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Neural Function: Metabolism and Actions of Inositol Metabolites in Mammalian Brain [and Discussion]

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Neural function: metabolism and actions of inositol metabolites in mammalian brain

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In the nervous system, a variety of cell types respond to external stimuli through the inositol lipid signalling pathways. The stimulus-coupled sequence of intracellular events has been investigated in a homogeneous model system, the cloned mammalian neural cell line NG115-401L. The neural peptide bradykinin stimulates a rapid production of identified inositol phosphate isomers and an intracellular Ca^{2+} discharge followed by a persistent plasma membrane influx. The temporal sequence suggests that Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 or both may coordinate these events in a neuronal cell, as has been suggested in other cell types. Thapsigargin, an irritant and tumour-promoting plant product, produces calcium transients in the absence of inositol phosphate production, and may provide a new tool for investigating the interactions between inositol phosphates and changes in cellular calcium homeostasis. In the 401L line, high levels of radiolabelled InsP_5 and InsP_6 have been detected, which has led to the evaluation of their possible occurrence and actions in normal brain. Both InsP_5 and InsP_6 are produced from a radiolabelled *myo*-inositol precursor in intact mature brain in a region-specific manner. This suggests that both inositol polyphosphates may be end products of regionally regulated biosynthetic pathways. When microinjected into a nucleus of the brainstem, or iontophoretically applied to the dorsal horn of the spinal cord, both InsP_5 and InsP_6, but not Ins(1,3,4,5)P_4 isomers, appear to be potent neural stimulants. These results suggest that the inositol lipid signalling pathways may generate both intracellular and extracellular signals in brain.

Introduction

Although the inositol metabolites and their associated proteins are highly enriched in neural tissues, it is only recently that much progress has been made in describing their functional significance. Indeed, the past five years have led to the recognition of the inositol lipid signalling pathways as one of the major topics in modern neuroscience. Accordingly, aspects of the operation of these pathways in the nervous system have been reviewed elsewhere (Nahorski *et al.* 1986; Downes 1986; Fisher & Agranoff 1987; Berridge 1987; Kikkawa *et al.*, this symposium). We will focus on three specific issues: the relation of identified inositol phosphates to intracellular calcium homeostasis; the description of pharmacological reagents for the intervention at discrete steps in the signalling sequence; and the possibility that the inositol polyphosphates, InsP_5 and InsP_6, may have special roles in the nervous system.

The nervous system is composed of an extraordinary diversity of cells classified into two

major categories: neurons and glia. The inositol lipid signalling pathways are expected to be expressed universally in these cell populations. However, the possibility of region-specific or cell-type specific specialization of these pathways poses a major analytical challenge in a tissue as complex and varied as mammalian brain. There are three levels of complexity that must be considered. First, the heterogeneity of the major cell populations of the brain creates an enormous phenotypic range of anatomical and physiological properties. Second, cell–cell interactions are extensive and include, but are not limited to, synaptic junctions between neurons. Third, spatial and temporal aspects of neuronal responsiveness include significant complications such as elaborate patterns of process branching; distinct cellular domains such as the soma, axon, dendrite, and presynaptic ending; and exceedingly rapid responses to stimulation. These factors conspire to make the investigation of the sequence of events after activation of inositol-lipid-coupled receptors virtually impossible in intact brain or conventional preparations. The use of primary cultures of astrocytes or neurons reduces, but does not eliminate, these complications. Thus, we and others have adopted the use of simplified models, clonal cell lines, which maintain a variety of differentiated neural characteristics, and can be examined in either their actively dividing or post-mitotic state. Table 1 indicates a representative range of neural cell lines that have been used to examine inositol lipid responses.

TABLE 1. REPRESENTATIVE NEURAL CELL LINES EXPRESSING INOSITOL LIPID-LINKED RECEPTORS

cell line	origin	receptors	references
NG108-15	mouse neuroblastoma × rat glioma hybrid	bradykinin	Yano *et al.* (1984), Higashida & Brown (1986*a*)
NG115-401L	mouse neuroblastoma × rat glioma hybrid	bradykinin	Hatanaka & Amano (1981), Jackson *et al.* (1987*a*)
NCB-20	Chinese hamster neuron × mouse neuroblastoma hybrid	bradykinin	Francel & Dawson (1986)
PC12	rat pheochromocytoma	angiotensin II	Van Calker *et al.* (1987)
		bradykinin	Van Calker *et al.* (1987)
		muscarinic	Pozzan *et al.* (1986) Van Calker *et al.* (1987)
NIE-115	mouse neuroblastoma	bradykinin	Higashida & Brown (1987) Tertoolen *et al.* (1987)
		H1-histamine	Cohen *et al.* (1983)
		muscarinic	Cohen *et al.* (1983)
		neurotensin	Amar *et al.* (1987)
C6	rat glioma	serotonin	Ananth *et al.* (1987)
1321N1	human astrocytoma	H1-histamine	Nakahata & Harden (1987)
		muscarinic	Nakahata & Harden (1987)

We have focused on the detailed analysis of the NG115-401L cell clone, which has many properties in common with the primary sensory neuron (Hanley 1987). In particular, the NG115-401L line is sensitive to the inflammatory mediator bradykinin, a recognized physiological stimulant of the peripheral ends of nociceptive sensory neurons. After bradykinin application, intracellular recording from 401L cells shows a biphasic membrane potential response (figure 1), which is likely to share similar ionic mechanisms to the biphasic bradykinin responses observed in the related neuronal cells NG108-15 (Higashida & Brown 1986*a*) and N1E-115 (Higashida & Brown 1987; Tertoolen *et al.* 1987). By investigating a genetically stable and homogeneous population, we are able to use a combination of single-cell and

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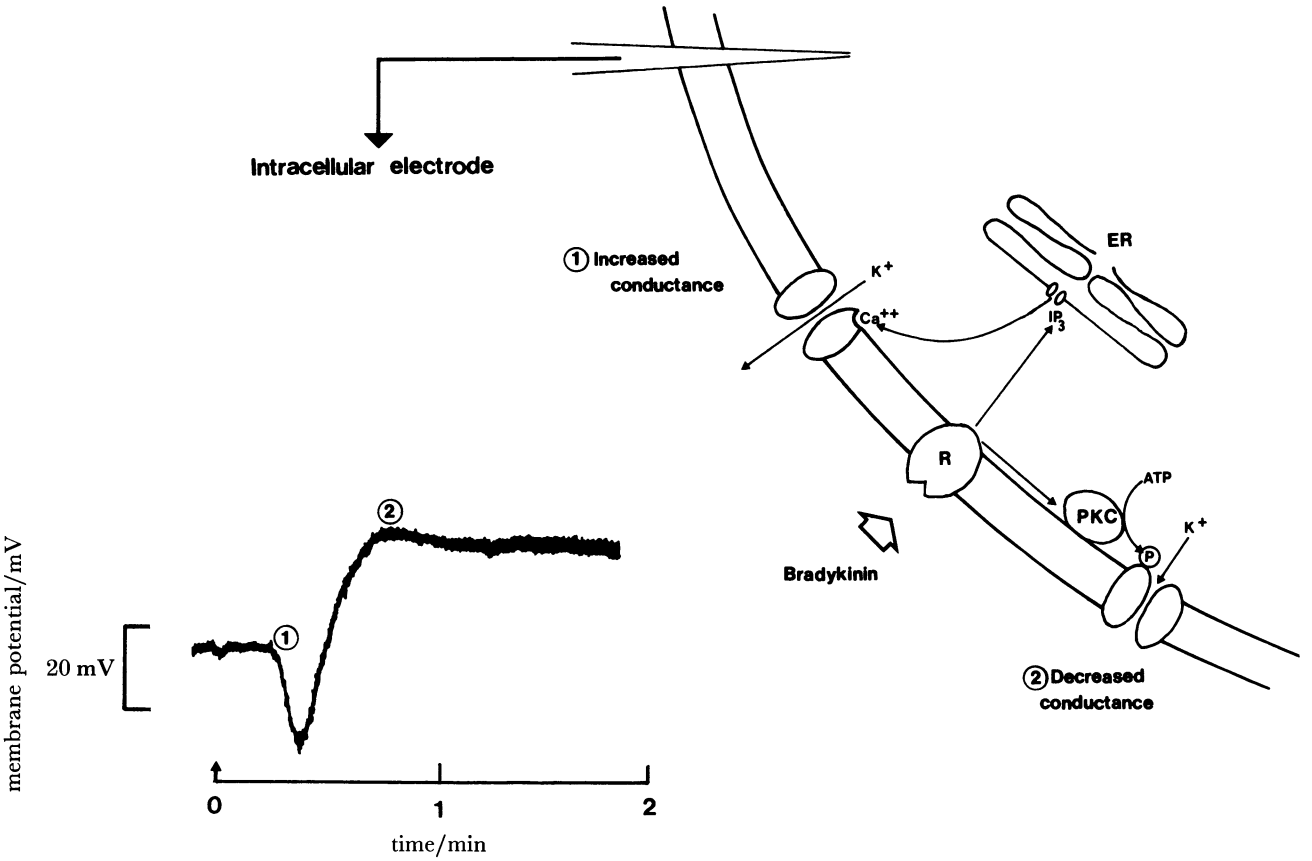

FIGURE 1. Summary of proposed ionic events after bradykinin stimulation of NG108-15 cells (Higashida & Brown 1986*a*). Bradykinin interaction with its receptor (R) is suggested to generate Ins(1,4,5)P_3, which liberates calcium from the endoplasmic reticulum (ER), and to activate protein kinase C (PKC). The electrogenic consequences are the activation of a calcium-sensitive potassium conductance to generate an initial hyperpolarization (①), followed by a PKC-mediated phosphorylation of a second potassium channel to generate a subsequent long-lived depolarization(②). Intracellular recording from differentiated NG115-401L cells shows a very similar biphasic response to bath-applied bradykinin (inset) which is likely therefore to have the same ionic basis.

population measurement techniques to identify the biochemical events that may underly the electrogenic and other responses to bradykinin. Not all of the propositions derived from the study of the 401L will be applicable to neurons as a class, nor necessarily to sensory neurons. The great strength of the clonal cell line approach is that it can assist the concerted genetic, biochemical and physiological investigation of the cellular mechanisms of inositol lipid pathways in what is essentially a permanent population derived from a single neuron.

STIMULATED INOSITOL PHOSPHATE PRODUCTION AND CALCIUM HOMEOSTASIS

It is now generally recognized that a variety of neural receptors elicit stimulated breakdown of inositol lipids in nerve cells (Downes 1986; Berridge 1987) and the pharmacological characteristics of the responses have been examined in some detail (Nahorski *et al.* 1986; Fisher & Agranoff 1987). However, the majority of the investigations use complex multicellular preparations, such as tissue slices in consort with the highly sensitive, but mechanistically

uninformative, lithium-amplification assay of the production of radiolabelled inositol phosphates (Berridge *et al.* 1982). Thus it remains a significant concern as to the nature of the informational events generated in neurons after stimulation of inositol lipid breakdown (Hawthorne 1986). For example, does Ins(1,4,5)P_3 couple surface stimulation to intracellular discharge, or is this type of calcium regulation inoperative in some or all neuronal populations, as has been suggested for cardiac myocytes (Movsesian *et al.* 1985)? To approach this issue, we developed methods for analysis of basal and stimulated production of radiolabelled inositol metabolites which could be applied to monolayer populations under physiological conditions used for other types of measurements (Jackson *et al.* 1987*a*). At the outset, the conditions for maximal cellular loading of [^{3}H]inositol were defined experimentally, and it was found that there was a strict dependence on provision of extracellular inositol for cell survival and proliferation (figure 2). Indeed, it has been recognized for some time that a variety of neural and neuroendocrine cell lines have an absolute dependence on medium inositol in culture (Jackson & Shin 1982). It is possible that this difference arises because of the presence or absence of the L-*myo*-inositol-1-phosphate synthase (Hasegawa & Eisenberg 1981), which is known to be absent from brain cells (Wong *et al.* 1987). The deleterious effects of inositol deprivation can be reversed by supplementation with as little as 10 μM inositol, although it is unclear what relation this has to the ambient inositol concentration in the brain. It will be an interesting topic for the future whether any form of developmentally triggered, or toxin-induced, neuronal death involves inositol starvation.

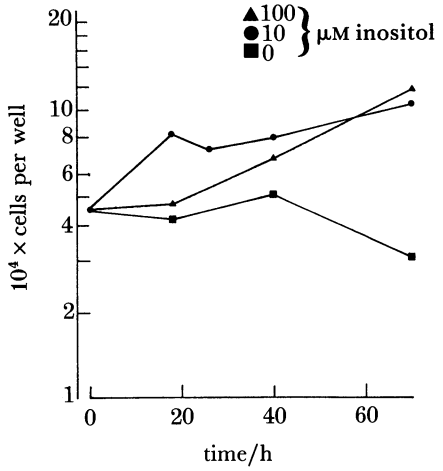

FIGURE 2. Dependence of NG115-401L cells on extracellular inositol for normal growth. Cells were incubated in inositol-free tissue culture medium supplemented with dialysed foetal calf serum, which in control experiments could sustain normal proliferation, and the indicated concentrations of added inositol. Cells were counted after trypsinization at the indicated time points.

Upon stimulation with a maximal dose of bradykinin, there is rapid generation of inositol phosphates (Jackson *et al.* 1987*a*), as has been seen in a number of cell types. The time-courses of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production (figure 3) show that the former appears to be produced in a biphasic fashion, with an early peak that may lie below 2 s, and a later peak between 7 and 20 s. By using fura-2/AM as a trapped fluorescent indicator of calcium concentration, the variations in cytoplasmic calcium after bradykinin activation were monitored by established methods (Grynkiewicz *et al.* 1985). The results are superimposed on

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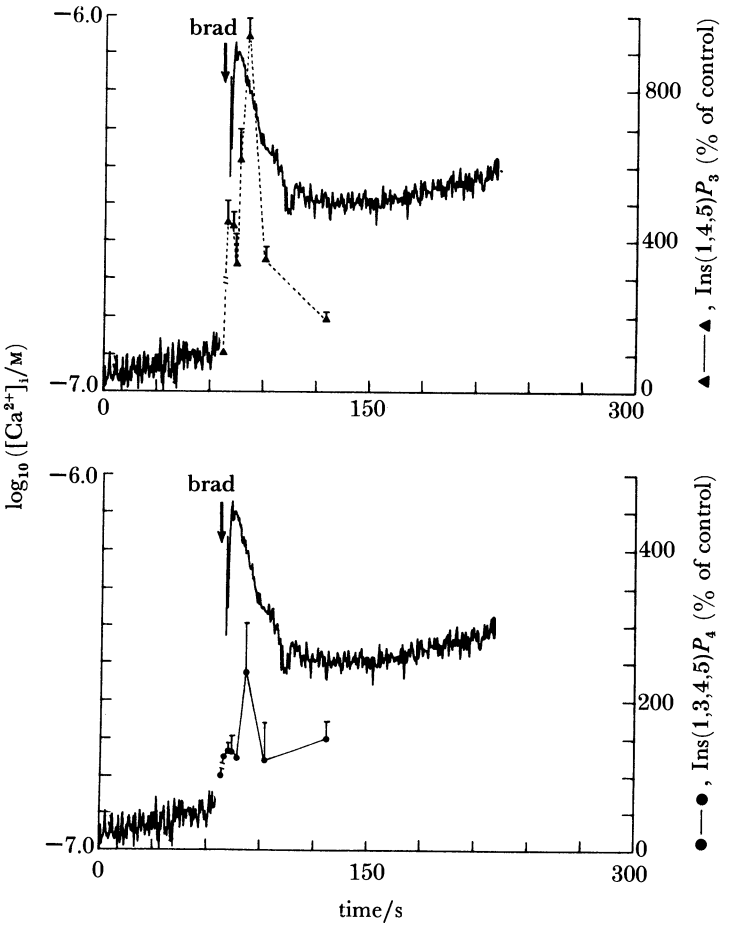

FIGURE 3. Comparison of the time-courses of Ins(1,4,5)P_3 production (upper trace) and Ins(1,3,4,5)P_4 (lower trace) after bradykinin (brad) stimulation of NG115-401L cells on coverslips to the time-course of cytosolic free calcium changes. Inositol phosphates are expressed as percentage of basal values, and the cytosolic calcium concentrations were determined by using calibrated Fura-2/AM fluorescence. The results are redrawn from Jackson *et al.* (1987*a*), wherein experimental details can be found.

the Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 time-courses in figure 3. The sequence of events appears to be: (*a*) the first peak of Ins(1,4,5)P_3; (*b*) a peak of intracellular calcium at 1–2 s; (*c*) a second peak of Ins(1,4,5)P_3 paralleled by a peak of Ins(1,3,4,5)P_4 on the declining phase of the calcium transient; and (*d*) sustained elevation of cytoplasmic calcium for several minutes after stimulation. By removal of extracellular calcium, it can be shown that the initial transient arises from an intracellular store, presumably endoplasmic reticulum, but that the long-lived elevation of cytoplasmic calcium is due to a sustained plasma membrane flux. This pattern of events is very similar to that observed in non-neuronal cell populations (Reynolds & Dubyak 1986; Pozzan *et al.* 1986), which argues that neurons share the fundamental mechanisms of calcium regulation with other cell populations. However, it is important to emphasize that these studies are done on actively mitotic 'undifferentiated' cells, which might be more properly regarded as a model for dividing neuroblasts, rather than mature neurons. Moreover, these results are correlative, so they do not argue that Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 necessarily act to coordinate the changes in cytoplasmic calcium levels. However, the ability of

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microinjected Ins(1,4,5)P_3 to elicit calcium discharge has been shown in the NG108-15 (Higashida & Brown 1986*b*) and N1E-115 (Tertoolen *et al.* 1987) neural lines, and more recently in a normal non-dividing neural population: the bag cells of *Aplysia* (Fink *et al.* 1988). On this basis, the first rise in Ins(1,4,5)P_3 might be suggested to be the stimulus inducing the calcium discharge. Indeed the production of calcium transients shows a dose–response curve to bradykinin (figure 4) that is very similar to that described for inositol phosphate production in the 401L line (Jackson *et al.* 1987*b*) and other neuronal lines (Yano *et al.* 1984; Francel & Dawson 1986). If Ins(1,4,5)P_3 produces the transient, what produces the sustained calcium influx from the cell exterior?

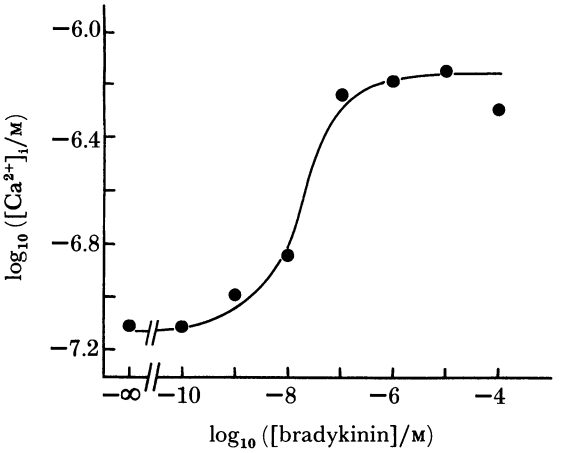

FIGURE 4. Concentration dependence of bradykinin-stimulated intracellular calcium transients. Results are from Fura-2-loaded NG115-401L cells on coverslips in a medium containing no calcium (1 mM EGTA added), by using the calibrated peak height of the rapid transient. Experimental details are described in Jackson *et al.* (1987*a*).

The product of the Ins(1,4,5)P_3-kinase, Ins(1,3,4,5)P_4, has been suggested to be a regulatory messenger in eliciting this flux (Irvine *et al.*, this symposium). It is interesting that the time course of its appearance appears consistent with this role in the 401L line. Moreover, the rise in cytoplasmic calcium immediately precedes the second Ins(1,4,5)P_3 peak and the Ins(1,3,4,5)P_4 peak. Thus it is also plausible that calcium might act to coordinate the activity of Ins(1,4,5)P_3 kinase, as has been suggested in other cells (Biden & Wollheim 1986; Zilberman *et al.* 1987), so that the combination of substrate availability and calcium regulation might create a strict temporal sequence of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 conversion, subject to calcium discharge. The alternative proposition is that calcium itself, either its elevation in the cytoplasm or its depletion from its store, might provide the stimulus for inducing the sustained calcium influx (Putney 1986).

Several factors regulate basal and stimulated cytosolic free-calcium levels: uptake into and discharge from endoplasmic reticulum stores; sequestration by mitochondria and possibly other organelles; immobilization by calcium-binding sites; and influx or efflux across the plasma membrane (McBurney & Neering 1987). Although the 401L results suggest that Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 or both may act on at least two of these regulatory processes, it should be considered whether inositol phosphates or other inositol lipid-derived mediators may participate in the other processes regulating cell calcium. This may prove a fruitful area

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for further consideration of the inositol polyphosphates InsP_5 and InsP_6, which are widely distributed among different cell types, and may therefore play housekeeping roles, in addition to the special possibilities discussed below.

Before extending these results to normal neurons, much more must be learned about stimulated inositol phosphate production and calcium homeostasis in the fully differentiated, post-mitotic 401L cell line. It may be that the Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 pathways operate during the proliferative phase of neuronal development, and the adult neuron switches to a greater reliance on extracellular calcium. Indeed, it need not be the case that all neurons must have the same calcium response and homeostasis mechanisms, so that the final resolution of how important these second messenger components may be *in vivo* may have several different answers for different regions of the central and peripheral nervous systems.

Another important parameter of normal nerve-cell function that is sacrificed *in vitro* is the functional geometry of the cell. These spatial factors may be of immense importance in the body. For example, the normal counterpart to the 401L cell, the primary afferent neuron, is bipolar, with processes that project both to peripheral tissues and into the spinal cord. These processes may extend for considerable distances, raising the question of whether all parts of the neuron, particularly the peripheral ends normally activated by bradykinin, have an intracellular calcium store available to them. Normal neuronal populations will therefore have to be analysed by single-cell imaging techniques (Williams *et al.* 1985; Fink *et al.* 1988), which are capable of resolving intracellular gradients and structural domains in calcium control.

Pharmacological tools for investigating calcium homeostasis

Several reagents have been suggested to interact with discrete steps in the inositol lipid signalling sequence. These are summarized in table 2. What is notably lacking is a reagent for the selective production of hormone-independent calcium transients which bypasses the early events. Such a reagent could distinguish between calcium-dependent or -independent modes of calcium influx induction. With this in mind, we were attracted to a detailed analysis of thapsigargin, the sesquiterpene lactone irritant shown in figure 5. Previous work has shown that thapsigargin (Tg) was able to induce a dose-dependent activation of several types of immune cell (Ali *et al.* 1985; Jacobsen *et al.* 1987) and that this activation appeared to be due to a discharge of intracellular calcium coupled with a transmembrane calcium flux (Thastrup *et al.* 1987). We examined this reagent for its possible application to stimulating calcium transients without a concomitant production of inositol phosphates. Figure 6 compares the results of Tg stimulation of platelets with the 401L cells. What is immediately apparent is that the responses in platelets are long-lived, but that the responses in 401L cells return to the pre-stimulation basal level, in spite of the presence of extracellular calcium. Moreover, the Tg transient can be produced in the absence of extracellular calcium, and can abolish a subsequent bradykinin response, suggesting that the calcium compartment whose discharge is activated by Tg includes the hormone-sensitive pool. However, as shown in table 3, there is no production of inositol phosphates by Tg. These results support the following interpretation: that hormone-dependent production of a signal (Ins(1,3,4,5)P_4?) is required for the sustained calcium flux in the 401L cell, and that calcium discharge alone is not sufficient. Moreover, this response pattern is different from platelets, suggesting that here, either calcium discharge may in itself be sufficient for eliciting a sustained calcium influx or that the mechanisms of calcium

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TABLE 2. SELECTED PHARMACOLOGICAL REAGENTS PROPOSED TO INTERACT ON EARLY EVENTS IN STIMULATED INOSITOL LIPID METABOLISM AND CALCIUM REGULATION

agent	effects	proposed sites of action	comments	references
maitotoxin	stimulates inositol phosphate production and calcium transients	phospholipase C	may activate calcium channels, causing indirect inositol phosphate production	Berta *et al.* (1986), Gusovsky *et al.* (1987)
monoalide	blocks phospholipases	phospholipases A2,C	may have multiple other sites of action	Bennett *et al.* (1987)
neomycin	blocks phosphatidyl-inositol-4,5-bisphos-phate breakdown	binding to phosphatidyl-inositol-4,5,-bisphosphate	may block ion channels	Siess & Lapetina (1986), Polascik *et al.* (1987)
ryanodine	prevents myocardial calcium-dependent intracellular calcium discharge	sarcoplasmic reticulum calcium channel	may be specific to muscle cells	Pessah *et al.* (1986)
TMB-8	blocks agonist-induced intracellular calcium discharge	endoplasmic reticulum calcium channel or pump	may be specific to limited variety of cell types	Vicentini & Villereal (1984)
vanadate	insulin mimetic; mitogenic; prolongs calcium transients	calcium pumps; protein phosphotyrosine phosphatases	may be toxic; slow entry into cells	Proffitt & Case (1984), Gill & Chueh (1985) Bosch *et al.* (1987), Thastrup *et al.* (1987)

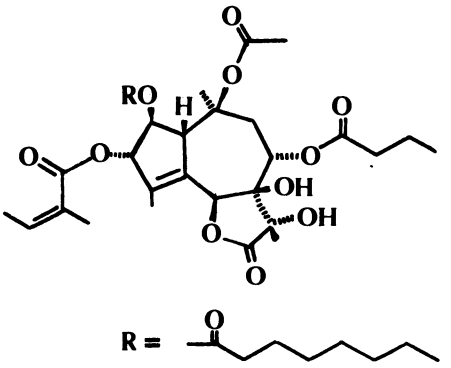

FIGURE 5. Structure of thapsigargin.

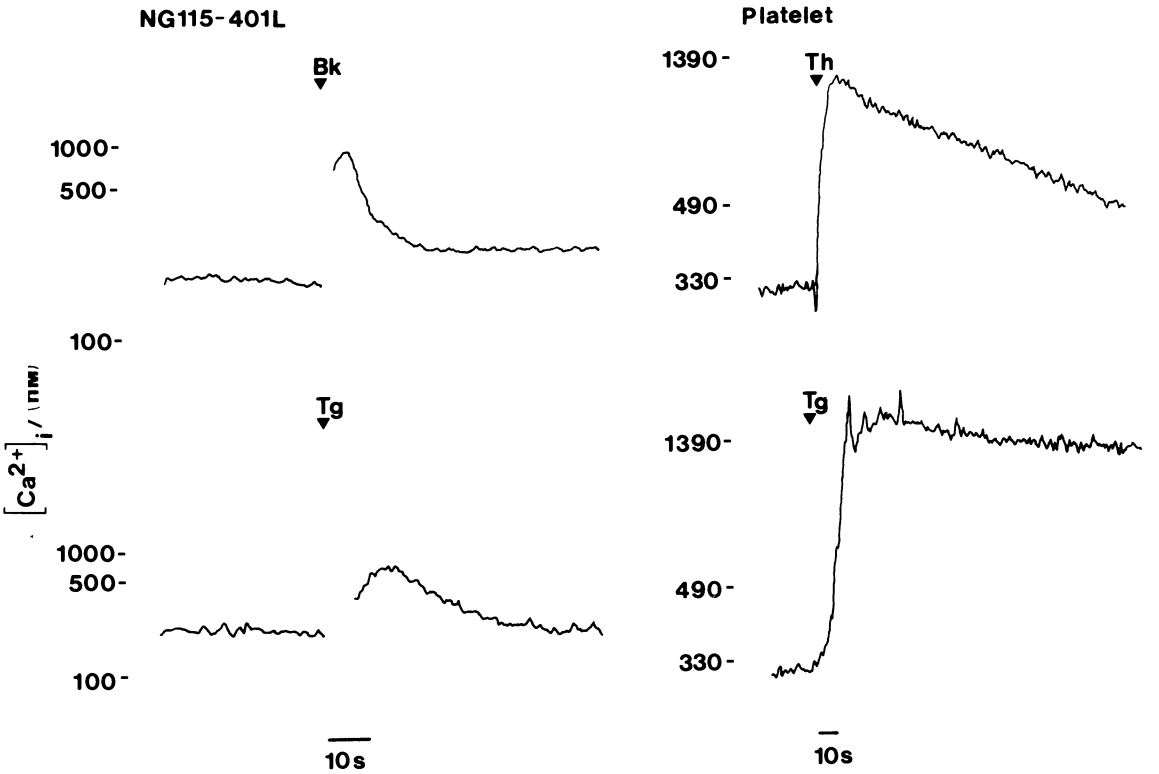

FIGURE 6. Comparison of the actions of thapsigargin (Tg) and receptor agonists in stimulating cytosolic calcium changes in NG115-401L cells and human platelets. The NG115-401L cells were stimulated with Tg (1.7 μM) or bradykinin (Bk, 1 μM) whereas platelets were stimulated with Tg (1 μM) or thrombin (Th, 0.2 U ml^{-1}). The results are redrawn from Thastrup *et al.* 1987 and Jackson *et al.* 1988 and show the time-course of Fura-2 fluorescence changes calibrated to free calcium levels.

homeostasis must be different in the two cell types. The conclusion to be drawn is that Tg may be selectively targeted to the production of intracellular calcium discharge, and thus provides a membrane-permeable tool for the activation of calcium transients in intact cells, complementary to fluoroaluminate, for activation of G-proteins, and phorbol diesters, for activation of protein kinase C.

The application of this reagent would be greatly enhanced by a knowledge of its mechanism

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Table 3. Thapsigargin does not elevate inositol phosphate levels in NG115-401L cells

	control levels of inositol phosphates (%)†
bradykinin (1 μM)	462 ± 147‡
thapsigargin (1.7 μM)	99 ± 10§
A23187 (2 μM)	405 ± 175‡

† Results are means ± s.e.m. for three separate experiments in triplicate. Incubations were for 30 min at 37 °C in the presence of 10 mM LiCl. Total inositol phosphates were determined. Under these conditions, control values were 709 ± 150 counts per minute. Data were taken from Jackson *et al.* (1988).
‡ Significant. $p \leqslant 0.05$ (Student's *t*-test).
§ Not statistically different from controls.

and specificity. Surprisingly, Tg shows activity as a tumour promoter (Hakii *et al.* 1986), but does not stimulate protein kinase C. We have confirmed that Tg does not have phorbol diester activities in the 401L cells by using two intact-cell assays: competition for [^{3}H]phorbol-12,13-dibutyrate binding and stimulation of cytoplasmic alkalinization. Thus Tg appears to have no activity on the protein kinase C forms in this population. Moreover, Tg is not an ionophore in that it cannot transfer $^{45}Ca^{2+}$ into an organic phase, does not release K^+ from erythrocytes, and cannot release histamine from isolated mast-cell granules (Ali *et al.* 1985; Thastrup *et al.* 1987). Tg therefore appears to have a novel mechanism for the induction of intracellular calcium release. Because of its very high potency, it is likely that this mechanism involves a specific protein interaction within the cell. The action of Tg resembles that of caffeine which induces a calcium leakage from microsomal stores in sensory neurons (Neering & McBurney 1984), although Tg is active at 10^6-fold lower doses (10 nM against 10 mM). Tg and caffeine may share possible sites of action, which include calcium pumps, or the cellular components involved in GTP- or Ins(1,4,5)P_3-induced calcium release.

InsP_5 and InsP_6 in the nervous system

The high-performance liquid chromatography (HPLC) fractionation of inositol-labelled compounds from the 401L cells revealed that there were two major peaks of material more polar than InsP_4 (Jackson *et al.* 1987*a*). These have now been assigned by co-chromatography with standards to Ins(1,3,4,5,6)P_5 (referred to hereafter as InsP_5) and InsP_6 (phytic acid). What attracted initial interest were the observations that the labelled levels of InsP_5 were surprisingly high, exceeding the stimulated levels of InsP_3, and that these levels seemed relatively insensitive to bradykinin stimulation. If anything, the levels of InsP_5 and InsP_6 appeared to decline slightly at 25–30 s after bradykinin addition (Jackson *et al.* 1987*a*), as has also been reported in A431 cells (Tilly *et al.* 1987). Moreover, in 401L cells and other cell lines, steady-state labelling takes longer for InsP_5 and InsP_6 than for other inositol metabolites. This implies that these compounds may be unrelated to the rapid events in the intracellular signalling sequence, and may not be in the same compartment as other inositol phosphates. It was unclear whether InsP_5 and InsP_6 might be produced only in proliferating cells, or as a result of the transforming events leading to the establishment of permanent cell lines. For this reason, we examined the possible occurrence of InsP_5 and InsP_6 in normal mature mammalian brain (Vallejo *et al.* 1987).

We adapted the same procedure used for analysing inositol metabolites in cells in culture, radioactive labelling with [^{3}H]-*myo*-inositol, which offers the advantage of specificity over

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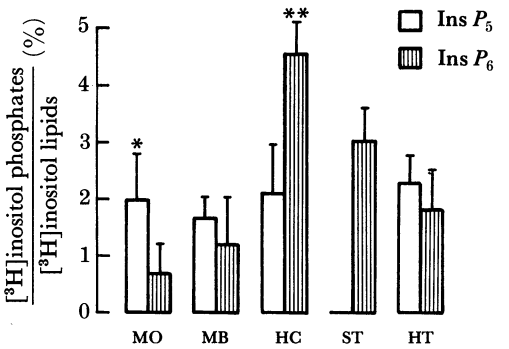

FIGURE 7. Normalized levels of radiolabelled inositol phosphates relative to labelled inositol lipids in different rat brain regions after micro-injection of [^{3}H]inositol. Inositol phosphates were separated by HPLC. The results are means $\pm$ s.e.m. ($n = 4$–7 separate determinations). The regions shown are medulla oblongata (MO), midbrain (MB), hippocampus (HC), striatum (ST) and hypothalamus (HT). The figure is reproduced from Vallejo *et al.* (1987), which should be consulted for experimental details.

chemical techniques which could not distinguish between *myo*- and *neo*-, *scyllo*- or *chiro*-inositol metabolites. Radioactive inositol was microinfused into the ventricles, and several brain regions were extracted for HPLC analysis of inositol-labelled products. The results, normalized to levels of labelled inositol lipids, are summarized in figure 7. InsP_5 and InsP_6 both show regional patterns of distribution that appear to be independent of one another. For example, there is no detectable InsP_5 in the striatum, whereas there is detectable InsP_6. In addition, the InsP_5 and InsP_6 peak can be measured under conditions wherein InsP_4 appears absent. This experimental design may favour the detection of accumulated products, rather than stimulated, transient metabolites (Lightman *et al.* 1987), providing a clue that InsP_5 and InsP_6 may exhibit a slower metabolic turnover because of storage. It can be concluded that InsP_5 and InsP_6 are normal neural metabolites, and are not exclusively associated with the special properties of cell lines or growing cells. At this stage, it is important to emphasize that the identification of InsP_5 and InsP_6 is not rigorous. InsP_6 does not have the problem of positional isomers, so its identification is not as problematical as that of a specific InsP_5 isomer. The major form of InsP_5 detected in brain and 401L cells co-migrates with a [^{32}P]Ins(1,3,4,5,6)P_5 standard prepared from avian erythrocytes (Stephens *et al.* 1988), but this does not constitute proof for structural assignment. However, a kinase that can produce this isomer of InsP_5 from Ins(3,4,5,6)P_4 can be detected at high levels in mammalian brain (Stephens *et al.* 1988), supporting the conclusion that Ins(1,3,4,5,6)P_5 is likely to exist in mammalian neural cells and may correspond to the major InsP_5 isomer detected in inositol-labelled intact brain.

The accumulation of InsP_5 and InsP_6 in neural cells, and their apparent metabolic independence from the rapid events in transmembrane signalling argue that they may act in another cellular context. In brain, several small molecules, such as ATP, glutamate and glycine, are thought to act as extracellular messengers in addition to subserving other biochemical roles. Thus, among the inositol metabolites, InsP_5 and InsP_6 might provide the best candidates for extracellular messengers. On this basis, we examined responses to exogenous InsP_5 and InsP_6 after microinjection into a nucleus of the brainstem, a region where both had been shown to occur. The nucleus selected, the nucleus tractus solitarius (NTS), is involved in cardiovascular and respiratory control and sensitive to sensory input from primary baroreceptors. Responses can be elicited by focal infusion of some of the neuroactive substances found in the NTS (see

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references in Vallejo *et al.* (1987)), so that this brain region provided a site of predicted sensitivity that could be monitored by changes in whole-animal physiological parameters. Figure 8 summarizes the results. A mixed Ins$(w,x,y,z)P_4$, prepared from a phytic acid hydrolysate, was used for comparison because the physical properties of this preparation were very similar to those of InsP_5 or InsP_6. Both InsP_5 and InsP_6 caused a reversible and dose-dependent reduction in arterial blood pressure and heart rate which was rapid in onset and persisted for some minutes. For comparison, microinjection of the transmitter candidate glutamic acid caused similar effects but was one to two orders of magnitude less effective. Indeed, one of the striking aspects of these results is that InsP_5 and InsP_6 are the most potent small molecules evaluated by this test.

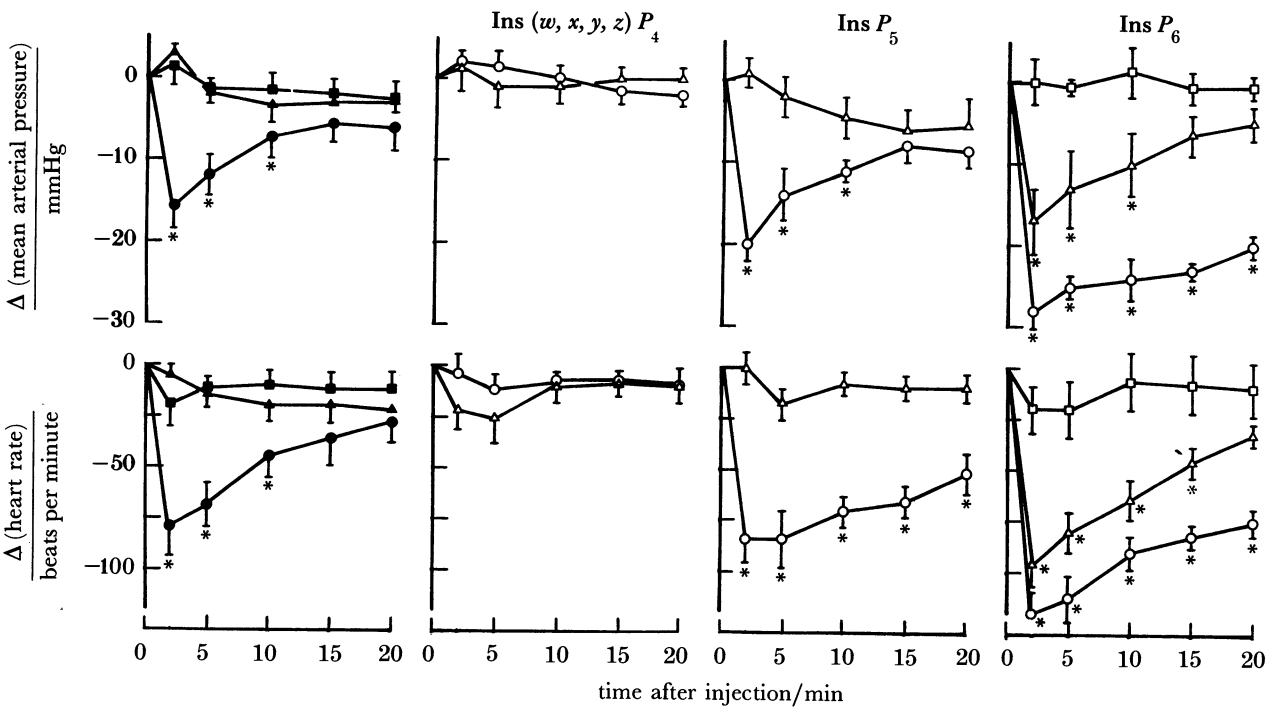

FIGURE 8. Changes in mean arterial pressure and heart rate after micro-injection of saline, glutamic acid (lefthand traces) or inositol phosphates into the nucleus tractus solitarius of rats. Each point represents the mean ± s.e.m. (4–7 experiments). $*p < 0.05$, Duncan's multiple-range test, compared with the saline-treated group. Concentrations were: glutamic acid, 2 nmol (▲), 5 nmol (●); inositol phosphates, 20 pmol (□), 40 pmol (△) and 200 pmol (○); and saline controls (■). The figure is reproduced from Vallejo *et al.* 1987, which should be consulted for experimental details.

One of the first concerns in considering the significance of these results is the underlying mechanism of the effect. To this end, we have explored two avenues. First, we have analysed the cellular basis of the physiological activation. Second, we have examined the physical properties of Ins(1,3,4,5)P_4, InsP_5 and InsP_6 to determine whether the observed actions could have a purely physical basis.

The nature of the cells responding in the NTS cannot be identified from the results described so far. Accordingly, we examined electrophysiological responses from a region anatomically closely related to the NTS that also receives sensory input: the dorsal horn of the spinal cord. In this instance, inositol phosphates and related substances were locally released by

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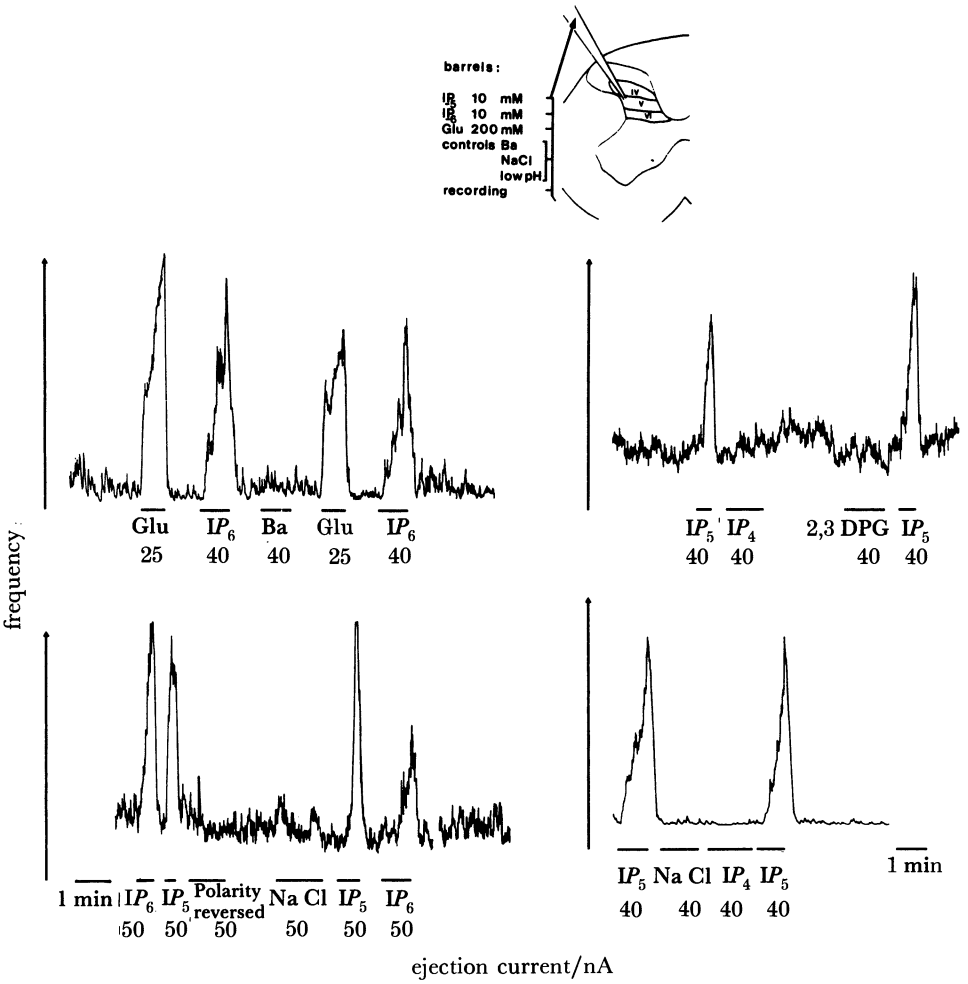

FIGURE 9. Extracellular recordings from the dorsal horn of rat spinal cord with iontophoretic application of glutamic acid (Glu), Ins(1,3,4,5,6)P_5, InsP_6, 2,3-diphosphoglycerate (2,3DPG), barium (Ba) or saline (NaCl). The experimental preparation is shown schematically above (Kelly 1975). Extracellular recordings were made from a minimum of 18 cells from 4 animals deeply anaesthetized with thiobutabarbital sodium (110 mg kg^{-1}). The spinal cord was transected in the lower thoracic region and electrode penetrations were made in the lumbar segments (L IV–VI) as indicated. All drugs were ejected iontophoretically as anions (pH 4), with barrel concentrations as indicated at the left. The time of application is shown by the bars. The firing frequency records for four separate neurons are shown. The apparent differences in levels of basal activity are in part due to changes in scale between the different records.

iontophoretic ejection from a multi-barrel pipette, and single-unit responses were recorded as action potential firing frequencies. After iontophoretic application, neurons were excited by either InsP_5 or InsP_6, but not by 2,3,-diphosphoglycerate, Ins$(w,x,y,z)P_4$ or Ins(1,3,4,5)P_4 (figure 9). When compared with the recognized neuronal excitant glutamic acid, both InsP_5 and InsP_6 had slower onsets, but firing reversed rapidly on cessation of the ejection current. The responses can be recorded for many minutes, suggesting that there is limited toxicity or local damage during the time-course of the experimental application of the inositol phosphates. Effective extracellular concentrations of the ejected compounds cannot be determined by using iontophoretic techniques *in vivo*, but it is worth noting that the electrodes were loaded with a twenty times lower concentration of InsP_5 or InsP_6 than for glutamic acid, but responses were

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approximately equivalent at the same ejection current. This might indicate that either InsP_5 or InsP_6 have high transport numbers for iontophoresis, owing to their high charge, or that they may be quite potent in stimulating their effects. Although it is unclear whether InsP_5 and InsP_6 are working through a common site, those neurons on which both substances have been tested do respond to both InsP_5 and InsP_6. In this experimental design, it is possible to drive spinal neurons physiologically, through sensory input, and to examine whether there is any correlation between the sensory responsiveness of a given cell and its pharmacological sensitivity. We found no evidence correlating InsP_5 or InsP_6 sensitivity with any sensory modality; rather it appears that these compounds share with acidic amino acids, such as glutamic acid, the ability to stimulate several neuronal populations. Thus it is likely that neuronal excitation, and not a non-neuronal local action such as alteration in regional blood flow, underlies the potent haemodynamic changes elicited by InsP_5 and InsP_6 upon administration within the NTS.

For some time it has been known that many of the inositol phosphates can chelate divalent cations to form soluble or insoluble complexes (Brown 1969; Cosgrove 1980). Thus, InsP_5 or InsP_6 might activate neurons through a physical mechanism involving the depletion of free cations or the formation of insoluble salts. For this reason, we have measured the solubility and chelation constants for Ins(1,3,4,5)P_4, InsP_5 and InsP_6 in media mimicking the ionic conditions and pH of the intracellular and extracellular environments. In experiments to be published in detail elsewhere, we have determined that Ins(1,3,4,5)P_4, InsP_5 and InsP_6 are very similar in their solubility and chelation behaviour so that, in all cases, significant depletion of divalent cations and insolubility of the products is a problem only above inositol phosphate concentrations of 0.05–0.1 mM. Inside the neuron, InsP_5 and InsP_6 are likely to be in compartments depleted of divalent cations, or complexed with suitable poly-cations. Alternatively, they may not be stored, but may instead be complexed with cellular proteins. In avian erythrocytes, wherein InsP_5 concentrations can be several millimolar, the InsP_5 has been suggested to be bound to haemoglobin, upon which it may act as a regulatory factor (Cosgrove 1980). One conclusion to be drawn is that the best control for purely physical interactions in extracellular tests is comparison with the effects, if any, of InsP_4, which exhibits the same chelation and solubility effects as InsP_5 and InsP_6. Thus, as InsP_4 is inactive in extracellular tests, the neuronal stimulation by InsP_5 and InsP_6 may be mediated through specific surface sites. Clearly, direct demonstration of surface recognition sites by radioligand binding approaches is an essential next step.

It must be emphasized that there is insufficient information to evaluate whether InsP_5 or InsP_6 or both might function physiologically in neural cell-to-cell communication. However, the demonstration that InsP_5 and InsP_6 have central biological activities, unrelated to intracellular signalling, may provide a useful lead for the design of novel neuropharmacological reagents. In addition, it should be borne in mind that InsP_5 or InsP_6 might be released as a consequence of neural damage, in which case these excitant actions might contribute to the resulting pathology. Another important line of enquiry will be whether lithium, proposed to exert its therapeutic actions in bipolar affective disorders via interactions with the enzymes of inositol metabolism (Berridge *et al.* 1982), can inhibit the phosphatases acting on InsP_5 and InsP_6. In this regard, chronic lithium treatment can dramatically potentiate cholinergic agonist-induced seizures in normal animals (Honchar *et al.* 1983). Could this potentiation be due to enhancement of the excitant effects of InsP_5 or InsP_6?

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Conclusions

The inositol lipid signalling pathways constitute a primary transduction system for neural responses to environmental stimulation. It is likely that these pathways may generate extracellular as well as intracellular messengers. Not surprisingly, a signalling system of this sophistication and complexity can be expected to be involved in several processes in the brain, some of which are summarized in table 4. Many of these processes are not exclusively neuronal

Table 4. Neural processes in which inositol lipid signalling pathways may be involved

receptor activation
presynaptic mechanisms and secretion
membrane electrical events
regulation of gene expression
metabolism
information storage and plasticity
nerve regeneration
cell proliferation and maturation

and span different cell types. For example, receptor-mediated activation of inositol lipid events has been shown in astrocyte populations (Pearce *et al.* 1986; Cholewinski *et al.* 1988) as well as neurons. Thus it can be envisioned that a combination of approaches, such as the use of stable cell lines on the one hand, and intact mammalian brain on the other, will be necessary to understand how the inositol lipid-derived mediators are produced, regulated and function in neural cells.

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References

Ali, H., Christensen, S. B., Foreman, J. C., Pearce, F. L., Piotrowski, W. & Thastrup, O. 1985 The ability of thapsigargin and thapsigargicin to activate cells involved in the inflammatory response. *Br. J. Pharmacol.* **85**, 705–712.

Amar, S., Kitabgi, P. & Vincent, J.-P. 1987 Stimulation of inositol phosphate production by neurotensin in neuroblastoma NIE115 cells: implication of GTP-binding proteins and relationship with cyclic GMP response. *J. Neurochem.* **49**, 999–1006.

Ananth, U. S., Leli, U. & Hauser, G. 1987 Stimulation of phosphoinositide hydrolysis by serotonin in C6 glioma cells. *J. Neurochem.* **48**, 253–261.

Bennett, C. F., Mong. S., Clarke, M., Kruse, L. I. & Crooke, S. T. 1987 Differential effects of monoalide on secreted and intracellular phospholipases. *Biochem. Pharmac.* **36**, 733–740.

Berridge, M. J. 1987 Receptor-stimulated inositol phospholipid hydrolysis and neural function. *Biochem. Soc. Symp.* **52**, 153–161.

Berridge, M. J., Downes, C. P. & Hanley, M. R. 1982 Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J.* **206**, 587–595.

Berta, P., Sladeczek, F., Derancourt, J., Durand, M., Travo, P. & Haiech, J. 1986 Maitotoxin stimulates the formation of inositol phosphates in rat aortic myocytes. *FEBS Lett.* **197**, 349–352.

Biden, T. J. & Wollheim, C. B. 1986 Ca^{2+} regulates the inositol tris/tetrakisphosphate pathway in intact and broken preparations of insulin-secreting RIN m5F cells. *J. biol. Chem.* **261**, 11931–11934.

Bosch, F., Arino, J., Gomez-Foix, A. M & Guinovart, J. J. 1987 Glycogenolytic, noninsulin-like effects of vanadate in rat hepatocyte glycogen synthase and phosphorylase. *J. biol. Chem.* **282**, 218–222.

Brown, D. M. 1969 Aspects of phosphate ester chemistry as applied to the phosphoinositides. *Ann. N.Y. Acad. Sci.* **165**, 687–694.

Cholewinski, A. J., Hanley, M. R. & Wilkin, G. P. 1988 A phosphoinositide-linked peptide response in astrocytes: evidence for regional heterogeneity. *Neurochem. Res.* (In the press.)

Cohen, N. M., Schmidt, D. M., McGlennen, R. C. & Klein, W. L. 1983 Receptor-mediated increases in phosphatidylinositol turnover in neuron-like cell lines. *J. Neurochem.* **40**, 547–554.

Cosgrove, D. J. 1980 *Inositol phosphates. Their chemistry, biochemistry, and physiology*. Amsterdam: Elsevier.

Downes, C. P. 1986 Agonist-stimulated phosphatidylinositol-4,5-bis-phosphate metabolism in the nervous system. *Neurochem. Int.* **9**, 211–230.

Fink, L. A., Connor, J. A. & Kaczmarek, L. K. 1988 Inositol trisphosphate modulates ion channels and intracellular calcium in molluscan neurones. *J Neurosci.* (In the press.)

Fisher, S. K. & Agranoff, B. W. 1987 Receptor activation and inositol lipid hydrolysis in neural tissues. *J. Neurochem.* **48**, 999–1017.

Francel, P. C. & Dawson, G. 1986 Bradykinin induces a rapid release of inositol trisphosphate from a neuroblastoma hybrid cell NCB-20 that is not antagonized by enkephalin. *Biochem. biophys. Res. Commun.* **135**, 507–514.

Gill, D. & Chueh, S.-H. 1985 An intracellular ($ATP + Mg^{2+}$)-dependent calcium pump within the NIE-115 neuronal cell line. *J. biol. Chem.* **260**, 9289–9297.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. biol. Chem.* **260**, 3440–3450.

Gusovsky, F., Yasumoto, T. & Daly, J. W. 1987 Maitotoxin stimulates phosphoinositide breakdown in neuroblastoma hybrid NCB-20 cells. *Cell. molec. Neurobiol.* **7**, 317–322.

Hakii, H., Fujiki, H., Suganuma, M., Nakayasu, M., Tahira, T., Sugimura, T., Scheuer, P. & Christensen, S. B. 1986 Thapsigargin, a histamine secretogogue, is a non-12-o-tetradecanoyl phorbol-13-acetate (TPA) type tumor promoter in two-stage mouse skin carcinogenesis. *Cancer Res. clin. Oncol.* **111**, 177–181.

Hanley, M. R. 1987 Analysis of receptor-coupled events in neuropeptide action using clonal cell lines. *Prog. Brain Res.* **72**, 189–196.

Hasegawa, R. & Eisenberg, F. 1981 Selective hormonal control of myoinositol biosynthesis in reproductive organs and liver of the male rat. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4863–4866.

Hatanaka, H. & Amano, T. 1981 A mouse neuroblastoma X rat glioma hybrid produces immunoreactive substance P-like material. *Brain Res.* **215**, 305–316.

Hawthorne, J. N. 1986 Does receptor-linked phosphoinositide metabolism provide messengers mobilizing calcium in nervous tissue? *Int. Rev. Neurobiol.* **28**, 241–273.

Higashida, H. & Brown, D. A. 1986*a* Two polyphosphoinositide metabolites control two K^+ currents in a neuronal cell. *Nature, Lond.* **323**, 333–335.

Higashida, H. & Brown, D. A. 1986*b* Membrane current responses to intracellular injections of inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate in NG108-15 hybrid cells. *FEBS Lett.* **208**, 283–286.

Higashida, H. & Brown, D. A. 1987 Bradykinin inhibits potassium (M) currents in NIE-115 neuroblastoma cells. *FEBS Lett.* **220**, 302–306.

Honchar, M. P., Olney, J. W. & Sherman, W. R. 1983 Systemic cholinergic agents induce seizures and brain damage in lithium-treated rats. *Science, Wash.* **220**, 323–325.

Jackson, T. R., Hallam, T. J., Downes, C. P. & Hanley, M. R. 1987*a* Receptor-coupled events in bradykinin action: rapid production of inositol phosphates and regulation of cytosolic free Ca^{2+} in a neural cell line. *EMBO J.* **6**, 49–54.

Jackson, T. R., Patterson, S. I., Wong, Y. H. & Hanley, M. R. 1987*b* Bradykinin stimulation of inositol phosphate and calcium responses is insensitive to pertussis toxin in NG115-401L neuronal cells. *Biochem. biophys. Res. Commun.* **148**, 412–416.

Jackson, T. R., Patterson, S. I., Thastrup, O. & Hanley, M. R. 1988 A novel tumour promoter, thapsigargin, transiently elevates cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115-401L neuronal cells. *Biochem. J.* 253.

Jackson, M. J. & Shin, S. 1982 Inositol as a growth factor for mammalian cells in culture. *Cold Spring Harb. Conf. Cell Prolif.* **9**, 75–86.

Jacobsen, S., Hansen, H. S. & Jensen, B. 1987 Synergism between thapsigargin and the phorbol ester 12-o-tetradecanoyl phorbol 13-acetate on the release of [^{14}C]-arachidonic acid and histamine from rat peritoneal mast cells. *Biochem. Pharmac.* **36**, 621–626.

Kelly, J. S. 1975 Microiontophoretic application of drugs onto single neurons. In *Handbook of psychopharmacology* (ed. L. L. Iversen, S. D. Iversen, & S. H. Snyder), vol. 2 (*Principles of receptor research*), pp. 29–67. New York & London: Plenum Press.

Lightman, S. L., Moratalla, R. & Vallejo, M. 1987 Vasopressin activates polyphosphoinositide metabolism in the brain stem of rats in vivo. *J. Physiol., Lond.* **396**, 125*P*.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

McBurney, R. N. & Neering, I. R. 1987 Neuronal calcium homeostasis. *Trends Neurosci.* **10**, 164–169.
Movsesian, M. A., Thomas, A. P., Selak, M. & Williamson, J. R. 1985 Inositol trisphosphate does not release Ca^{2+} from permeabilized cardiac myocytes and sarcoplasmic reticulum. *FEBS Lett.* **185**, 328–332.
Nahorski, S. R., Kendall, D. A. & Batty, I. 1986 Receptors and phosphoinositide metabolism in the central nervous system. *Biochem. Pharmac.* **35**, 2447–2453.
Nakahata, N. & Harden, T. K. 1987 Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H1-histamine receptors on human astrocytoma cells. *Biochem. J.* **241**, 337–344.
Neering, I. R. & McBurney, R. N. 1984 Role for microsomal Ca storage in mammalian neurones? *Nature, Lond.* **309**, 158–160.
Pearce, B., Morrow, C. & Murphy, S. 1986 Receptor-mediated inositol phospholipid hydrolysis in astrocytes. *Eur. J. Pharmacol.* **121**, 231–243.
Pessah, I. N., Fracini, A. O., Scales, D. J., Waterhouse, A. L. & Casida, J. E. 1986 Calcium–ryanodine receptor complex. *J. biol. Chem.* **261**, 8643–8648.
Polascik, T., Godfrey, P. P. & Watson, S. P. 1987 Neomycin cannot be used as a selective inhibitor of inositol phospholipid hydrolysis in intact or semi-permeabilized human platelets. *Biochem. J.* **243**, 815–819.
Pozzan, T., Di Virgilio, F., Vicentini, L. M. & Meldolesi, J. 1986 Activation of muscarinic receptors in PC12 cells: stimulation of Ca^{2+} influx and redistribution. *Biochem. J.* **234**, 547–553.
Proffitt, R. & Case. R. M. 1984 Vanadate stimulates rat pancreatic enzyme secretion through the release of calcium from an intracellular store. *Cell Calcium* **5**, 335–350.
Putney, J. W. 1986 A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1–12.
Reynolds, E. E. & Dubyak, G. R. 1986 Agonist-induced calcium transients in cultured smooth muscle cells: measurements with fura-2 loaded monolayers. *Biochem. biophys. Res. Commun.* **136**, 927–934.
Siess, W. & Lapetina, E. G. 1986 Neomycin inhibits inositol phosphate formation in human platelets stimulated by thrombin but not other agonists. *FEBS Lett.* **207**, 53–57.
Stephens, L. R., Hawkins, P. T., Morris, A. J. & Downes, C. P. 1988 L-*myo*-inositol 1,4,5,6-tetrakisphosphate (3-hydroxy) kinase. *Biochem. J.* **249**, 283–292.
Tertoolen, L. G. J., Tilly, B. C., Irvine, R. F. & Moolenaar, W. H. 1987 Electrophysiological responses to bradykinin and microinjected inositol polyphosphates in neuroblastoma cells. *FEBS Lett.* **214**, 365–369.
Thastrup, O., Foder, B. & Scharff, O. 1987 The calcium mobilizing and tumor promoting agent, thapsigargin, elevates the platelet cytoplasmic free calcium concentration. *Biochem. biophys. Res. Commun.* **142**, 654–660.
Tilly, B. C. Paridon, P. A., Verlaan, I., Wirtz, K. W. A., DeLaat, S. W. & Moolenaar, W. H. 1987 Inositol phosphate metabolism in bradykinin-stimulated human A431 carcinoma cells. *Biochem. J.* **244**, 129–135.
Vallejo, M., Jackson, T. R., Lightman, S. L. & Hanley, M. R. 1987 Occurrence and extracellular actions of inositol pentakis- and hexakisphosphate in mammalian brain. *Nature, Lond.* **330**, 656–658.
Van Calker, D., Assmann, K. & Greil, W. 1987 Stimulation by bradykinin, angiotensin II, and carbachol of the accumulation of inositol phosphates in PC12 pheochromcytoma cells: differential effects of lithium ions on inositol mono- and polyphosphates. *J. Neurochem.* **49**, 1379–1385.
Vicentini, L. M. & Villereal, M. L. 1984 Serum, bradykinin, and vasopressin stimulate release of inositol phosphates from human fibroblasts. *Biochem. biophys. Res. Commun.* **123**, 663–670.
Williams, D. A., Fogarty, K. E., Tsien, R. Y. & Fay, F. S. 1985 Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. *Nature, Lond.* **318**, 558–561.
Wong, Y.-H., Kalmbach, S. J., Hartman, B. K. & Sherman, W. R. 1987 Immunohistochemical staining and enzyme activity measurements show myo-inositol-1-phosphate synthase to be localized in the vasculature of brain. *J. Neurochem.* **48**, 1434–1442.
Yano, K., Higashida, H., Inoue, R. & Nozawa, Y. 1984 Bradykinin-induced rapid breakdown of phosphatidylinositol-4,5-bisphosphate in neuroblastoma X glioma hybrid NG108-15 cells. *J. biol. Chem.* **259**, 10201–10207.
Zilberman, Y., Howe, L. R., Moore, J. P., Hesketh, T. R. & Metcalfe, J. C. 1987 Calcium regulates inositol 1,3,4,5-tetrakisphosphate production in lysed thymocytes and in intact cells stimulated with concanavalin A. *EMBO J.* **6**, 957–962.

Discussion

C. P. Downes (*Smith Kline & French Research Ltd, Welwyn, U.K.*). In considering the possible ways in which inositol pentakisphosphate and hexakisphosphate may be compartmentalized in cells, Dr Hanley mentioned storage in vesicles and protein binding. Another possibility is metabolic compartmentation. Studies in our laboratory by L. Stephens and P. T. Hawkins have demonstrated that D-inositol(1,3,4,5)P_4 is not phosphorylated directly to inositol pentakisphosphate by cellular homogenates, but that two novel inositol tetrakisphosphates, D-inositol(3,4,5,6)P_4 and the meso-compound inositol(1,3,4,6)P_4 are both phosphorylated to the

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same inositol pentakisphosphate isomer(Ins(1,3,4,5,6)P_5). The product precursor relations among these different inositol polyphosphates and the subcellular distribution of the enzymes involved in their metabolism may effectively segregate functionally discrete families of inositol phosphates.

O. Thastrup (*Department of Clinical Chemistry, University Hospital, Rigshospitalet, Copenhagen, Denmark*). I shall try to give a short introduction into the history of the rather strange compound, thapsigargin (Tg). It is a naturally occurring sesquiterpene lactone that originates from the umbelliferous plant *Thapsia garnica*. Tg was isolated and its structure elucidated at the Royal Danish School of Pharmacy by S. B. Christensen and co-workers. From pharmacological studies we found that Tg was able to activate a broad spectrum of cells including different cells involved in the inflammatory response, platelets and lymphocytes. In addition, it was found that Tg constitutes a non-TPA type tumour promoter. Measurements of the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in platelets revealed that the Tg induced activation was associated with a transient rise in $[Ca^{2+}]_i$ that was followed by a minor decline leading to a new elevated steady state level of $[Ca^{2+}]_i$. Protein phosphorylation studies led us to suggest that the Tg-induced rise in $[Ca^{2+}]_i$ is a direct effect on intracellular Ca^{2+} pools independent of hydrolysis of phosphoinositides. The sustained elevated $[Ca^{2+}]_i$ is maintained by an increased influx (inositol phosphate independent) from the extracellular space. $[Ca^{2+}]_i$ stabilizes on a rather high level because the Ca^{2+} extrusion systems are not in their fully activated state. I believe that Tg will provide a new valuable tool for analysis of the initial intracellular regulation systems.

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