Davidson, 1984*b*). However, the effective pore size estimated for the electropermeabilized adrenal medullary cell is consistent with the failure to observe leakage of cytosolic proteins ranging in molecular mass from 63 kDa (enolase) to 125 kDa (lactate dehydrogenase) from the electropermeabilized platelet (Knight *et al.*, 1982). The stability of the lesions created appears to vary in different cells. Several exposures of bovine adrenal medullary cells (Baker & Knight, 1978, 1981), sea urchin eggs (Baker *et al.*, 1980), pancreatic acinar and β-cells (Yaseen *et al.*, 1982; Knight & Koh, 1984) or platelets (Knight & Scrutton, 1980) to fields adequate to allow free access of low-M_r solutes to the cytosol gives permeabilized preparations which are stable in this state for at least 1 h in some cases. Other cells, e.g. erythrocytes (Kinoshita & Tsong, 1977), macrophages and Hela cells (Baker *et al.*, 1985) are unstable in the permeabilized state and appear capable of repairing the membrane lesions to at least some extent. No clear rationale exists at present which explains these differences, and it is therefore necessary to define the period over which an electropermeabilized preparation is stable when no prior studies exist.

Applications of electropermeabilized cells

Early studies demonstrated that plasma membrane breakdown occurred on exposure of cells to an intense electric field (Zimmermann *et al.*, 1974), while more recently conditions have been defined under which this breakdown was specific for the plasma membrane while leaving the cytoarchitecture and the integrity of the intracellular organelles unperturbed (Baker & Knight, 1978). Thus far this technique has mainly been used to investigate responses involving Ca^{2+}, especially those such as secretion which are dependent on the integrity of the plasma membrane and of the cytoskeletal architecture. More recently, electropermeabilized cell preparations have been used in other ways, e.g. in studies of membrane transduction mechanisms, of intracellular Ca^{2+} sequestration and release, and of the autophagic response. We will examine some of the data obtained in such studies in order to illustrate the potential of the electropermeabilized preparation.

Ca^{2+}-dependent secretory responses

It was widely inferred on the basis of indirect evidence that changes in cytosolic [Ca^{2+}] played an important role in initiation of the secretory responses in many cells (Douglas, 1974). In the case of the bovine adrenal medullary cell direct evidence for such a role came from the demonstration that an increase in cytosolic [Ca^{2+}] to the micromolar range induced catecholamine secretion (Baker & Knight, 1978). Since this initial demonstration, many other cells have been permeabilized and their secretory response(s) characterized by using this technique. Fig. 3 shows the collected data from a number of these studies. In all cases it is notable that little, if any, response is observed if the Ca^{2+} concentration is fixed at 0.1 μM, a value approximating the resting cytosolic [Ca^{2+}] in many intact cells (see, for example, Rink *et al.*, 1982; Tsien *et al.*, 1983; Knight & Kesteven, 1983; Baker, 1972). Furthermore, for all these sytems a maximal secretory response can be induced if the Ca^{2+} concentration is raised to 5–10 μM, and the $EC_{50}(Ca^{2+})$ is always in the range 1–2 μM (Fig. 3). [In studies using electropermeabilized cell preparations Ca^{2+}-buffer systems are used to define the Ca^{2+} concentration in the system and to prevent its alteration by the Ca^{2+}-sequestering or -mobilizing capacity of intracellular organelles such as the endoplasmic reticulum or the mitochondria. Hence the Ca^{2+} concentrations cited here and in all such studies are calculated on the basis of the stability constants published for the buffer system and for other components of the suspending buffer, e.g. ATP, which bind Ca^{2+}. Direct measurements of Ca^{2+} concentration in such systems would be desirable in order to confirm the calculated values.] When Ca^{2+} concentrations in excess of 10 μM are used inhibition of the secretory response is often observed, possibly due to activation of Ca^{2+}-dependent proteinases. This inhibition may be a pathological response, since measurements of cytosolic [Ca^{2+}] in functional cells do not suggest that the average

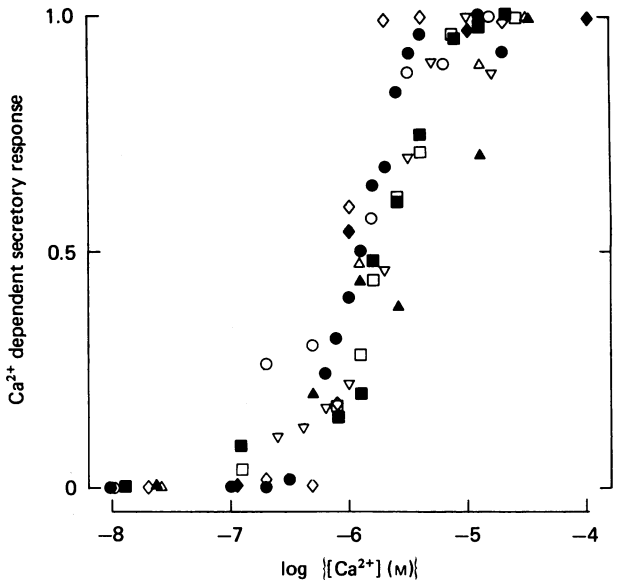

Fig. 3. Ca^{2+}-dependent secretory responses from various electropermeabilized cell preparations

The data have been drawn from six different studies and normalized so that the maximal extent of the response induced by Ca^{2+} is given a value of 1.0. This maximal extent varies widely when expressed as a percentage of total cellular content, from 4% in the case of amylase secretion (pancreatic acinar cells) to 80–90% in the case of 5-hydroxy[^{14}C]tryptamine secretion (platelets). The points shown relate to catecholamine (●) and Met-enkephalin (◇) secretion from bovine adrenal medullary cells (Baker & Knight, 1981; Knight & Baker, 1982), cortical granule discharge from sea urchin eggs (○) (Baker *et al.*, 1980), insulin secretion from pancreatic β-cells ((◆) (Jones *et al.*, 1985), 5-hydroxy[^{14}C]tryptamine (■) and β-*N*-acetylglucosaminidase (□) secretion from human platelets (Knight *et al.*, 1982), amylase secretion from pancreatic acinar cells (▽) (Knight & Koh, 1984), and acetylcholine (△) and ATP (▲) secretion from *Torpedo* synaptosomes (D. E. Knight & H. Zimmerman, unpublished work).

concentration of this cation normally increases beyond the micromolar range (Rink *et al.*, 1982; Tsien *et al.*, 1983). It is possible, however, that the measured Ca^{2+} transients are not uniform across the cell but are localized to an area just beneath the plasma membrane (Rose & Lowenstein, 1975). In such cases the *localized* Ca^{2+} concentration may exceed 10 μM.

In two of the systems for which data is shown in Fig. 3 the secretory response observed is clearly an exocytotic event rather than being due to granule destabilization in the cytosol. In these cases large proteins present in a secretory granule (dopamine β-hydroxylase in the chromaffin granule of the adrenal medullary cell; β-*N*-acetylglucosaminidase in the platelet lysosome) are released preferentially as compared with a cytosolic protein of lower M_r (lactate dehydrogenase) when the electropermeabilized cell is exposed to micromolar [Ca^{2+}] (Baker & Knight, 1981; Knight *et al.*, 1982).

The postulates that charge neutralization (Blioch *et al.*, 1968) or induction of osmotic instability in the secretory granule (the chemiosmotic hypothesis) (Pollard *et al.*, 1977) might explain the role of Ca^{2+} in secretion have been directly examined by using electropermeabilized preparations. Since Mg^{2+} cannot replace Ca^{2+}, but rather inhibits the response to this latter cation (Knight & Baker, 1982), simple charge neutralization is unlikely to provide an explanation. According to the chemiosmotic hypothesis, granule swelling brought about by an influx of either anions or cations facilitates exocytosis. The hypothesis predicts a strong correlation between exocytosis and the source of the extra-granular anion or cation, and also between exocytosis and the pH gradient and potential gradient across the granule membrane (Pollard *et al.*, 1977). These parameters can be measured and/or manipulated directly and independently in an electropermeabilized cell, and hence the hypothesis can be tested. Such studies using adrenal medullary cells show little, if any, correlation between the properties of the Ca^{2+}-dependent secretory response and the pH or potential gradient across the chromaffin granule membrane. The secretory response shows no obvious dependence on the nature of the monovalent cation and can occur in the virtual absence of such cations. Furthermore, addition of certain anions, e.g. Cl^- or SCN^-, causes inhibition of secretion rather than facilitation as predicted by the model (Baker & Knight, 1984; Knight & Baker, 1985*a*). Thus, if osmotic instability plays any role it must operate via another mechanism.

The ability of Ca^{2+} to induce secretory responses in all electropermeabilized preparations studied thus far is lost quite rapidly if $MgATP^{2-}$ is omitted from the suspending medium. This loss of responsiveness is assumed to result from dilution of the endogenous nucleotides (Baker & Knight, 1978; Knight & Scrutton, 1980) and for the adrenal medullary cell the rate of loss of responsiveness to Ca^{2+} in such a medium is that expected on the basis of the calculated rate of efflux of ATP from the electropermeabilized preparation (Knight & Baker, 1982). In some systems, e.g. adrenal medullary cells, responsiveness can be restored by addition of $MgATP^{2-}$ (Knight & Baker, 1982) whereas in others, e.g. platelets, a wider range of nucleoside triphosphates is effective (Knight *et al.*, 1984) due possibly to more rapid nucleotide transphosphorylation. Analogues of ATP which are either not readily hydrolysed, e.g. adenosine 5′-[β,γ-imido]triphosphate, or which donate a thiophosphate, e.g. adenosine 5′-[γ-thio]triphosphate are ineffective in supporting Ca^{2+}-dependent secretion from the electropermeabilized preparations (Knight & Baker, 1982; Knight *et al.*, 1984), thus suggesting that ATP-dependent phosphorylation, or more probably ATP hydrolysis, is required. The nature of the role for ATP in the secretory response is however unclear. Enhanced phosphorylation of specific polypeptides has been shown to accompany Ca^{2+}-dependent secretion in several electropermeabilized preparations, but the properties of such phosphorylation suggest that this may not play a direct role in the exocytotic event (Niggli *et al.*, 1984; Knight *et al.*, 1984; Haslam & Davidson, 1984*a*). If therefore energy provision by ATP hydrolysis is required it may be needed to bring the secretory vesicle and the plasma membrane into contact with each other, either by physical movement within the cytosol, or by removal of water molecules from the contact surfaces. Any physical movement may not however involve microfilaments or microtubules, since the Ca^{2+}-dependent secretory response is insensitive to addition of substances which are known to disrupt these elements of the cytoskeleton, e.g. colchicine or cytochalasins (Knight & Baker, 1982).

Selective secretion and factors enhancing the response to Ca^{2+}

Comparison of the secretory properties of intact and electropermeabilized cells has yielded important information and has led, in at least one instance, to identification of factors modulating the response to Ca^{2+}. It is apparent from Fig. 3 that 5-hydroxytryptamine and β-*N*-acetylglucosaminidase secretion from electropermeabilized platelets occurs over the same range of [Ca^{2+}] in the absence of additions other than $MgATP^{2-}$. This relationship does not replicate the situation observed in the intact platelet which, when stimulated by an agonist such as thrombin, secretes 5-hydroxytryptamine over a lower range of agonist concentration than β-*N*-acetylglucosaminidase (Knight *et al.*, 1982). Such a differential release can however be achieved in the electropermeabilized platelet with respect to variation of [Ca^{2+}] if thrombin is added to the incubation medium. Such an addition also increases the maximal extent of β-*N*-acetylglucosaminidase secretion to a level comparable with that which can be attained in the intact cell (Fig. 4) (Knight *et al.*, 1984). Thus observation of a selective secretory response at least in the platelet depends on an additional factor which modulates the sensitivity of the response to Ca^{2+} rather than operating via a distinct Ca^{2+}-independent pathway (Fig. 4). Other platelet excitatory agonists which resemble thrombin in having the capacity to activate phospholipase C (Brydon *et al.*, 1984; Pollock *et al.*, 1984) also decrease the Ca^{2+} concentration required to observe half-maximal 5-hydroxytryptamine secretion, although their effect is less marked than that caused by addition of thrombin, as would be expected on the basis of results obtained with intact platelets (Table 2) (Brydon *et al.*, 1984).

Although it is not possible for technical reasons to test the effect of natural 1,2-diacylglycerol (predominantly 1-stearoyl-2-arachidonyl-*sn*-glycerol) (Mauco *et al.*, 1984) in this system, the response of the electropermeabilized platelet to effective excitatory agonists (Table 2*b*; Fig. 4) can be mimicked by addition of OAG but not of inositol 1,4,5-trisphosphate (Table 2*d*) (Knight *et al.*, 1984; Scrutton *et al.*, 1985). Thus production of

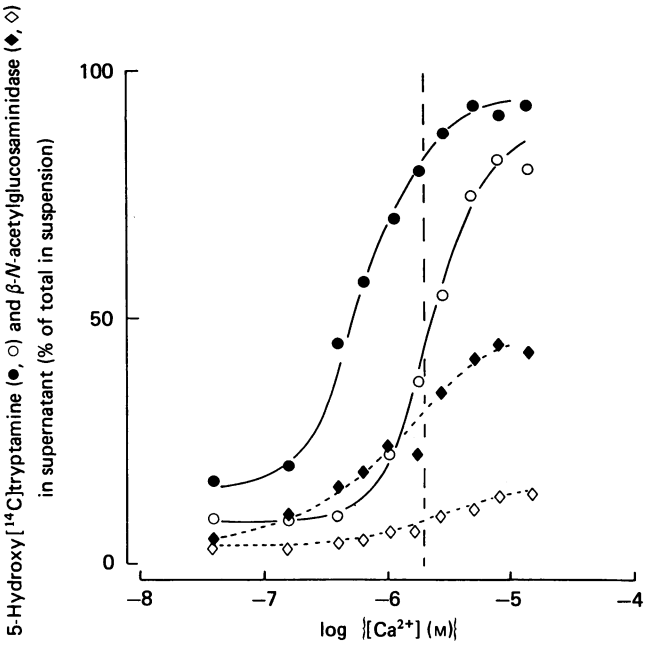

Fig. 4. Properties of 5-hydroxy[^{14}C]tryptamine (○, ●) and β-N-acetylglucosaminidase (◇, ◆) secretion from electropermeabilized human platelets induced by Ca^{2+} in the absence (○, ◇) and the presence (●, ◆) of thrombin

Modified from Fig. 2 of Knight *et al.* (1984).

1,2-diacylglycerol appears to be responsible for the effect of the excitatory agonists. Furthermore, the secretory response to Ca^{2+} in the electropermeabilized platelet is also enhanced by addition of nanomolar concentrations of TPA (Table 2*d*) and of other phorbol esters (Knight *et al.*, 1984) which activate protein kinase C (Castagna *et al.*, 1982). Since OAG activates this enzyme (Mori *et al.*, 1982) these data suggest a role for protein kinase C in the mechanism by which excitatory agonists enhance the secretory response. It is important to note in the context of current discussions (see, for example, Di Virgilio *et al.*, 1984) that the effects of excitatory agonists, OAG and TPA on the electropermeabilized platelet can be eliminated at low [Ca^{2+}] if sufficient Ca^{2+}-buffering capacity is added to the electropermeabilized preparation (Knight *et al.*, 1984). Addition of TPA enhances the secretory response induced by Ca^{2+} in other electropermeabilized preparations. However, where this has been tested OAG does not have a similar effect (Table 3), although at the concentrations required, which approach 100 μM (see Table 2), problems of solubility cannot be excluded as a explanation for this finding. Even in the platelet the relationship between activation of protein kinase C and modulation of the secretory response is far from clearly established, since a 40–47 kDa polypeptide (P47) which is the major substrate for this enzyme (Imoaka *et al.*, 1983) can be extensively phosphorylated on addition of a Ca^{2+} concentration which supports only a minimal secretory response from the electropermeabil-

Table 2. Effect of excitatory agonists, guanine nucleotides and activators of protein kinase C on the properties of 5-hydroxy[^{14}C]tryptamine secretion from electropermeabilized platelets induced by Ca^{2+}

The EC_{50} values shown are calculated from the data of Knight *et al.* (1984), Knight & Scrutton (1985) and Scrutton *et al.* (1985) (I), or from the data of Haslam & Davidson (1984*b*, *c*) (II). The data in (I) were obtained at pH 6.6 with a platelet preparation which was used without further treatment following electropermeabilization and a Ca–EGTA or Ca–BAPTA buffer. The data in (II) were obtained at pH 7.4 with a platelet preparation which was gel-filtered following electropermeabilization to remove released small molecules, and a Ca–EDTA/EGTA buffer system. The difference in EC_{50} for Ca^{2+} between (I) and (II) observed in the absence of additions is not attributable to the difference in pH used in the two studies since values similar to those shown in section (*a*) of the Table for system (I) were obtained with a Ca–EDTA/EGTA buffer at pH 7.4. The difference appears to result from the use of different values for the stability constants in the calculation of Ca^{2+} concentration by the two groups involved.

	Addition	EC_{50} (μM) for Ca^{2+} I	II
(*a*)	None	1.8±0.2	5.0±1.0
	GTP (100 μM)	1.2	1.5
	Guanosine 5′-[β,γ-imido]triphosphate (100 μM)	—	0.09
	Guanosine 5′-[γ-thio]triphosphate (100 μM)	—	0.01
(*b*)	Thrombin	0.39*	0.25†
	Thrombin+GTP	0.09*	0.12†
	11,9-Epoxymethanoprostaglandin H_2(U-46619) (10 μM)	—	0.8
	PAF (1 μM)	—	1.2
	PAF (1 μM)+GTP (4 μM)	—	0.4
	Vasopressin (0.2 μM)	—	1.5
	5-Hydroxytryptamine (10 μM)	1.3	—
	5-Hydroxytryptamine (10 μM)+GTP (10 μM)	0.6	—
(*c*)	ADP (20 μM)±GTP (20 μM)	1.4	—
	Adrenaline (20 μM)±GTP (20 μM)	1.5	
(*d*)	OAG (67 μM)±GTP (20 μM)	0.43	—
	TPA (16 nM)±GTP (20 μM)	0.39	—
	Inositol 1,4,5-trisphosphate (20 μM)	1.6	—

* 6 nM-thrombin + 10 μM-GTP † 20 nM-thrombin + 4 μM-GTP

Table 3. Effect of TPA on the properties of secretory responses induced by Ca^{2+} in different electropermeabilized cell preparations

+ indicates an enhancement in sensitivity (S) or maximal extent (E) of the response to Ca^{2+}; 0 indicates no detectable effect; n.d., not determined.

Cell	Response	Effect of TPA	Effect of OAG	Reference
Bovine adrenal medullary	Catecholamine release	+ (S)	0	Baker & Knight (1983), Knight & Baker (1985*b*)
Human platelet	5-Hydroxytryptamine release	+ (S)	+ (S)	Knight *et al.* (1984)
	β-*N*-Acetylglucosaminidase release	+ (E)	+ (E)	
Pancreatic acinar cell	Amylase release	+ (E and S)	n.d.	Knight & Koh (1984)
Pancreatic *β*-cell	Insulin release	+ (E and S)	n.d.	Jones *et al.* (1985)
Chicken adrenal medullary	Catecholamine release	+ (E and S)	0	Knight & Baker (1985*b*)

ized preparation (Haslam & Davidson, 1984*a*; Knight *et al.*, 1984). Other data are also difficult to reconcile with the postulate that protein kinase C has a primary role in modulation of secretion. For example, purified protein kinase C exhibits a specific requirement for ATP as phosphate donor with an apparent K_m of 6 μM (Kikkawa *et al.*, 1983), whereas in electropermeabilized cell preparations secretion induced by Ca^{2+} in the presence or absence of an agonist requires millimolar levels of a nucleoside triphosphate, which at least for the platelet need not be added as ATP (Knight & Baker, 1982; Knight *et al.*, 1984). Furthermore, 1,2-diacylglycerol is known to have activity as a membrane fusogen (Allan & Michell, 1979), and enhancement of a secretory response by TPA could arise as a consequence of 1,2-diacylglycerol production (Weinstein, 1981) rather than as a direct effect of this phorbol ester on protein kinase C (Castagna *et al.*, 1982).

Analysis of transmembrane coupling mechanisms

Investigations of the mechanisms by which agonists cause activation of plasma membrane-bound enzymes, e.g. adenylate cyclase, and cytosolic components modulate the properties of receptors have traditionally used isolated membrane fractions (Lefkowitz *et al.*, 1983). However, such studies can also be performed using an electropermeabilized cell preparation with the advantages that plasma membrane structure is less extensively disrupted and the organization of the cytosol is preserved in a form which more closely resembles that present in the intact cell. Furthermore, if the transmembrane coupling mechanism involves enhancement of the binding of a cytosolic enzyme to the plasma membrane on agonist stimulation, as proposed for example for phospholipase C by Berridge & Irvine (1984), and the response to the agonist is voltage-independent, then the electropermeabilized cell is a particularly valuable preparation. Useful insights into transmembrane coupling mechanisms have however only been obtained thus far using the electropermeabilized platelet in which enhanced production of 1,2-diacylglycerol occurs as a consequence of stimulation of thrombin, vasopressin and PAF (Haslam & Davidson, 1984*a*, *b*). This effect is observed at 0.1 μM-Ca^{2+}, indicating directly that phospholipase C has significant activity at a [Ca^{2+}] typical of the resting cell. A further increase in 1,2-diacylglycerol formation can however be obtained if Ca^{2+} is increased to the micromolar range (Haslam & Davidson, 1984*a*), thus suggesting the possibility of a positive feedback loop involving this cation (cf. Whitaker & Irvine, 1984). It is presumed on the basis of studies using intact platelets (Rittenhouse-Simmons, 1979) that the 1,2-diacylglycerol is formed by hydrolysis of a phosphoinositide although this has not yet been established for the electropermeabilized preparation. Furthermore, these studies provided one of the first indications that a guanine nucleotide binding (N) protein was involved in receptor–phospholipase C coupling, since 1,2-diacylglycerol production can also be stimulated by GTP or more effectively by the metabolically stable analogue guanosine 5′-[γ-thio]triphosphate (see also Cockcroft & Gomperts, 1985), and stimulation by thrombin is enhanced in the presence of a non-saturating concentration of GTP (Haslam & Davidson, 1984*b*). These effects generally parallel those observed for the effects of guanine nucleotides on Ca^{2+}-induced 5-hydroxytryptamine secretion from the electropermeabilized platelet (Table 2), thus strengthening the postulate that enhancement of the secretory response results from 1,2-diacylglycerol production. In this context it is important to note that addition of GTP has no effect on the enhancement of Ca^{2+} sensitivity induced by OAG or TPA (Table 2*d*).

Less insight into transmembrane coupling mechanisms has been obtained in other systems examined thus far, since agonists fail to alter the Ca^{2+}-dependence of the secretory response when added to an electropermeabilized preparation, e.g. acetylcholine $\pm$ GTP for catecholamine release from bovine or chicken adrenal medullary cells (Knight & Baker, 1982; Knight & Baker, 1985*b*), and glucose for insulin release from pancreatic *β*-cells (P. M. Jones & S. L. Howell, personal communication). The agonists could fail to activate phospholipase C in these electropermeabilized preparations because in the intact cell this response may involve modulation of the plasma membrane potential. Moreover, guanine nucleotides have apparently paradoxical effects. For example, addition of guanosine 5′-[γ-thio]triphosphate respectively enhances or inhibits Ca^{2+}-dependent secretion in electropermeabilized chicken and bovine adrenal medullary cells, whereas addition of GTP prevents both these effects (Knight & Baker, 1985*b*). The different effect of guanosine 5′-[γ-thio]triphosphate in these two preparations may relate to the difference in the acetylcholine receptor subtype present and could suggest the invol-

vement of both stimulatory and inhibitory guanine nucleotide binding proteins in receptor–phospholipase C coupling.

Interaction between the Ca^{2+} and cyclic AMP signal transduction systems

The basis for interaction between the Ca^{2+} and cyclic AMP signal transduction systems has been examined in several electropermeabilized preparations. In the platelet, where an increase in cyclic AMP mediates the response to inhibitory agonists, e.g. prostaglandin I_2 (Haslam *et al.*, 1978), addition of micromolar concentrations of this cyclic nucleotide to the electropermeabilized preparation prevents enhancement of the Ca^{2+} sensitivity of 5-hydroxytryptamine secretion induced by thrombin and other effective agonists (Table 2*b*) in the presence or absence of GTP. Under these conditions there is little effect on the Ca^{2+}-induced response itself or on the enhancement of this response induced by OAG or TPA (Knight & Scrutton, 1984; Haslam & Davidson, 1985). These data indicate that, in accord with results obtained using intact platelets (Rittenhouse-Simmons, 1979), cyclic AMP acts on, or prior to, phospholipase C in the signal transduction pathway. The presence of micromolar cyclic AMP also inhibits Ca^{2+} mobilization induced by addition of inositol 1,4,5-trisphosphate to electropermeabilized platelets (Moos & Goldberg, 1985). Hence the inhibitory action of cyclic AMP depends on interaction with at least two sites in the Ca^{2+} signal transduction mechanism.

In the pancreatic acinar cell the secretory response induced by certain agonists, e.g. acetylcholine, is proposed to be mediated by an increase in cytosolic [Ca^{2+}] whereas that induced by others, e.g. vasoactive intestinal peptide, is suggested to occur by a separate pathway involving an increase in cyclic AMP (Gardner & Jensen, 1981). This concept is not however supported by studies using electropermeabilized acinar cells which demonstrate that secretion can be induced by an increase in Ca^{2+} to the micromolar range but not by addition of cyclic AMP in the presence of 0.1 μM-Ca^{2+} (Knight & Koh, 1984). Such data may suggest that the effect of cyclic AMP is indirect although other explanations, e.g. species differences or loss from the permeabilized cell of a component required for the action of cyclic AMP, cannot at present be excluded.

Uptake into intracellular organelles

Electropermeabilized cell preparations have been used to examine directly the uptake of various substances into intracellular organelles. For Ca^{2+} such studies have shown that uptake observed in the electropermeabilized preparation has properties similar to those which characterize the isolated intracellular organelle (see, for example, Menashi *et al.*, 1984; Moos & Goldberg, 1985). A more interesting application of the method concerns introduction of [^{14}C]sucrose into the cytosol as a probe for the autophagic response in hepatocytes (Gordon & Seglen, 1982). In this latter study the hepatocytes were electropermeabilized using the standard methodology (see above) and then held at 0 °C to maintain the permeabilized state during loading with [^{14}C]sucrose. Resealing is induced by incubation at 37 °C and then, following appropriate experimental manipulation, the plasma membrane is selectively destroyed by exposure of the cells suspended in iso-osmotic electrolyte-free sucrose to a single electric discharge (electrodisruption). The contents of the cytosol are released by this treatment and the intracellular organelles can then readily be separated from the remnants of the plasma membrane (Gordon & Seglen, 1982). Seglen and his co-workers have used this approach to show that [^{14}C]sucrose is taken up by both lysosomes and mitochondria, and to probe the factors which influence uptake. For example lysosomal sucrose uptake is enhanced by conditions which increase autophagy, e.g. amino acid starvation, and depressed by an inhibitor of autophagic sequestration (3-methyladenine) (Seglen & Gordon, 1984; Tolleshaug *et al.*, 1984). The method has been used to demonstrate that a decrease in autophagic protein degradation occurs as a consequence of sequential treatments with carcinogens (Schwarze & Seglen, 1985).

Conclusions

We have attempted in this article to outline the basis for, and the advantage of, the electropermeabilized cell preparation and to illustrate its uses by reference to some studies in which this technique has been used most extensively. The method is however very versatile and is likely to find many other applications in addition to analysis of cellular signal transduction systems, which is our particular area of interest. For example, it has been employed to demonstrate an ATP requirement for axonal transport in crab nerves (Adams *et al.*, 1982).

In addition readers should appreciate that electropermeabilization is based on the same fundamental principles as the elegant electrofusion procedure described by Pilwat *et al.* (1981).

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