

## PHORBOL ESTERS, PROTEIN PHOSPHORYLATION AND THE REGULATION OF NEURONAL ION CHANNELS

By L. K. KACZMAREK

*Departments of Pharmacology and Physiology, Yale University School of Medicine,  
New Haven, CT 06510, USA*

### SUMMARY

Protein kinase C is an enzyme whose activity is modulated by its lipid environment and which is fully activated by diacylglycerol in the presence of phosphatidyl serine and calcium ions. This kinase is highly enriched in the nervous systems of both vertebrates and invertebrates. The activity of protein kinase C can be stimulated in intact cells by certain synthetic diacylglycerols as well as by phorbol esters which substitute for endogenous diacylglycerol. The effects of such activators on the endogenous electrical properties of neurones, as well as on synaptic transmission, have recently been investigated in several vertebrate and invertebrate preparations of neurones. One example is that of the bag cell neurones of *Aplysia* which, in response to brief stimulation, generate a prolonged discharge during which the height of their action potentials is increased. Exposure of isolated bag cell neurones to activators of protein kinase C results in the enhancement of their action potentials through an increase in the amplitude of their voltage-dependent calcium current. This is caused by the unmasking of a previously inactive species of calcium channel in the plasma membrane.

### INTRODUCTION

One well characterized effect of synaptic stimulation of a neurone is to produce a transient depolarization, or hyperpolarization, which triggers or inhibits the occurrence of action potentials. A second, and more pervasive, way that synaptic or hormonal stimulation influences many neurones is to produce long-lasting changes in their electrical properties – changes which substantially outlast the duration of stimulation. Such changes, which frequently result from modifications in the properties of voltage-dependent ion channels in the plasma membrane of a neurone, are of cardinal importance to the functioning of the nervous system and allow it to generate and control long-lasting changes in animal behaviour.

A biochemical mechanism that produces prolonged changes in neuronal excitability is the post-translational modification of ion channels, or of proteins that regulate ion channel activity, by the activation of protein kinases. The nervous system is the richest source of many of the different classes of protein kinases. The activity of several types of protein kinase can be stimulated by second messenger substances which are formed as a result of synaptic stimulation. This article deals

Key words: protein kinase C, phorbol esters, calcium channels.

with the characteristics of one class of protein kinase, the calcium/diacylglycerol/phospholipid-dependent protein kinase, also termed protein kinase C, and its possible roles in the regulation of neuronal excitability. These topics will be illustrated using the example of the bag cell neurones of *Aplysia*, a group of neurones which are particularly suited to combined biochemical-electrophysiological studies and which undergo a series of long-lasting changes in their electrical properties in response to brief synaptic stimulation.

#### PROTEIN KINASE C

Protein kinase C was first described as an enzyme, present in soluble fractions prepared from rat and bovine brain, which could catalyse the phosphorylation of serine residues in histones (Takai, Kishimoto, Inoue & Nishizuka, 1977; Inoue, Kishimoto, Takai & Nishizuka, 1977). It was shown to be distinct from both the cyclic-AMP-dependent and the cyclic-GMP-dependent protein kinases. The activity of protein kinase C was subsequently shown to depend on its lipid environment and, in particular, to require phosphatidylserine and diacylglycerol, as well as calcium ions, for its full activation (see Fig. 1). Diacylglycerols are generally found in significant quantities in cellular membranes only when cells are exposed to stimuli which cause the hydrolysis of membrane phosphatidylinositides. The transient production of diacylglycerol in response to such stimuli is believed to be the major physiological pathway for the activation of protein kinase C (for reviews see Nishizuka, 1984*a,b*; Berridge, 1984).

In many different species the highest concentrations of protein kinase C have been found in the nervous system (Kuo *et al.* 1980; Minakuchi, Takai, Yu & Nishizuka, 1981; Wrenn, Katoh & Kuo, 1981; Girard, Mazzei, Wood & Kuo, 1985). The enzyme has been purified from rat brain and found to comprise a single polypeptide chain with an apparent  $M_r$  of approximately 82 000 (Kikkawa *et al.* 1982). Diacylglycerol has been shown to increase the apparent affinity of the enzyme for phospholipids and to decrease the concentration of calcium ions that is needed to activate the enzyme fully. Thus, in the presence of diacylglycerol and phosphatidylserine, the activity of the enzyme can be stimulated maximally at a calcium concentration of about  $0.1 \mu\text{mol l}^{-1}$ , which is within the range of basal calcium concentrations of many cells.

Protein kinase C can be cleaved into two fragments by an endogenous calcium-dependent protease in rat brain (Kajikawa, Kishimoto, Shiota & Nishizuka, 1983). The larger fragment generated by such proteolysis is catalytically active in the absence of phospholipids, diacylglycerol or calcium ions. Although the proteolytic activation of protein kinase C was known before its activation by lipids came to be appreciated, the physiological significance of proteolytic activation is not yet known. One attractive possibility is that such irreversible activation may be responsible for some prolonged neuronal responses to stimulation (Lovinger *et al.* 1985).

It has been reported that protein kinase C can also be activated in cell-free extracts, and in intact cells, by unsaturated long-chain fatty acids such as arachidonic acid,

linolenic acid, linoleic acid and oleic acid. This has led to the suggestion that the stimulus-induced production of these fatty acids within cells may constitute an alternative physiological pathway for the activation of this kinase (McPhail, Clayton & Snyderman, 1985; Murakami, Chan & Routtenberg, 1985).

Protein kinase C activity can be detected in both cytoplasmic and membrane fractions from vertebrate and invertebrate nervous tissue (Kikkawa *et al.* 1982; DeRiener, Greengard & Kaczmarek, 1985a). In intact cells the activation of this enzyme by phorbol esters (see below) results in its translocation from cytoplasm to the plasma membrane (Kraft & Anderson, 1983). Such translocation may also be promoted by an increase in the intracellular calcium ion concentration (Wolf *et al.*

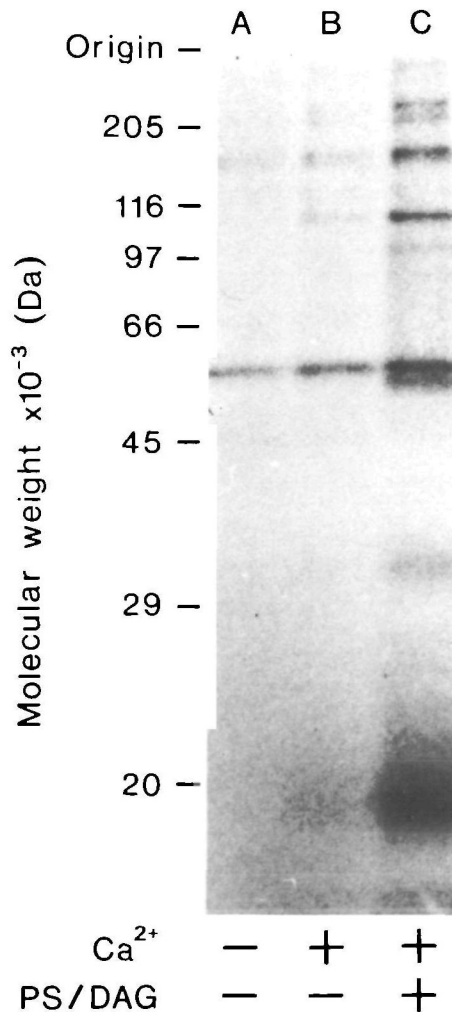


Fig. 1. An autoradiogram showing phosphorylation by protein kinase C in cytosolic fractions prepared from the bag cell neurones of *Aplysia*. Endogenous substrate proteins were phosphorylated in the presence of [<sup>32</sup>P]ATP in the absence (—) or presence (+) of calcium, phosphatidylserine (PS) and 1,3-diolein (DAG) (DeRiener, Greengard & Kaczmarek, 1985a).

1985; May, Sahyoun, Wolf & Cuatrecasas, 1985). It has recently been reported that electrical stimulation of an afferent input to the hippocampus results in significant translocation of protein kinase C into membrane fractions (Akers *et al.* 1985).

#### INHIBITORS OF PROTEIN KINASE C

A number of agents can inhibit the activity of protein kinase C. These include several polypeptide toxins (Kuo *et al.* 1983) as well as many antipsychotic agents (Mori *et al.* 1980; Schatzman, Wise & Kuo, 1981). Most of the agents which bind to calmodulin, and are thereby effective antagonists of calmodulin-dependent enzymes, also inhibit protein kinase C activity. For example, the 'calmodulin antagonists' trifluoperazine and W7 inhibit protein kinase C activity in the *Aplysia* nervous system at the same concentrations that inhibit calcium/calmodulin-dependent protein phosphorylation in these cells (DeRiemer *et al.* 1984, 1985a). The calmodulin antagonist, calmidazolium, is however a slightly more effective inhibitor of calmodulin-dependent protein phosphorylation than of protein kinase C (DeRiemer *et al.* 1985a). Because of the lack of specificity of most of the inhibitors of protein kinase C, pharmacological inhibition has not yet proved to be a useful tool for investigation of the role of protein kinase C in neuronal function.

Interestingly, protein kinase C can also be inhibited by calmodulin. Such inhibition has been demonstrated in cell-free extracts of rat brain (Wu, Walaas, Nairn & Greengard, 1982) and of *Aplysia* nervous system (DeRiemer *et al.* 1985a) and has also been demonstrated using purified protein kinase C and several of its substrate proteins (Albert *et al.* 1984b). These data have raised the possibility that calmodulin may be a physiological modulator of protein kinase C. A recent report has also suggested that there may exist other endogenous protein inhibitors of this enzyme (Chan, Murakami & Routtenberg, 1985).

#### ACTIVATORS OF PROTEIN KINASE C

As described above, under normal conditions protein kinase C is believed to be activated by physiological stimuli which result in the catabolism of phospholipids in plasma membranes. There also exist pharmacological techniques for the activation of protein kinase C which can stimulate this enzyme directly in intact cells or in broken cell preparations. These can activate protein kinase C without influencing other second messenger systems which may be normal components of the full physiological response. Such pharmacological agents, which include phorbol esters and synthetic diacylglycerols, have therefore been used to test the possible roles of this enzyme in a variety of physiological processes, including changes in neuronal excitability.

Phorbol esters, such as TPA (12-*O*-tetradecanoylphorbol-13-acetate), were first described as potent tumour promoters and inflammatory agents when applied to mouse skin. (For an account of the structure-activity relationships of phorbol esters see Hecker, 1971.) Within mouse skin different classes of phorbol esters have been

shown to exert different biochemical and cellular effects. In particular, the inflammatory and tumour-promoting effects can be distinguished by differing structure-activity relationships. This heterogeneity of biological response has been correlated with the existence of multiple classes of binding sites for phorbol esters in preparations of mouse skin (Dunn & Blumberg, 1983).

In contrast to mouse skin, equilibrium binding data from many other tissues, including particulate fractions and cytoplasmic fractions prepared from the nervous system, are consistent with a homogeneous class of high affinity binding sites for phorbol esters (Shoyab & Todaro, 1980; Dunphy, Delclos & Blumberg, 1980). In common with the activation of protein kinase C, the binding of phorbol esters requires the presence of phospholipids. The distribution of phorbol ester binding sites in different tissues (Shoyab, Warren & Todaro, 1981; Hergenhahn & Hecker, 1981) closely matches that of protein kinase C activity (Minakuchi *et al.* 1981; Wrenn *et al.* 1981), highest concentrations being found in the nervous system (Murphy *et al.* 1983). Furthermore the phorbol ester binding sites in brain tissue have been shown to co-purify with protein kinase C (Leach, James & Blumberg, 1983; Nidel, Kuhn & Vandebark, 1983). These data strongly suggest that protein kinase C is the major target of phorbol esters in many tissues and, in particular, in the nervous system (Blumberg *et al.* 1984). It should be noted, however, that the kinetics of binding of phorbol esters to brain membranes display two distinct phases (Dunphy, Kochenburger, Castagna & Blumberg, 1981). It is not known whether these phases represent binding to protein kinase C in different lipid environments, complex kinetics of binding to the enzyme, or whether they imply the existence of other binding proteins.

Active phorbol esters contain a diacylglycerol-like structure. These agents have been shown to bind to protein kinase C, substituting for diacylglycerol to produce a potent stimulation of enzyme activity (Castagna *et al.* 1982; Kikkawa *et al.* 1983). Because phorbol esters are lipophilic, readily entering membranes of intact cells, and because they are not rapidly catabolized, they have been widely used as activators of protein kinase C and their effects on neuronal excitability have recently begun to be investigated. In contrast to exogenous phorbol esters, the endogenously produced diacylglycerols, which generally contain arachidonate at the 2-position, are rapidly degraded in cells, cannot readily cross cellular membranes and cannot be used as exogenous activators of protein kinase C. A number of synthetic diacylglycerols, including 1-oleoyl-2-acetyl glycerol, have however been found to activate the enzyme when applied to intact cells (Kaibuchi *et al.* 1983; Lapetina, Reep, Ganong & Bell, 1985). Although these synthetic analogues may also be metabolized within cells and generally appear to be less potent activators than phorbol esters, they have been used in studies on intact non-neuronal and neuronal cells.

How specific are phorbol esters and synthetic diacylglycerols for the activation of protein kinase C in tissues such as the nervous system? Although this enzyme is, at present, the only known target for these agents, the concentrations that are used in physiological experiments are frequently greater than those needed to activate protein kinase C in cell-free extracts. It is likely, therefore, that these agents will turn

out to have other cellular effects. For example, it has been proposed that changes in diacylglycerol concentrations may directly influence the association of plasma membranes with components of the cytoskeleton (Burn, Rotman, Meyer & Burger, 1985). It is also possible that some of these agents can be metabolized to other biologically active substances. At present, however, they are the most powerful probes available for testing the involvement of protein kinase C in biological responses.

#### SUBSTRATES FOR PROTEIN KINASE C

In common with the cyclic-AMP-dependent protein kinase and the calcium/calmodulin-dependent protein kinases, protein kinase C phosphorylates substrate proteins at serine and threonine residues and undergoes autophosphorylation when activated (Kikkawa *et al.* 1982). The substrate specificity of this enzyme is, however, distinct from that of the other protein kinases and substrates have been found that are phosphorylated only by protein kinase C.

Proteins which influence neuronal excitability, and which have been shown to act as substrates for protein kinase C *in vitro*, include the nicotinic acetylcholine receptor (Huganir, Albert & Greengard, 1983), the voltage-dependent sodium channel (Costa & Catterall, 1984) and tyrosine hydroxylase (Albert *et al.* 1984a). It should be remarked, however, that the demonstration that a kinase can phosphorylate a given protein *in vitro* does not necessarily imply that the enzyme acts on the protein in intact cells. Other protein kinase C substrate proteins of specific interest to neurobiologists include a 87 kDa phosphoprotein in brain synaptosomes which does not appear to act as a substrate for any of the other known kinases (Wu *et al.* 1982) and a 47 kDa protein, named F1, which undergoes an increase in phosphorylation during long-term potentiation of synaptic responses in the hippocampus (Routtenberg, Lovinger & Steward, 1985). The latter protein is apparently identical to a previously described protein, termed B50, which may play a role in phosphoinositide metabolism (Oestreicher, Van Dongen, Zwiers & Gispen, 1983).

#### ELECTROPHYSIOLOGICAL EFFECTS OF PHORBOL ESTERS, SYNTHETIC DIACYLGLYCEROLS AND PROTEIN KINASE C

The cellular effects of agents that activate protein kinase C have been investigated in a wide variety of cell types. These effects include an elevation of intracellular pH (Hesketh *et al.* 1985) and the promotion of cell proliferation (Nishizuka, 1984a). In both non-neuronal and neuronal cells, these agents may evoke or potentiate secretion (Knight & Baker, 1983; Rink, Saez & Hallam, 1983; Kojima, Lippes, Kojima & Rasmussen, 1983; Nichols, Haycock, Wang & Greengard, 1985; Publicover, 1985; Zurgil & Zisapel, 1985). The following brief account of their effects, however, is confined to effects that have been observed on the electrical properties of neurones.

The effects of activators of protein kinase C on endogenous ionic currents in neurones are either to enhance or suppress excitability, depending on the type of neurone under investigation. Examples of neurones in which a net enhancement

of excitability is observed include hippocampal pyramidal cells, photoreceptors of *Hermisenda* and the bag cell neurones of *Aplysia* (see below).

Phorbol esters evoke a marked change in the pattern of firing of pyramidal neurones recorded in slices of hippocampus (Baraban, Gould, Peroutka & Snyder, 1985a; Baraban, Snyder & Alger, 1985b). A prominent effect at lower concentrations is the depression of a slow afterhyperpolarization that follows a burst of action potentials, and which may be generated by a calcium-activated potassium current. When sodium currents and potassium currents have been attenuated pharmacologically, an enhancement of the calcium action potentials by phorbol esters is observed. This may therefore represent an enhancement of calcium current. In addition to these actions on intrinsic membrane currents, phorbol esters influence several of the synaptic responses observed in hippocampal neurones and, in particular, produce a large increase in the amplitude of the fast excitatory postsynaptic potential (EPSP) evoked by orthodromic electrical stimulation (Nicoll, Madison, Malenka & Andrade, 1985). It has been reported that the application of an active phorbol ester *in vivo* can prolong the duration of the long-term potentiation of synaptic responses that follows repetitive stimulation of the perforant path (Lovinger *et al.* 1985).

In photoreceptors of the mollusc *Hermisenda*, the application of a phorbol ester, as well as intracellular injection of protein kinase C, have been reported to reduce the amplitudes of the calcium-activated potassium current and the transient inactivating potassium current (A-current) (Fisher, Auerbach & Farley, 1985; Farley & Auerbach, 1986). The same manipulations also increase the magnitude of the voltage-dependent calcium current. Similar changes in the amplitudes of these ionic currents can be evoked by application of serotonin to the photoreceptors.

A suppressive effect of activators of protein kinase C on a neuronal calcium current occurs in primary cultures of neurones from the dorsal root ganglion. In these cells, norepinephrine causes calcium action potentials to shorten in duration by attenuating the amplitude of the voltage-dependent calcium current. This effect is mimicked by 1-oleoyl-2-acetyl glycerol, as well as by phorbol esters (Rane & Dunlap, 1985; Werz & Macdonald, 1985). A suppression of calcium current, as well as changes in some other voltage-dependent currents which produce an inhibition of cell firing, has also been observed in neurones of the rat dorsal raphe when exposed to phorbol esters (Freedman & Aghajanian, 1985).

A number of effects of phorbol esters on transmission at peripheral synapses have been noted (Baraban *et al.* 1985b). One interesting observation, in view of the fact that the acetylcholine receptor acts as a substrate for protein kinase C *in vitro*, is that a phorbol ester and glyceryl dioleate reduce the sensitivity of cultured myotubes to acetylcholine (Eusebi, Molinaro & Zani, 1985).

#### THE BAG CELL NEURONES

The abdominal ganglion of mature *Aplysia* contains two large clusters of neurones, situated at the junctions of the pleuroabdominal connective nerves and the ganglion.

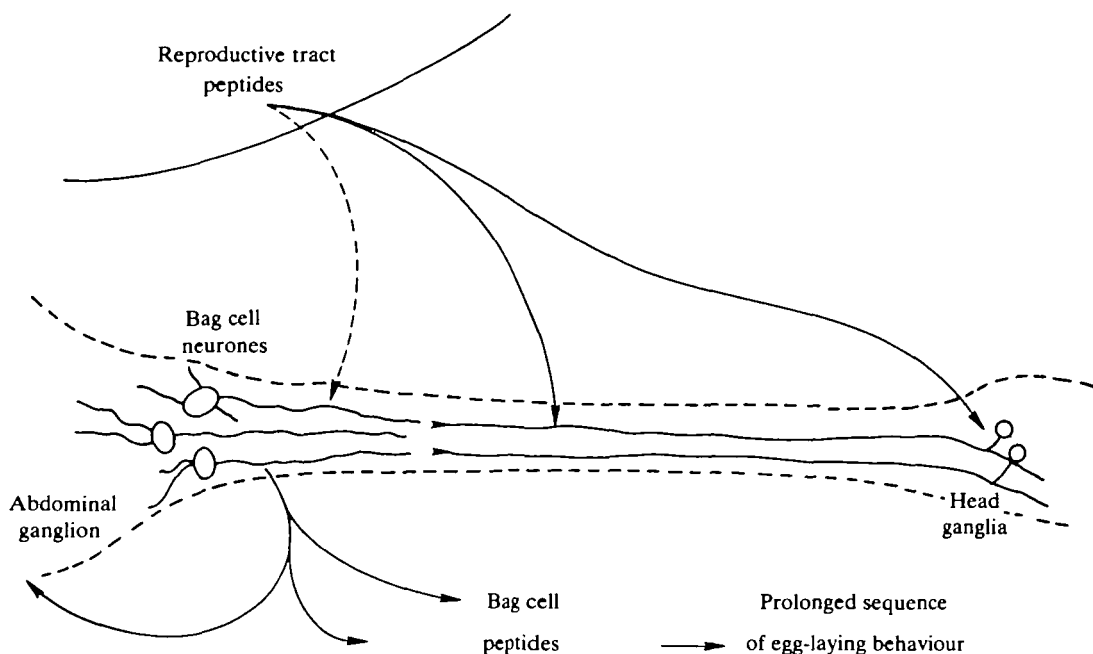


Fig. 2. Schematic drawing of the bag cell neuronal system.

The neurones in these clusters appear to be entirely homogeneous by several electrical and biochemical criteria. Moreover, they are coupled electrically by gap junctions on their processes, which ensures that, when stimulated, these neurones always discharge in synchrony (Kaczmarek, Finbow, Revel & Strumwasser, 1979; Blankenship & Haskins, 1979). These cells have been termed the bag cell neurones and they play a pivotal role in the generation of the prolonged sequence of behaviour that constitutes egg-laying behaviour in the animal.

In response to brief (approx. 15 s) electrical stimulation, or to peptides from the reproductive tract, the bag cell neurones generate a prolonged discharge of action potentials (Kupfermann & Kandel, 1970; Heller *et al.* 1980). This discharge continues after the stimulation has stopped and generally lasts for about 30 min. During this afterdischarge the action potentials of these neurones can be observed to increase in height and in width (Kaczmarek, Jennings & Strumwasser, 1982). This enhancement of action potentials promotes calcium entry into the cells. The discharge leads to the release of several neuroactive peptides which act on other neurones within the nervous system, as well as on peripheral targets to induce the sequence of egg laying behaviour (Fig. 2) (Arch, 1972; Dudek, Cobbs & Pinsker, 1979; Mayeri, Brownell, Branton & Simon, 1979; Schlesinger, Babirack & Blankenship, 1981; Rothman *et al.* 1983; Scheller *et al.* 1983).

The clusters of bag cell neurones can readily be dissected from the remainder of the abdominal ganglion as a homogeneous population of neurones for biochemical analysis. For the analysis of transmembrane ionic currents, individual neurones can also be isolated and maintained in primary cell culture, where they retain many of the



properties of neurones within intact clusters (Kaczmarek *et al.* 1979; Kaczmarek & Strumwasser, 1981).

The pharmacological properties of protein kinase C in the bag cell neurones have been characterized in cell-free extracts both by studying the phosphorylation of endogenous substrates for this enzyme (Fig. 1) and by measuring the incorporation of phosphate into the  $M_r \approx 87\,000$  protein from rat brain, which appears to be a specific substrate for this enzyme (DeRiemer *et al.* 1985a). Using these techniques the enzyme has been shown to be present in both membrane and cytosolic fractions from these neurones. Its activity can be stimulated by TPA at concentrations of  $10\text{--}100\text{ nmol l}^{-1}$  and is unaffected by inactive phorbols such as 4- $\alpha$ -phorbol.

Although, as in other types of cells, a number of substrates for protein kinase C can be detected in cell-free extracts of bag cell neurones, it is unlikely that all of these proteins act as physiological substrates for the enzyme in intact cells. An alternative approach to identifying substrates in intact cells is to label intact clusters of cells with radiolabelled inorganic phosphate that is incorporated into ATP and is then transferred by the kinase into phosphoproteins. In such 'pre-labelled' cells, the enzyme may be activated by TPA and those proteins whose phosphorylation state is enhanced can subsequently be identified after separation by gel electrophoresis. Fig. 3 shows a two-dimensional autoradiogram of [ $^{32}\text{P}$ ]phosphoproteins labelled in intact cell clusters. Fig. 4 shows enlargements of the region corresponding to molecular weights of  $\approx 50\,000\text{--}190\,000$  from control clusters and from clusters treated with  $100\text{ nmol l}^{-1}$  TPA. A dramatic increase in  $^{32}\text{P}$  incorporation into a protein of apparent  $M_r$  140 000, labelled X, can be detected. This protein cannot be detected in control autoradiograms but appears as a relatively intense spot in autoradiograms from TPA-treated cells. A smaller change in incorporation may also occur in some other proteins (e.g. those marked B in Fig. 4), but the vast majority of phosphoproteins labelled in this homogeneous population of neurones are unaffected by TPA.

Because, in intact cells, activation of protein kinase C may be associated with indirect alterations of other protein kinases (Gilmore & Martin, 1983; Haycock, Browning & Greengard, 1985) it is not yet known whether the  $M_r$  140 000 phosphoprotein is a direct substrate for protein kinase C. These data indicate however that, in contrast to results obtained using cell-free extracts, the number of phosphoproteins affected by activation of this enzyme in intact cells is likely to be rather small.

#### REGULATION OF CALCIUM CHANNELS IN BAG CELL NEURONES

At the onset of an afterdischarge in bag cell neurones, there is an increase in the height of action potentials. An increase in the height of action potentials evoked by depolarizing current pulses is also observed when an isolated bag cell neurone maintained in cell culture is exposed to TPA at concentrations which activate protein kinase C in these neurones ( $10\text{--}100\text{ nmol l}^{-1}$ ) (DeRiemer *et al.* 1985b). A similar effect is seen within minutes of exposure of the cells to 1,2 dioctanoyl glycerol (K. Bley & L. K. Kaczmarek, unpublished results). Inactive analogues, such as

4- $\alpha$ -phorbol, are without effect. The enhancement of action potential amplitude by TPA and the synthetic diacylglycerol can be mimicked by direct microinjection of purified protein kinase C into such isolated bag cell neurones (DeRiemer *et al.* 1985b). Both the activators of protein kinase C and the enzyme itself exert their effects without a significant change in the width of the evoked action potentials. The data are therefore consistent with the hypothesis that the phorbol ester exerts its effects through the activation of protein kinase C.

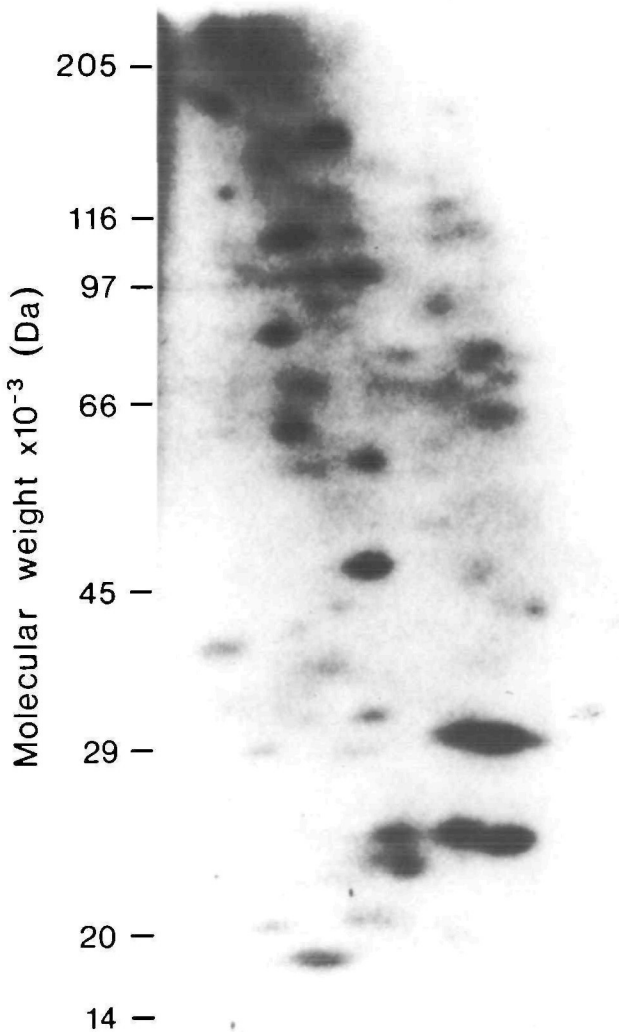


Fig. 3. A two-dimensional autoradiogram of [<sup>32</sup>P]labelled phosphoproteins in an isolated cluster of bag cell neurones (A. Knorr, E. Azhderian & L. K. Kaczmarek, unpublished results). Proteins were labelled by incubation of isolated clusters of cells in medium containing [<sup>32</sup>P]orthophosphate. Separation of proteins was carried out using nonequilibrium pH gradient electrophoresis (O'Farrell, Goodman & O'Farrell, 1977).

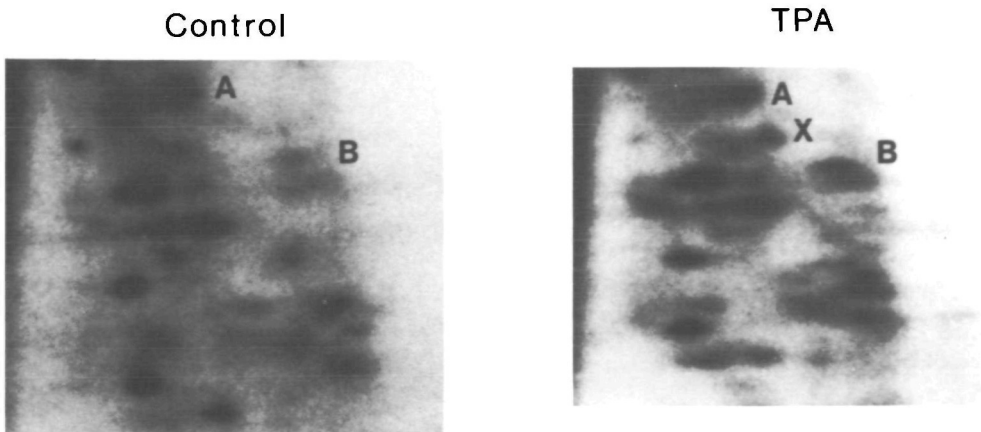


Fig. 4. Autoradiograms of [ $^{32}\text{P}$ ]labelled phosphoproteins in isolated clusters of bag cell neurones. Only that portion of the complete autoradiogram (Fig. 3), comprising molecular weights of approximately 60 000–150 000 is shown. The left autoradiogram shows labelled phosphoproteins in a control unstimulated cluster. Two 'landmark' proteins, A and B, are indicated. The right-hand autoradiogram shows labelled phosphoproteins in a bag cell cluster incubated with  $100 \text{ nmol l}^{-1}$  12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 30 min. A very marked increase in  $^{32}\text{P}$  incorporation into a protein of  $M_r \approx 140\,000$ , marked X, is observed.

A change in the shape of action potentials can, in theory, be brought about by changes in the properties of any one of the voltage-dependent ionic conductances which are activated on depolarization. The ionic basis of the enhanced action potentials has been examined in isolated bag cell neurones, using the whole-cell, patch-clamp technique to dialyse the cells internally with solutions of different ionic composition. When internal solutions which abolish all potassium conductances are used, the inward calcium current can be measured in these neurones without interference from voltage- or calcium-activated outward currents. Under these conditions TPA can be shown to induce a significant increase in the amplitude of the calcium current without any marked change in its voltage-dependence or kinetics (Fig. 5) (DeRiemer *et al.* 1985*b*). In contrast to the effects of activation of the cyclic-AMP-dependent second messenger system (see below), TPA has no effect on the voltage-dependent potassium currents in these neurones.

One interesting aspect of the modulation of the calcium current by TPA is its sensitivity to internal dialysis of the cells. Although TPA consistently increases the calcium current when cells are treated with TPA before internal dialysis is begun, and consistently enhances action potentials in cells that have been penetrated by microelectrodes, no effects of TPA are observed if TPA is administered after internal dialysis has begun. Internal dialysis may therefore disrupt some intracellular process which is required for an initial step in the increase of the calcium current by TPA but does not affect the current once it has become enhanced. One plausible hypothesis is that intracellular dialysis prevents the translocation of protein kinase C from cytoplasm to the plasma membrane.

An enhancement of calcium current in a neurone may, in theory, be brought about either by some alteration in the properties of pre-existing calcium channels or by the recruitment of additional channels. This question has been examined in the bag cell neurones using cell-attached patch pipettes to record the activity of single calcium channels (Strong, Fox, Tsien & Kaczmarek, 1986). Fig. 6 shows recordings made in

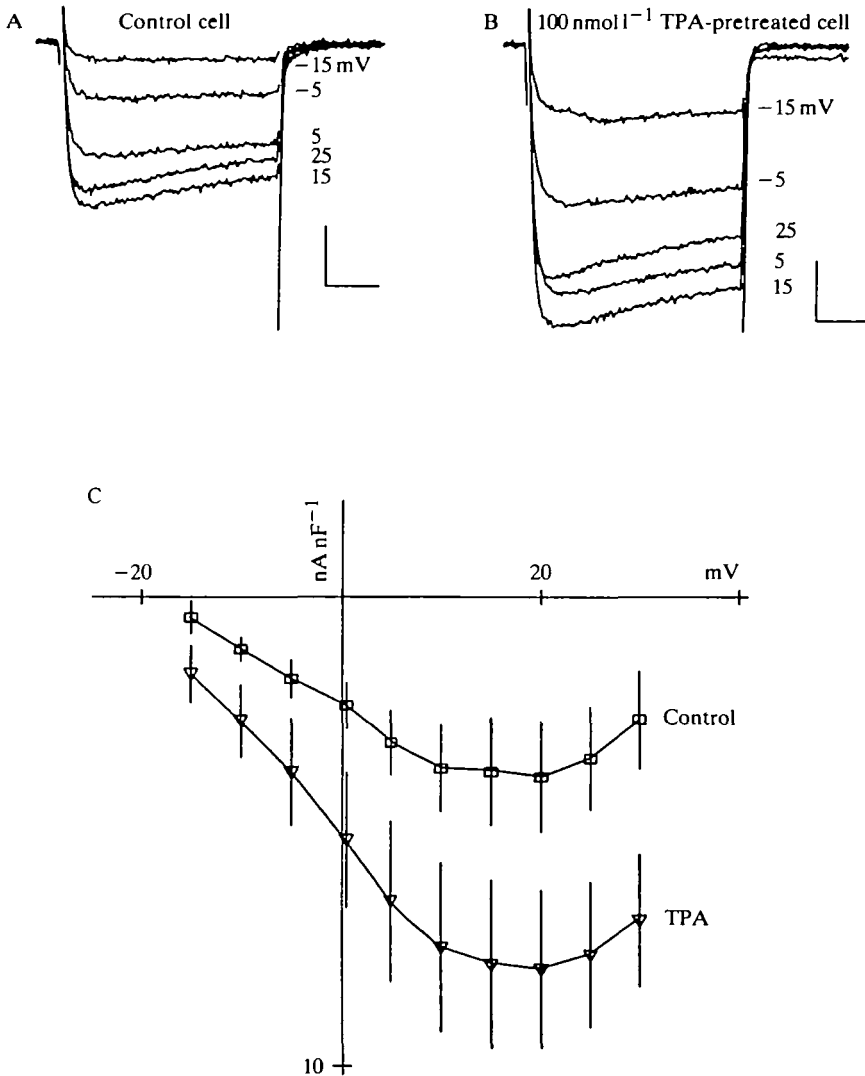


Fig. 5. Enhancement of calcium current by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in isolated, internally dialysed, bag cell neurones. (A) Calcium currents, which were evoked by stepping the membrane potential from  $-60$  mV to the indicated potentials in a control neurone exposed to the inactive 4- $\alpha$ -phorbol. (B) The calcium currents were recorded in a cell which had been exposed to  $100 \text{ nmol l}^{-1}$  TPA. (C) A graph of the mean calcium current density as a function of membrane voltage for seven control and seven TPA-treated neurones taken from the same cluster of bag cell neurones (DeRiemer *et al.* 1985b).

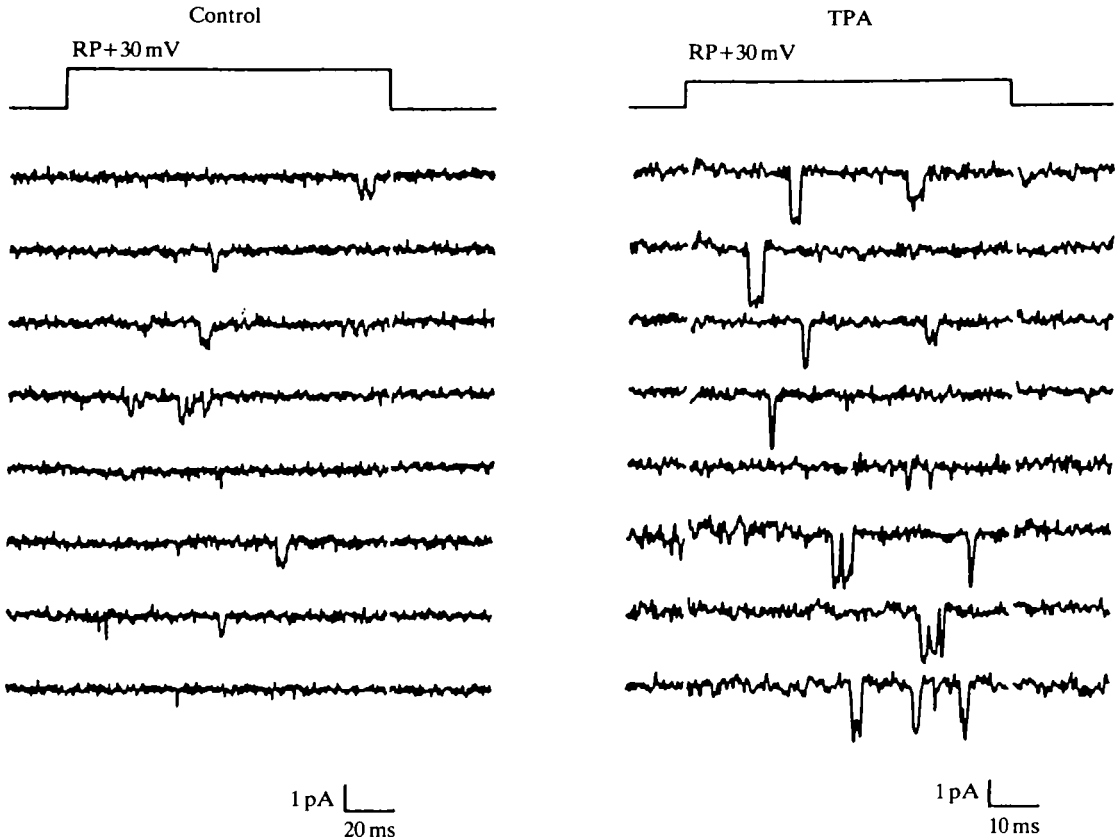


Fig. 6. Unmasking of a second species of calcium channels in bag cell neurones by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The left-hand traces show representative unitary currents recorded on depolarization of the membrane in a cell treated with the inactive phorbol, 4- $\alpha$ -phorbol. The right-hand traces are from a cell exposed to TPA. The traces show openings of a channel with a larger unitary conductance than that observed in the control recordings, in addition to openings which are similar to those seen in the control (Strong, Fox, Tsien & Kaczmarek, 1986). RP, resting potential.

control and TPA-treated cells using  $330 \text{ mmol l}^{-1} \text{ Ba}^{2+}$  within the pipettes to amplify the currents flowing through calcium channels. In control cells, relatively small unitary currents, with an open channel conductance of  $\approx 14 \text{ pS}$ , are observed. This species of calcium channel appears to be sparsely distributed over the plasma membrane of the cell, but patches containing multiple channels are occasionally recorded, suggesting that these channels form clusters in the membrane. In contrast, TPA-treated cells contain, in addition to this small-conductance channel, a second class of channel with a larger unitary conductance ( $\approx 23 \text{ pS}$ ). This TPA-induced channel is found in 80 % of patches recorded on cells exposed to  $100 \text{ nmol l}^{-1}$  TPA but is rare or absent in control cells. The voltage dependence of the control and TPA-induced channels is similar. It appears likely therefore that the appearance of the covert calcium channel in response to TPA fully accounts for the enhancement of calcium current seen in whole-cell recordings.

The actions of activators of protein kinase C on ionic currents in the bag cell neurones are distinct from those of any other second messenger pathway. The onset of afterdischarge is associated with an elevation of cyclic AMP levels (Kaczmarek, Jennings & Strumwasser, 1978). Activation of the cyclic-AMP-dependent protein kinase in these neurones results in a prolongation of action potential duration and repetitive firing through the modification of the properties of three distinct voltage-dependent potassium currents (Kaczmarek & Strumwasser, 1984; Strong, 1984; Strong & Kaczmarek, 1986). These changes occur with no apparent alteration of calcium current. Conversely, TPA has no effect on voltage-dependent potassium currents (DeRiemer *et al.* 1985b). It is possible, therefore, that the cyclic-AMP-dependent protein kinase and protein kinase C act synergistically to transform the electrical properties of the bag cell neurones at the onset of afterdischarge.

#### CONCLUSIONS

The high concentrations of protein kinase C in the nervous system relative to those in many other tissues suggest that this enzyme plays an important role in the regulation of neuronal activity. Studies using activators of this enzyme have recently begun and several effects on excitability have been described in different types of neurones. It remains to be determined whether these changes result directly or indirectly from alterations in the function of ion channels by protein kinase C and to what extent the activators of this enzyme also act on other, as yet unknown, metabolic pathways.

#### REFERENCES

- AKERS, R. F., COLLEY, P., LINDEN, D., LOVINGER, D. M., MURIKAMI, K. & ROUTTENBERG, A. (1985). Protein kinase C (PKC) activity is translocated to the membrane following hippocampal synaptic plasticity. *Fedn Proc. Fedn Am. Socs exp. Biol.* **44**, 1421.
- ALBERT, K. A., HELMER-MATYJEK, E., NAIRN, A. C., MULLER, T. H., HAYCOCK, J., GREENE, L. A., GOLDSTEIN, M. & GREENGARD, P. (1984a). Calcium/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7713–7717.
- ALBERT, K. A., WU, W. C.-S., NAIRN, A. C. & GREENGARD, P. (1984b). Inhibition by calmodulin of calcium phospholipid-dependent protein phosphorylation. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3622–3625.
- ARCH, S. (1972). Polypeptide secretion from the isolated parietovisceral ganglion of *Aplysia californica*. *J. gen. Physiol.* **59**, 47–59.
- BARABAN, J. M., GOULD, R. J., PEROUTKA, S. J. & SNYDER, S. H. (1985a). Phorbol ester effects on neurotransmission: interaction with neurotransmitters and calcium in smooth muscle. *Proc. natn. Acad. Sci. U.S.A.* **82**, 604–607.
- BARABAN, J. M., SNYDER, S. H. & ALGER, B. E. (1985b). Protein kinase C regulates ionic conductance in hippocampal pyramidal neurons: electrophysiological effects of phorbol esters. *Proc. natn. Acad. Sci. U.S.A.* **82**, 2538–2542.
- BERRIDGE, M. J. (1984). Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**, 345–360.
- BLANKENSHIP, J. E. & HASKINS, J. T. (1979). Electrotonic coupling among neuroendocrine cells in *Aplysia*. *J. Neurophysiol.* **42**, 347–355.

- BLUMBERG, P. M., JAKEN, S., KONIG, B., SHARKEY, W. A., LEACH, K. L., YENG, A. Y. & YEH, E. (1984). Mechanism of action of the phorbol ester tumor promoters: specific receptors for lipophilic ligands. *Biochem. Pharmac.* **33**, 933–940.
- BURN, P., ROTMAN, A., MEYER, R. K. & BURGER, M. M. (1985). Diacylglycerol in large  $\alpha$ -actinin/actin complexes and in the cytoskeleton of activated platelets. *Nature, Lond.* **314**, 469–472.
- CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. biol. Chem.* **257**, 7847–7851.
- CHAN, S. Y., MURAKAMI, K. & ROUTTENBERG, F. (1985). Purification of a kinase C substrate: brain phosphoprotein F1 and the discovery of an endogenous kinase C inhibitory factor. *Soc. Neurosci. Abstr.* **11**, 926.
- COSTA, M. R. & CATTERALL, W. A. (1984). Phosphorylation in the  $\alpha$ -subunit of the sodium channel by protein kinase C. *Cell. molec. Neurobiol.* **4**, 291–297.
- DERIEMER, S. A., GREENGARD, P. & KACZMAREK, L. K. (1985a). Calcium/phosphatidylserine/diacylglycerol-dependent protein phosphorylation in the *Aplysia* nervous system. *J. Neurosci.* **5**, 2672–2676.
- DERIEMER, S. A., KACZMAREK, L. K., LAI, Y., MCGUINNESS, T. L. & GREENGARD, P. (1984). Calcium/calmodulin-dependent protein phosphorylation in the nervous system of *Aplysia*. *J. Neurosci.* **4**, 1618–1625.
- DERIEMER, S. A., STRONG, J. A., ALBERT, K. A., GREENGARD, P. & KACZMAREK, L. K. (1985b). Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. *Nature, Lond.* **313**, 313–316.
- DUDEK, F. E., COBBS, J. S. & PINSKER, H. M. (1979). Bag cell electrical activity underlying spontaneous egg laying in freely behaving *Aplysia brasiliensis*. *J. Neurophysiol.* **42**, 804–817.
- DUNN, J. A. & BLUMBERG, P. M. (1983). Specific binding of [20- $^3$ H]12-deoxyphorbol 13-isobutyrate to phorbol ester receptor subclasses in mouse skin particulate preparations. *Cancer Res.* **3**, 4632–4637.
- DUNPHY, W. G., DELCLOS, K. B. & BLUMBERG, P. M. (1980). Characterization of specific binding of  $^3$ H-phorbol 12,13-dibutyrate and  $^3$ H-phorbol 12-myristate 13-acetate to mouse brain. *Cancer Res.* **40**, 3635–3641.
- DUNPHY, W. G., KOCHENBURGER, R. J., CASTAGNA, M. & BLUMBERG, P. M. (1981). Kinetics and subcellular localization of specific  $^3$ H-phorbol 12,13-dibutyrate binding by mouse brain. *Cancer Res.* **41**, 2640–2647.
- EUSEBI, F., MOLINARO, M. & ZANI, B. M. (1985). Agents that activate protein kinase C reduce acetylcholine sensitivity in cultured myotubes. *J. Cell Biol.* **100**, 1339–1342.
- FARLEY, J. & AUERBACH, S. (1986). Reductions of potassium currents and enhancement of calcium current in *Hermisenda* photoreceptors by phorbol ester and protein kinase C. *Nature, Lond.* (in press).
- FISHER, D., AUERBACH, S. & FARLEY, J. (1985). Protein kinase C reduces  $K^+$  currents and enhances a  $Ca^{2+}$  current in *Hermisenda* type B cells. *Soc. Neurosci. Abstr.* **11**, 788.
- FREEDMAN, J. E. & AGHAJANIAN, G. K. (1985). Phorbol esters and alpha 1-adrenoceptors: differing effects on outward currents in rat dorsal raphe neurons. *Soc. Neurosci. Abstr.* **11**, 1094.
- GILMORE, T. & MARTIN, G. S. (1983). Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. *Nature, Lond.* **306**, 487–490.
- GIRARD, P. R., MAZZEI, G. J., WOOD, J. G. & KUO, J. F. (1985). Polyclonal antibodies to phospholipid/ $Ca^{2+}$ -dependent protein kinase and immunocytochemical localization of the enzymes in rat brain. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3030–3034.
- HAYCOCK, J. W., BROWNING, M. D. & GREENGARD, P. (1985). Phorbol dibutyrate increased protein III phosphorylation in bovine adrenal chromaffin cells. *Soc. Neurosci. Abstr.* **11**, 846.
- HECKER, E. (1971). New phorbol esters and related carcinogens. In *Proceedings 10th International Congress*, vol. 5 (ed. R. L. Clark, R. W. Cumley, J. E. McCoy & M. M. Copeland), pp. 213–224. Chicago: Yearbook Medical Publishers.
- HELLER, E., KACZMAREK, L. K., HUNKAPILLER, M. W., HOOD, L. E. & STRUMWASSER, F. (1980). Purification and primary structure of two neuroactive peptides that cause bag cell afterdischarge and egg-laying in *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2328–2332.

- HERGENHAHN, M. & HECKER, E. (1981). Specific binding of the tumor promoter TPA in various mouse organs as measured by a 'cold acetone-filter assay'. *Carcinogenesis* **2**, 1277-1281.
- HESKETH, T. R., MOORE, J. P., MORRIS, J. D. H., TAYLOR, M. V., ROGERS, J., SMITH, G. A. & METCALFE, J. C. (1985). A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. *Nature, Lond.* **313**, 481-484.
- HUGANIR, R. L., ALBERT, K. A. & GREENGARD, P. (1983). Phosphorylation of the nicotinic acetylcholine receptor by Ca/phospholipid-dependent protein kinase, and comparison with its phosphorylation by cAMP-dependent protein kinase. *Soc. Neurosci. Abstr.* **9**, 578.
- INOUE, M., KISHIMOTO, A., TAKAI, Y. & NISHIZUKA, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J. biol. Chem.* **252**, 7610-7616.
- KACZMAREK, L. K., FINBOW, M., REVEL, J. P. & STRUMWASSER, F. (1979). The morphology and coupling of *Aplysia* bag cells within the abdominal ganglion and in cell culture. *J. Neurobiol.* **10**, 535-550.
- KACZMAREK, L. K., JENNINGS, K. & STRUMWASSER, F. (1978). Neurotransmitter modulation, phosphodiesterase inhibitor effects, and cAMP correlates of afterdischarge in peptidergic neurites. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5200-5204.
- KACZMAREK, L. K., JENNINGS, K. R. & STRUMWASSER, F. (1982). An early sodium and a late calcium phase in the afterdischarge of peptide secreting neurons of *Aplysia*. *Brain Res.* **238**, 105-115.
- KACZMAREK, L. K. & STRUMWASSER, F. (1981). The expression of long-lasting afterdischarge by isolated *Aplysia* bag cell neurons. *J. Neurosci.* **1**, 626-634.
- KACZMAREK, L. K. & STRUMWASSER, F. (1984). A voltage-clamp analysis of currents underlying cyclic-AMP induced membrane modulation in isolated peptidergic neurons of *Aplysia*. *J. Neurophysiol.* **52**, 340-349.
- KAIUCHI, K., TAKAI, Y., SAWAMURA, M., HOSHII, M., FUJIKURA, T. & NISHIZUKA, Y. (1983). Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J. biol. Chem.* **258**, 6701-6704.
- KAJIKAWA, N., KISHIMOTO, A., SHIOTA, M. & NISHIZUKA, Y. (1983).  $\text{Ca}^{2+}$ -dependent neutral protease and proteolytic activation of  $\text{Ca}^{2+}$ -activated phospholipid-dependent protein kinase. *Meth. Enzym.* **102**, 279-290.
- KIKKAWA, U., TAKAI, Y., MINAKUCHI, R., INOHARA, S. & NISHIZUKA, Y. (1982). Calcium-activated phospholipid-dependent protein kinase from rat brain: subcellular distribution, purification and properties. *J. biol. Chem.* **257**, 13 341-13 348.
- KIKKAWA, U., TAKAI, Y., TANAKA, Y., MIYAKE, R. & NISHIZUKA, Y. (1983). Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J. biol. Chem.* **258**, 11 442-11 445.
- KNIGHT, D. E. & BAKER, P. F. (1983). The phorbol ester TPA increases the affinity of exocytosis for calcium in 'leaky' adrenal medullary cells. *FEBS Letts* **160**, 98-100.
- KOJIMA, A., LIPPES, H., KOJIMA, K. & RASMUSSEN, H. (1983). Aldosterone secretion: effect of phorbol ester and A23187. *Biochem. biophys. Res. Commun.* **116**, 555-562.
- KRAFT, A. S. & ANDERSON, W. B. (1983). Phorbol esters increase the amount of  $\text{Ca}^{2+}$ -phospholipid-dependent protein kinase associated with plasma membrane. *Nature, Lond.* **301**, 621-623.
- KUO, J. F., ANDERSSON, R. G. G., WISE, B. C., MACKERLOVA, L., SALOMONSSON, I., KATOH, N., SHOJI, M. & WRENN, R. W. (1980). Calcium-dependent protein kinase: widespread occurrence in various tissues and phyla of the animal kingdom and comparison of the effects of phospholipid, calmodulin and trifluoperazine. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7039-7043.
- KUO, J. F., RAYNOR, R. L., MAZZEI, G. J., SCHATZMAN, R. C., TURNER, R. S. & KEM, W. R. (1983). Cobra polypeptide cytotoxin I and marine worm polypeptide cytotoxin a-IV are potent and selective inhibitors of phospholipid-sensitive  $\text{Ca}^{2+}$ -dependent protein kinase. *FEBS Letts* **153**, 183-186.
- KUPFERMANN, I. & KANDEL, E. R. (1970). Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of *Aplysia*. *J. Neurophysiol.* **33**, 865-876.
- LAPETINA, E. G., REEP, B., GANONG, B. R. & BELL, R. M. (1985). Exogenous sn-1,2-diacylglycerols containing saturated fatty acids function as bioregulators of protein kinase C in human platelets. *J. biol. Chem.* **260**, 1358-1361.



- LEACH, K. L., JAMES, M. L. & BLUMBERG, P. M. (1983). Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4208–4212.
- LOVINGER, D., CALLEY, P., LINDEN, D. J., MURAKAMI, K. & ROUTTENBERG, A. (1985). Phorbol ester, which induces protein kinase C (PKC) translocation to the membrane, prevents decay of long-term potentiation. *Soc. Neurosci. Abstr.* **11**, 927.
- MCPHAIL, L. C., CLAYTON, C. C. & SNYDERMAN, R. (1985). A potential second messenger role for unsaturated fatty acids: activation of  $\text{Ca}^{2+}$ -dependent protein kinase. *Science* **224**, 622–625.
- MAY, W. S., SAHYOUN, N., WOLF, M. & CUATRECASAS, P. (1985). Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. *Nature, Lond.* **317**, 549–551.
- MAYERI, E., BROWNELL, P., BRANTON, W. D. & SIMON, S. B. (1979). Multiple prolonged actions of neuroendocrine bag cells on neurons in *Aplysia*: effects on bursting pacemaker neurons. *J. Neurophysiol.* **42**, 1165–1184.
- MINAKUCHI, R., TAKAI, Y., YU, B. & NISHIZUKA, Y. (1981). Widespread occurrence of calcium-activated phospholipid dependent protein kinase in mammalian tissues. *J. Biochem.* **89**, 1651–1654.
- MORI, T., TAKAI, Y., MINAKUCHI, R., YU, B. & NISHIZUKA, Y. (1980). Inhibitory action of chlorpromazine, dibucaine and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. *J. biol. Chem.* **255**, 8378–8380.
- MURAKAMI, K., CHAN, S. Y. & ROUTTENBERG, A. (1985). Direct stimulation of protein kinase C (PKC): unsaturated fatty acids (oleate, arachidonate) are sufficient. *Soc. Neurosci. Abstr.* **11**, 927.
- MURPHY, K. M. M., GOULD, R. J., OSTER-GRANITE, M. L., GEARHART, J. D. & SNYDER, S. H. (1983). Phorbol ester receptors: autoradiographic identification in the developing rat. *Science* **222**, 1036–1038.
- NICHOLS, R. A., HAYCOCK, J. W., WANG, T. K. T. & GREENGARD, P. (1985). Phorbol ester enhances calcium-dependent neurotransmitter release from rat brain synaptosomes. *Soc. Neurosci. Abstr.* **11**, 846.
- NICOLL, R. A., MADISON, D. V., MALENKA, R. C. & ANDRADE, R. (1985). Actions of phorbol esters on synaptic transmission and membrane currents in hippocampal CA1 pyramidal neurons. *Soc. Neurosci. Abstr.* **11**, 151.
- NIEDEL, J. E., KUHN, L. J. & VANDENBARK, G. R. (1983). Phorbol diester receptor copurifies with protein kinase C. *Proc. natn. Acad. Sci. U.S.A.* **80**, 36–40.
- NISHIZUKA, Y. (1984a). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature, Lond.* **308**, 693–698.
- NISHIZUKA, Y. (1984b). Turnover of inositol phospholipids and signal transduction. *Science* **225**, 1365–1370.
- OESTREICHER, A. B., VAN DONGEN, C. J., ZWIERS, H. & GISPEN, W. H. (1983). Affinity purified anti B-50 protein antibody: interference with the function of the phosphoprotein B-50 in synaptic plasma membranes. *J. Neurochem.* **41**, 331–340.
- O'FARRELL, P. Z., GOODMAN, H. M. & O'FARRELL, P. H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133–1142.
- PUBLICOVER, S. J. (1985). Stimulation of spontaneous transmitter release by the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, an activator of protein kinase C. *Brain Res.* **333**, 185–187.
- RANE, S. G. & DUNLAP, K. (1985). The C kinase activator 1,2-oleoyl acetyl glycerol mimicks norepinephrine's effect on the voltage dependent calcium current of embryonic chick dorsal root ganglion neurons. *Soc. Neurosci. Abstr.* **11**, 748.
- RINK, T. J., SACHEZ, A. & HALLAM, T. J. (1983). Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature, Lond.* **305**, 317–319.
- ROTHMAN, B. S., MAYERI, E., BROWN, R. O., YUAN, P. & SHIVELY, J. (1983). Primary structure and neuronal effects of  $\alpha$ -bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5733–5757.
- ROUTTENBERG, A., LOVINGER, D. M. & STEWARD, O. (1985). Selective increase in phosphorylation of a 47-kDa protein (F1) directly related to long-term potentiation. *Behav. Neurol. Biol.* **43**, 3–11.

- SCHATZMAN, R. C., WISE, B. C. & KUO, J. F. (1981). Phospholipid-sensitive calcium-dependent protein kinase: inhibition by antipsychotic drugs. *Biochem. biophys. Res. Commun.* **98**, 669–676.
- SCHELLER, R. H., JACKSON, J. F., McALLISTER, L. B., ROTHMAN, B., MAYERI, E. & AXEL, R. (1983). A single gene encodes multiple neuropeptides mediating a stereotyped behavior. *Cell* **32**, 7–22.
- SCHLESINGER, D. H., BABIRACK, S. P. & BLANKENSHIP, J. E. (1981). In *A Symposium on Neurohypophyseal Peptide Hormones and Other Biologically Active Peptides* (ed. D. H. Schlesinger), pp. 137–150. New York: Elsevier North-Holland.
- SHOYAB, M. & TODARO, G. J. (1980). Specific high affinity cell membrane receptors for biologically active phorbol and ingenol esters. *Nature, Lond.* **288**, 451–455.
- SHOYAB, M., WARREN, T. C. & TODARO, G. J. (1981). Tissue and species distribution and developmental variation of specific receptors for biologically active phorbol and ingenol esters. *Carcinogenesis* **2**, 1273–1276.
- STRONG, J. (1984). Modulation of potassium current kinetics in bag cell neurons of *Aplysia* by an activator of adenylate cyclase. *J. Neurosci.* **4**, 2772–2783.
- STRONG, J. A., FOX, A. P., TSIEN, R. W. & KACZMAREK, L. K. (1986). Phorbol ester promotes a large conductance Ca channel in *Aplysia* bag cell neurons. *Biophys. J.* **49**, 430a.
- STRONG, J. A. & KACZMAREK, L. K. (1986). Multiple components of delayed potassium current in peptidergic neurons of *Aplysia*: modulation by an activator of adenylate cyclase. *J. Neurosci.* (in press).
- TAKAI, Y., KISHIMOTO, A., INOUE, M. & NISHIZUKA, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. *J. biol. Chem.* **252**, 7603–7609.
- WERZ, M. A. & MACDONALD, R. L. (1985). Phorbol esters: opposing effects on calcium-dependent action potentials of dorsal root ganglion cells. *Soc. Neurosci. Abstr.* **11**, 747.
- WRENN, R. W., KATOH, N. & KUO, J. F. (1981). Stimulation by phospholipid of calcium-dependent phosphorylation of endogenous proteins from mammalian tissues. *Biochim. biophys. Acta* **676**, 266–269.
- WOLF, M., LEVINE, H., MAY, W. S., CUATRECASAS, P. & SAHYOUN, N. (1985). A model for intracellular translocation of protein kinase C involving synergism between  $\text{Ca}^{2+}$  and phorbol esters. *Nature, Lond.* **317**, 546–549.
- WU, W. C. S., WALAAS, S. I., NAIRN, A. C. & GREENGARD, P. (1982). Calcium/phospholipid regulates phosphorylation of a  $M_r$  '87k' substrate protein in brain synaptosomes. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5249–5253.
- ZURGIL, N. & ZISAPEL, N. (1985). Phorbol ester and calcium act synergistically to enhance neurotransmitter release by brain neurons in culture. *FEBS Letts* **185**, 257–261.

Photographs taken at the  
Discussion Meeting held at Titisee  
in March 1986



Treherne McCleskey Moolenaar

Drummond

Adams

Gerschenfeld

Gerschenfeld

Neyton

Beltz

Speck Siegelbaum Kaczmarek

Evans

Levitan

Jessell

Petersen

Miller

Lemos

Marty



Levitan

Evans



Moolenaar

Adams



Jessel

Dodd



Gerschenfeld

Neyton

Marty



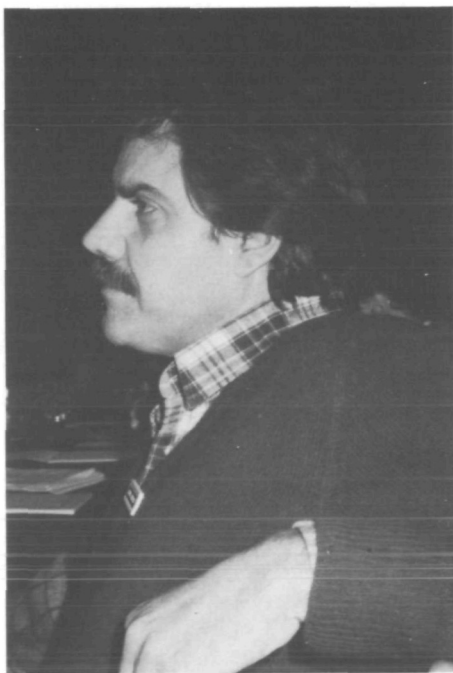
Kaczmarek



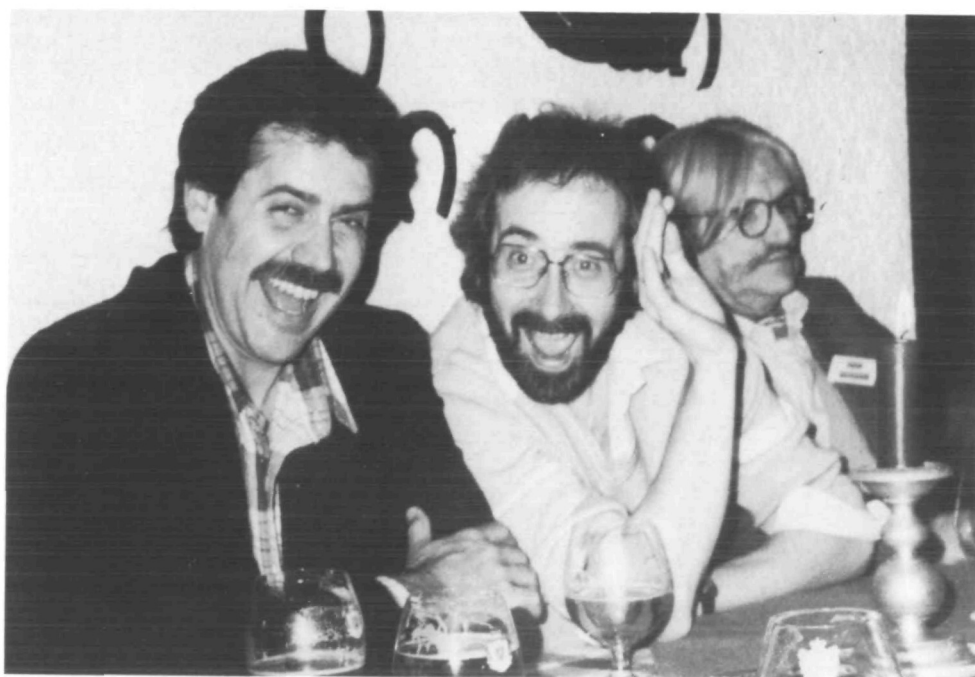
Dodd



Petersen



Lemos



Lemos

Drummond

Gerschenfeld



Speck

Nancy

McCleskey





Miller discoursing



Treherne conducting



Reuter lecturing



Beltz bowling



On thin ice