Accumulation of inositol phosphates in sympathetic ganglia

Effects of depolarization and of amine and peptide neurotransmitters

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Depolarization of isolated [^3H]inositol-labelled rat superior cervical sympathetic ganglia in a high- K^+ medium stimulates an accumulation of labelled inositol phosphates. This accumulation occurs only when ganglia are incubated in a Ca^{2+} -containing medium, suggesting that it represents a receptor-stimulated hydrolysis of inositol lipid(s) activated by an endogenously released neurotransmitter. A minor fraction of this accumulation appears to be activated by intraganglionically released acetylcholine, since it is slightly reduced by atropine. The accumulation of inositol phosphates is unaffected by blockade of appropriate catecholamine, histamine and 5-hydroxytryptamine receptors and also by aspirin and indomethacin. This response to depolarization is potentiated by incubation with proteinase inhibitors, suggesting that it might be caused by an endogenously released peptide neurotransmitter. However, it is not prevented by a V_1 -vasopressin receptor antagonist, and none of the peptides tested so far fully reproduces the response: these include a stable substance P analogue, physalaemin, neurokinin α , bradykinin, angiotensin, pancreaticozym, bombesin and luteinizing-hormone-releasing hormone. Stimulated inositol lipid breakdown in depolarized sympathetic ganglia seems likely to be activated by an as-yet-unidentified peptide neurotransmitter: this might serve as an intraganglionic mediator of postsynaptic excitation by employing the same signalling mechanism as muscarinic cholinergic and V_1 -vasopressin receptors.

Stimulated phospholipase C-mediated hydrolysis of inositol phospholipid(s) is intimately associated with activation of receptors for which Ca^{2+} and diacylglycerol are intracellular messengers (for reviews, see Michell, 1975, 1979, 1982a; Michell & Kirk, 1981; Michell *et al.*, 1981, 1984b; Berridge, 1980, 1981, 1984; Putney, 1981; Nishizuka, 1984). One of the stimuli that provokes very rapid inositol lipid breakdown is vasopressin acting on isolated superior cervical sympathetic ganglia from the rat (Hanley *et al.*, 1984; Bone *et al.*, 1984; Michell *et al.*, 1984a). These ganglia contain a vasopressin-like peptide (VLP) in their principal noradrenergic

neurons and a plexus of nerve fibres (Hanley *et al.*, 1984).

Some years ago, Nagata *et al.* (1973) reported that increasing the K^+ concentration in the medium bathing isolated superior cervical ganglia causes an increase in the incorporation of ^{32}P into PtdIns, a response that is often an indication of the occurrence of receptor-stimulated inositol lipid breakdown. This response was maintained in axotomized ganglia, suggesting that it could not be solely located in membranes of the major postsynaptic neurons. Since incubating ganglia in a medium of high K^+ concentration in the presence of extracellular Ca^{2+} is an effective method for evoking neurotransmitter release from intraganglionic nerve terminals (Blaustein, 1976; Drapeau & Blaustein, 1983; Floor, 1983), it seemed likely that the stimulation of PtdIns labelling was the result of receptor activation by a neurotransmitter released within the ganglia. Axotomization would have removed the postsynaptic muscarinic cholinergic receptors of the ganglion, whose activation can

Abbreviations used: PtdIns, phosphatidylinositol; InsP, InsP₂, InsP₃, the mono-, bis- and tris-phosphates of inositol; VLP, vasopressin-like peptide; LHRH, luteinizing-hormone-releasing hormone (luliberin).

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stimulate ganglionic PtdIns metabolism (Hokin *et al.*, 1960; Lapetina *et al.*, 1976; Pickard *et al.*, 1977), so it appeared that acetylcholine was unlikely to be the neurotransmitter responsible for these effects.

Upon its discovery, VLP became an obvious candidate as mediator of the ganglionic response to depolarization. In addition, a variety of other peptide neurotransmitters and their receptors have been detected in ganglia in the past few years (Schultzberg *et al.*, 1979; Jan *et al.*, 1980; Konishi *et al.*, 1980; Lundberg *et al.*, 1982a,b), some of which were also possible mediators of the stimulation of inositol lipid metabolism in depolarized ganglia. The experiments reported here demonstrate that inositol phosphates are produced in depolarized ganglia, confirming the occurrence of inositol lipid hydrolysis. In addition, we report attempts to identify the ganglionic neurotransmitter responsible for activating this reaction.

Materials and methods

Materials

Most materials were from sources previously specified (Bone *et al.*, 1984). Pancreozymin was a gift from Professor V. Mutt, Karolinska Institute, Stockholm, Sweden, LHRH a gift from Dr. R. Clayton of the Department of Medicine of this University, and a metabolically stabilized substance P analogue {[5-pyroglutamyl, 8-*N*-methylphenylalanine, 9-*N*-methylglycine]substance P-(5-11)-heptapeptide} a gift from Dr. M. R. Hanley, Department of Biochemistry, Imperial College of Science and Technology, London, U.K. Physalamin, bradykinin, γ -aminobutyric acid, bombesin, somatostatin, antipain, amastatin and bestatin were from Sigma, and neurokinin α (substance K) from Bachem.

Labelling of ganglia with [^3H]inositol, depolarization with K^+ and measurement of water-soluble inositol phosphates

The procedures used to label ganglionic inositol phospholipids and to extract and separate the inositol phosphates have been fully described previously (Bone *et al.*, 1984). In summary, individual superior cervical ganglia were dissected from Wistar rats and incubated in Krebs-Ringer bicarbonate medium, pH 7.2, containing 0.9 mM- Ca^{2+} , 10 mM-glucose and 7.5 μCi of *myo*-2-[^3H]inositol (16.9 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) for 2.5 h at 37°C. Incubation mixtures were gassed with O_2/CO_2 (19:1) every 30 min. Ganglia were then incubated for a further 1 h in similar medium containing no inositol. LiCl (10 mM) was added to inhibit inositol 1-phosphate phosphatase (Berridge *et al.*, 1982),

together with 10 μM -atropine (unless otherwise stated) to inhibit any response to intrinsically released acetylcholine. After 5 min, ganglia were transferred to portions of medium, warmed to 37°C, containing Li^+ (10 mM) and atropine (10 μM) in which 80 mmol $\cdot \text{l}^{-1}$ of the NaCl was replaced by KCl. Other receptor antagonists were added with the Li^+ and atropine, and were present throughout the period of depolarization. Incubations were terminated after the appropriate period by the addition of HClO_4 . The acid extract was processed and the inositol phosphates separated as previously described (Bone *et al.*, 1984).

Extraction of lipids

Lipids were extracted from the acid-treated ganglia with chloroform/methanol/12 M-HCl (100:200:1, by vol.). The solvent was evaporated and the incorporation of [^3H]inositol into the lipids was determined. The total amounts of labelled inositol phosphates ($\text{InsP}_3 + \text{InsP}_2 + \text{InsP}$) produced have usually been expressed relative to every 10^2 d.p.m. incorporated into the ganglionic lipids (i.e. as a percentage conversion).

Results

As described previously (Bone *et al.*, 1984), we have assayed stimulated inositol lipid breakdown in terms of the accumulation of labelled inositol phosphates in [^3H]inositol-labelled ganglia when they are stimulated in the presence of Li^+ . Li^+ inhibits InsP phosphatase (Hallcher & Sherman, 1980) and thus traps the phosphates liberated during inositol lipid hydrolysis as InsP (Berridge *et al.*, 1982). In a previous study (Bone *et al.*, 1984) we demonstrated that InsP_3 and InsP_2 accumulate most rapidly on stimulation and that these are probably the precursors of the accumulated InsP . Most of the results presented here come from ganglia stimulated for 90 min, by which time the major accumulated product is InsP .

Muscarinic stimulation of inositol phosphate accumulation in ganglia

Bethanechol, a muscarinic agonist, stimulates inositol phosphate accumulation in ganglia, but is considerably less potent than vasopressin (Bone *et al.*, 1984). However, a recent report has shown that bethanechol is only a partial agonist in stimulating ganglionic PtdIns labelling (Horwitz *et al.*, 1984). We have therefore done additional experiments which demonstrate that both bethanechol and muscarine can stimulate accumulation of inositol phosphates in ganglia, with muscarine displaying both greater affinity and greater efficacy than bethanechol (Fig. 1): the concentration of muscar-

Fig. 1. Concentration-dependence of muscarine- and bethanechol-stimulated accumulation of inositol phosphates in rat superior cervical ganglia

Isolated ganglia were labelled as described in the text. After incubation in Krebs-Ringer medium containing 10 mM-LiCl for 5 min, ganglia were stimulated with muscarine or bethanechol for 90 min. Incubations were terminated and inositol phosphates separated as described in the text. The radioactivity of the phosphate esters is expressed per 10² d.p.m. incorporated into ganglionic lipids. The results presented are means $\pm$ S.E.M. Values in parentheses represent the numbers of ganglia analysed.

ine giving a half-maximal response was approx. 1 μ M, as compared with a value of approx. 10 μ M for bethanechol. However, even muscarine, the more potent cholinergic stimulus, stimulated the accumulation of only about one-third the quantity of inositol phosphates accumulated in response to vasopressin (compare Fig. 1 with Fig. 1 of Bone *et al.*, 1984).

Since acetylcholine is known both to be released within stimulated ganglia and to stimulate inositol lipid hydrolysis therein, 10 μ M-atropine was routinely included in most incubations involving depolarization-induced transmitter release, in order to prevent the activation of muscarinic receptors by endogenously released acetylcholine (see below).

Accumulation of inositol phosphates in ganglia depolarized by incubation in a medium of high K⁺ concentration

Ganglia were labelled with [³H]inositol and were then incubated in a depolarizing medium in which 80 μ M-LiCl of the Na⁺ was iso-osmotically replaced by K⁺, in the presence of 10 mM-Li⁺ and 10 μ M-atropine. Depolarization induced approximately a doubling in the amounts of inositol phosphates accumulated during a 90 min incuba-

Table 1. Effect of atropine on high K⁺-induced accumulation of inositol phosphates

Isolated ganglia were labelled with [³H]inositol as described in the text. After a 5 min preincubation with 10 mM-LiCl, with or without 10 μ M-atropine, ganglia were incubated in the presence or absence of 80 mM-K⁺ for 90 min. Values represent means $\pm$ S.E.M. (no. of ganglia analysed) of the combined inositol phosphates expressed relative to an incorporation of 10⁵ d.p.m. into total ganglionic lipids.

Conditions	³ H in inositol phosphates (d.p.m./10 ⁵ d.p.m. in lipids)	
	Controls	High K ⁺
Atropine absent	14700 $\pm$ 2200 (6)	36700 $\pm$ 1600 (6)
With 10 μ M-atropine	14400 $\pm$ 2100 (5)	31000 $\pm$ 2100 (6)

tion (Fig. 2, Tables 1 and 2). This increase was initiated rapidly and began to decline after about 30 min (Fig. 2). After approx. 45 min, the rate of accumulation of inositol phosphates was no greater than in control ganglia.

The response to depolarization in the absence of atropine was only slightly greater than in its presence (Table 1). This confirms that a small

Fig. 2. Time course of the accumulation of inositol phosphates in depolarized sympathetic ganglia

Ganglia were labelled with [^3H]inositol as described in the text. After preincubation with 10 mM-LiCl and 10 μM -atropine for 5 min, ganglia were incubated for the specified period in the presence or absence of 80 mM- K^+ . Inositol phosphates were separated as described in the text. Data have been expressed as total inositol phosphates accumulated for every 10^2 d.p.m. incorporated into ganglionic lipids. The results presented are means $\pm$ S.E.M. ($n=4-5$).

Table 2. Dependence of high- K^+ -induced inositol phosphate accumulation on Ca^{2+} in the extracellular medium

Isolated ganglia were labelled with [^3H]inositol as described in the text. After a 5 min preincubation with 10 mM-LiCl and 10 μM -atropine, ganglia were incubated for 90 min in the presence or absence of 0.9 mM- Ca^{2+} and 80 mM- K^+ . The values represent the means $\pm$ S.E.M. ($n=9-11$) of the combined inositol phosphates, corrected to an incorporation of 10^5 d.p.m. into ganglionic lipids.

Conditions	^3H in inositol phosphates (d.p.m./ 10^5 d.p.m. in lipids)	
	Controls	+ 80 mM- K^+
With 0.9 mM- Ca^{2+}	9680 $\pm$ 1020	19450 $\pm$ 900
Ca^{2+} -free	7800 $\pm$ 1020	9390 $\pm$ 860

proportion of the total response to depolarization is evoked by endogenously released acetylcholine, but that most of the ganglionic response to depolarization must be caused by another endogenously released ligand.

Depolarization-induced accumulation of inositol phosphates requires extracellular Ca^{2+}

Neurotransmitter release from nerve terminals is normally stimulated by an influx of extracellular Ca^{2+} that is initiated by membrane depolarization. In the absence of extracellular Ca^{2+} there was a slight decrease in the quantity of inositol phosphates accumulated in control ganglia (Table 2),

possibly reflecting a reduced rate of spontaneous neurotransmitter release within these ganglia.

Depolarization with high K^+ failed to evoke a significant accumulation of inositol phosphates in ganglia incubated in Ca^{2+} -free medium (Table 2). This is in sharp contrast with the observation that vasopressin-stimulated accumulation of inositol phosphates is unimpaired by the absence of extracellular Ca^{2+} (Bone *et al.*, 1984).

The dependency of depolarization-induced inositol lipid hydrolysis on extracellular Ca^{2+} is consistent with the idea that release of an intrinsic ganglionic neurotransmitter mediates this response. Additional experiments were undertaken with the aim of identifying this agent.

Are classical amine neurotransmitters involved?

The major ganglionic neurotransmitter is believed to be acetylcholine, which is released from nerve terminals by a Ca^{2+} -dependent mechanism (e.g. Blaustein, 1976). As described above, 10 μM -atropine reduces the accumulation of inositol phosphates in response to depolarization only slightly.

Another obvious candidate for the mediation of the non-cholinergic component of the depolarization-induced inositol lipid metabolism is noradrenaline, which is present within the major ganglionic neurons. Noradrenaline stimulates inositol lipid breakdown only through activation of α_1 -receptors (Jones & Michell, 1978; Fain & Garcia-Sainz, 1980). Added noradrenaline did not stimulate inositol phosphate accumulation in ganglia (results not shown), and the response of ganglia to depolarization was not reduced by preincubation with 10 μM -phenoxybenzamine, a high concentration of a potent and irreversible antagonist of α -receptors.

Other agents that failed to modify the accumulation of inositol phosphates induced by depolarization included antagonists for H_1 -histamine receptors (10 μM -mepyramine) and for 5-hydroxytryptamine receptors (10 μM -methysergide) (results not shown).

Are prostaglandins involved?

Another possibility is that the rise in cytosolic Ca^{2+} concentration induced by neuronal depolarization might activate phospholipase A_2 and mobilize arachidonate for prostanoid synthesis. However, the accumulation of inositol phosphates in depolarized ganglia was unaffected by two cyclo-oxygenase inhibitors (50 μM -aspirin and 10 μM -indomethacin) (results not shown).

Are peptide neurotransmitters involved?

VLP is present both in the major ganglionic neurons and in a plexus of fibres within the

ganglion (Hanley *et al.*, 1984). Vasopressin produces a striking stimulation of inositol lipid breakdown in ganglia (Hanley *et al.*, 1984; Bone *et al.*, 1984), so it initially seemed likely that VLP would be the mediator of at least a part of the ganglionic response to depolarization. However, a selective V_1 -receptor antagonist ([1- β -mercapto- β , β -cyclopentamethylenepropionic acid, 8-arginine]vasopressin), which abolishes the response of ganglia to exogenous vasopressin (Hanley *et al.*, 1984; Bone *et al.*, 1984), produced no significant decrease in the accumulation of inositol phosphates in response to depolarization: the response was 94% of that seen in control ganglia ($n=32$). In order to check that this insensitivity to the V_1 -antagonist was not caused by a depolarization-induced modification to the characteristics of V_1 -receptors, the response to vasopressin was re-examined in depolarized ganglia: this response, and its inhibition by the V_1 -antagonist, both occurred normally in the depolarized ganglia (results not shown).

A variety of other peptides have been shown either to be present in sympathetic ganglia or to affect the electrophysiological characteristics of these ganglia (Lewis & Reit, 1965, 1966; Konishi *et al.*, 1979; Jan *et al.*, 1980; Jan & Jan, 1983; Kuffler, 1980; Medgett, 1983; Kessler *et al.*, 1983; Schultzberg, 1983), and some of these are known to stimulate inositol lipid breakdown in other tissues. Incubation of ganglia with a 'cocktail' of proteinase inhibitors (10 μ g/ml each of antipain, amastatin and bestatin) modestly potentiated and also prolonged the accumulation of inositol phosphates in response to depolarization (Fig. 3), as might be expected if this response is mediated by an endogenously released peptide neurotransmitter. Moreover, the proteinase inhibitors alone caused an accumulation of inositol phosphates in control ganglia, probably by protecting a small quantity of spontaneously released transmitter from rapid degradation (Fig. 3). The fact that at early times the accumulations of inositol phosphates produced by depolarization and by the proteinase inhibitor 'cocktail' were much less than additive would support the idea that the same peptide transmitter was responsible for both responses.

A variety of candidate peptides were tested to see whether they would stimulate an accumulation of inositol phosphates in ganglia (Table 3). LHRH and angiotensin had no effect. Pancreozymin and bradykinin produced small stimulations that were statistically significant, whereas bombesin appeared to evoke a similar, but statistically borderline, response. When incubated with depolarized ganglia, maximally active concentrations of both pancreozymin and bradykinin produced accumulations of inositol phosphates that appeared to be

Fig. 3. *Effect of proteinase inhibitors on inositol phosphate accumulation in control and depolarized ganglia* After labelling with [3 H]inositol, ganglia were preincubated for 5 min with 10 mM-LiCl and antipain, amastatin and bestatin (10 μ g/ml of each). Ganglia were then incubated for a further 90 min, in the presence or absence of 80 mM-KCl, together with the inhibitors.

Table 3. *Effects of neuropeptides on the accumulation of inositol phosphates in rat superior cervical ganglia* Ganglia were labelled as described in the text. After preincubation with 10 mM-LiCl for 5 min, ganglia were incubated in the presence of the appropriate neuropeptide for 90 min. The reactions were terminated and the inositol phosphates extracted and separated as described in the text. Abbreviation used: N.S., not significant. Values in parentheses represent the number of ganglia analysed.

Peptide	Stimulation of inositol phosphate accumulation (%)
LHRH (10 μ M)	5 (29) (N.S.)
Angiotensin (10 μ M)	0 (13)
Bombesin (1 μ M)	44 (13) (N.S.)
Pancreozymin (3 ivy dog units/ml)	41 (13) ($P<0.01$)
Bradykinin (10 μ M)	86 (20) ($P<0.001$)
Substance P analogue (10 μ M)	66 (44) ($P<0.05$)
Physalaemin (10 μ M)	20 (13) (N.S.)
Neurokinin α (10 μ M)	31 (10) ($P<0.05$)

additive with those produced by high K^+ alone (results not shown). A stable agonist analogue of substance P (Lee *et al.*, 1982; for its structure, see the Materials and methods section), which is active at SP_P and SP_E receptor subtypes, also stimulated an accumulation of inositol phosphates (Table 3).

Though statistically significant, this stimulation was inconsistent, was too small to account for the depolarization-induced response and was additive with the response to high K^+ (results not shown). Neither physalaemin nor neurokinin α (substance K), which show some selectivity for the SP_p and SP_E receptor subtypes respectively, produced greater stimulation than the substance P analogue (Table 3).

Effect of inhibitory neurotransmitters

Somatostatin and γ -amino-n-butyric acid are believed to be involved in inhibition of ganglionic neurotransmission. Neither had any effect on basal, vasopressin-stimulated, or muscarine-stimulated inositol phosphate accumulation in sympathetic ganglia (results not shown).

Discussion

In the present study we have confirmed and extended the observation of Nagata *et al.* (1973) that depolarization stimulates inositol lipid metabolism in sympathetic ganglia. The accumulation of inositol phosphates demonstrates that this process involves a phosphodiesterase-catalysed hydrolysis of inositol lipids, as in the responses of many tissues to a variety of receptor-directed stimuli (Michell, 1975; Michell *et al.*, 1981, 1984b; Berridge, 1984).

Early studies of electrically stimulated and acetylcholine-stimulated inositol lipid metabolism in sympathetic ganglia emphasized the probable localization of the response in the major neurons (Larrabee *et al.*, 1963; Larrabee & Leicht, 1965; Hokin, 1965). However, the only previous study of artificially depolarized ganglia included the observation that much of this response survived in axotomized ganglia, in which the postsynaptic neurons degenerate and non-neuronal elements proliferate (Nagata *et al.*, 1973). It seems likely, therefore, that appropriate stimuli may cause stimulation of inositol lipid breakdown in a variety of the cellular elements of sympathetic ganglia.

A major characteristic that distinguishes between the response of ganglia to depolarization and the response to direct stimulation of receptors (e.g. muscarinic cholinergic or V_1 -vasopressin) is that the former response requires the presence of extracellular Ca^{2+} . The most likely reason for this requirement is that depolarization leads to a Ca^{2+} -dependent release within the ganglia of an as-yet-unidentified neurotransmitter that stimulates inositol lipid breakdown.

The initial rate of accumulation of inositol phosphates during the first 15 min of depolarization in the presence of extracellular Ca^{2+} is approximately one-half that induced by a maxi-

mally active concentration of exogenous vasopressin (Bone *et al.*, 1984), suggesting that the endogenously released ligand is highly potent. However, the high rate of this stimulated phosphoinositide metabolism is transient, and inositol phosphate accumulation has returned to control rates within 45 min. It is possible that, on depolarization, either the potential-controlled Ca^{2+} channels inactivate or the effective neurotransmitter is rapidly released and the releasable intracellular stores become exhausted.

Many of the experiments reported here have been undertaken in an attempt to identify this potent endogenously released agonist. Acetylcholine, noradrenaline, histamine, 5-hydroxytryptamine, prostanooids, LHRH, angiotensin, bombesin, bradykinin, pancreozymin, substance P, neurokinin α and VLP (or vasopressin) all appear to have been eliminated as sole mediators of the atropine-resistant response to depolarization. However, there are a number of other putative peptide neurotransmitters in sympathetic ganglia which still might be responsible for the depolarization-induced inositol phosphate accumulation. These include neuropeptide Y (Lundberg *et al.*, 1982b), enkephalins (Schultzberg *et al.*, 1979) and neurotensin (Lundberg *et al.*, 1982a; Goedert & Emson, 1983). A possibility that cannot be excluded by our experimental protocols is that the large response to depolarization is the sum of the smaller responses to several endogenously released ligands.

The two receptor-directed stimuli (muscarinic cholinergic and V_1 -vasopressin) already known to stimulate inositol lipid breakdown in ganglia both cause a slow and sustained depolarization of some or all ganglionic neurons by inhibition of the neuronal M-current (an outward K^+ current) (Brown & Adams, 1980; Peters & Kreulen, 1984). Although we cannot yet identify the potent endogenously released neurotransmitter that is responsible for stimulation of inositol lipid breakdown in depolarized ganglia, it seems possible that this molecule might also inhibit the M-current and hence initiate a slow excitatory postsynaptic potential within intact ganglia. Its demonstrated ability to evoke a readily measured biochemical response may be an important aid in future approaches to its isolation and characterization.

Some years ago, this laboratory reported that inositol lipid metabolism is enhanced when ileum smooth muscle is depolarized in the presence of atropine, and we interpreted this result as evidence that inositol lipid breakdown is involved in the mechanism of action of potential-sensitive Ca^{2+} 'gates' at the plasma membrane (Jafferji & Michell, 1976). The recognition since then of many new peptide neurotransmitters almost certainly

renders this conclusion incorrect (Michell, 1982b), and Watson & Downes (1983) have noted that this response of ileum muscle to depolarization, like the response of sympathetic ganglia reported in the present paper, requires extracellular Ca^{2+} and is probably a response to an endogenously released neurotransmitter. Perhaps the clearest evidence that inositol lipids are not implicated in the mechanism of action of potential-controlled Ca^{2+} 'gates' comes from the observation that depolarization with high K^{+} of cloned tissue-culture cells that contain such ion channels does not modify inositol lipid metabolism (e.g. Schlegel *et al.*, 1981).

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