K⁺-stimulation of the phosphoinositide pathway in guinea-pig ileum longitudinal smooth muscle is predominantly neuronal in origin and mediated by the entry of extracellular Ca²⁺

¹Stephen P. Watson, Jeremy Lai & ²Toshiyuki Sasaguri

Department of Pharmacology, South Parks Road, Oxford OX1 3QT

- 1 K⁺ and scorpion toxin stimulate formation of inositol phosphates in guinea-pig ileum longitudinal smooth muscle slices. The response to these two agents is not additive.
- 2 The response to K⁺ is inhibited partially by nifedipine and partially by ω -conotoxin. When given together the effect of these two Ca²⁺ channel blockers is additive and the response to K⁺ is reduced by more than 80%.
- 3 The response to scorpion toxin is inhibited completely by tetrodotoxin, partially by ω -conotoxin but not by atropine or nifedipine. Scorpion toxin induces a similar formation of inositol phosphates in collagenase-dispersed cells to that seen in cross-chopped slices.
- 4 The responses to scorpion toxin and K⁺ are inhibited completely when the extracellular Ca²⁺ concentration is reduced to below cytosolic levels (<100 nM).
- 5 Neither nifedipine nor ω -conotoxin, either alone or in combination, inhibited formation of inositol phosphates by substance P or carbachol. Both of these agonists induced a significant formation of inositol phosphates even when the extracellular Ca²⁺ concentration was reduced to 10 nM.
- 6 These results indicate that K⁺ and scorpion toxin induce formation of inositol phosphates through the mobilisation of extracellular Ca²⁺. The response to K⁺ appears to occur predominantly in neuronal cells.

Introduction

In guinea-pig ileum longitudinal smooth muscle elevation of the extracellular K⁺ concentration induces hydrolysis of inositol phospholipids (Jafferji & Michell, 1976a; Best & Bolton, 1986; Sasaguri & Watson, 1988). Jafferji & Michell (1976a) proposed that the response to K⁺ is mediated by depolarization of the cell membrane leading to the entry of Ca²⁺ through voltage-operated channels. Support for this hypothesis was provided by the studies of Sasaguri & Watson (1988). These authors demonstrated that the response to K⁺ (without equimolar replacement of Na⁺) is inhibited completely in the presence of Cd²⁺ ions, which block voltage-operated Ca²⁺ channels non-selectively, and is significantly reduced in the presence of the dihydropyridine Ca²⁺ antagonist, nifedipine. Since the response to K⁺ is inhibited by these two Ca²⁺ channel blockers the activation of phospholipase C by K⁺ cannot be due to membrane depolarization as suggested by Best & Bolton (1986). It is also unlikely that the release of neurotransmitters has a significant role in this response as the formation of inositol phosphates by high K⁺ was similar in dispersed cells, which lack close synaptic contact, to that obtained with cross-chopped slices (Sasaguri & Watson, 1988). Moreover, Sasaguri & Watson (1988) were unable to detect a significant effect of atropine on this response. Thus it would seem that high K⁺-induced inositol phospholipid hydrolysis in guinea-pig ileum is attributable to voltage-operated Ca²⁺ entry, part of which is through dihydropyridine-sensitive channels.

Both Jafferji & Michell (1976b) and Sasaguri & Watson (1988) found that Ca²⁺ entry through voltage-operated channels does not contribute significantly to the formation of inositol phosphates by the muscarinic receptor agonist, carbachol. This is surprising since carbachol induces depolarization in this tissue (Bolton, 1972) and Ca²⁺ antagonists block carbachol-induced contraction implying an increase in intracellular Ca²⁺. Further the response to carbachol and K⁺

is slightly greater than additive (Sasaguri & Watson, 1988) thereby implying that these two stimuli induce formation of inositol phosphates either by distinct mechanisms or in different cell populations. (The guinea-pig ileum longitudinal smooth muscle contains additional cell types to smooth muscle cells e.g. neurones, fibroblasts and mast cells).

The aim of this work therefore has been to investigate the mechanism of the nifedipine-insensitive component of the response to K⁺ in the guinea-pig ileum and to identify the cell types in which the response to K⁺ takes place. Part of this work has been presented in preliminary form (Lai *et al.*, 1989).

Methods

Preparation of ³H-inositol-labelled tissue slices

The whole length of the ileum was removed from guinea-pigs (300–500 g) of either sex into pregassed (100% O₂) solution A (composition, mM: HEPES/Tris buffer 10, NaCl 135, KCl 4, MgCl₂ 2, CaCl₂ 2, KH₂PO₄ 1, glucose 12, pH 7.4) at 37°C. The ileum was perfused with solution A and longitudinal smooth muscle strips prepared by passing small segments over a glass rod and gently stroking the muscle layer away with moist cotton wool. Cross-chopped slices (350 μ m by 350 μ m) of the muscle were prepared with a McIlwain tissue chopper. The slices were washed four times with 20 ml of solution A over a period of 15 min, then incubated in 5 ml of solution A containing 30 μ Ci of myo-[³H]-inositol under O₂ for 1 h in a shaking water bath at 37°C.

The pre-labelled tissue slices were washed four times with 10 ml of solution A and then three times in a modified buffer containing EGTA (0.5 mM) and no added CaCl₂ over a period of 40 min in order to remove unincorporated [³H]-inositol and extracellular Ca²⁺. At zero time 35 μ l portions of the tissue were transferred to plastic flat-bottomed tubes containing 215 μ l of solution A with the Ca²⁺ concentration buffered using EGTA (see below), LiCl (12 mM) and other reagents as indicated. Tubes were gassed with O₂ and incubated in a shaking water bath at 37°C for 30 min. In experiments where ω -conotoxin was used the tissue slices were pre-incubated with

¹ Author for correspondence.

² Present address: Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan.

ω -conotoxin for 15 min at 37°C before addition of stimulatory agents.

Experiments on collagenase-dispersed cells were carried out as described previously (Sasaguri & Watson, 1988).

Assay of inositol phosphates

Reactions were terminated by addition of 940 μ l of chloroform/methanol/HCl (50:100:1 v/v); chloroform (310 μ l) and water (310 μ l) were added, the samples vortex-mixed and phases separated by centrifugation at 1000 g for 5 min. The upper aqueous phase (700 μ l) was diluted with 1 ml water and applied to a Dowex column. Inositol was removed with 16 ml of 5 mM sodium tetraborate/60 mM ammonium formate. Total [3 H]-inositol phosphates (inositol mono-, bis-, and tris-phosphates) were eluted with 6 ml of 0.8 M ammonium formate/0.1 M formic acid (Watson & Downes, 1983). The eluate was collected in scintillation vials and, after addition of 10 ml of Liquiscint, was counted in the gel phase for radioactivity. A portion of the lower organic phase (300 μ l) was transferred to scintillation vials, evaporated and, after addition of 2 ml Liquiscint, counted for radioactivity.

Data for [3 H]-inositol phosphates were normalised to total lipid d.p.m. (Watson & Downes, 1983). The combined amounts of inositol mono-, bis-, and tris-phosphates were measured rather than the individual phosphates since, after a 30 min incubation with excess KCl or carbachol, more than 90% of the stimulated increase can be found in the inositol monophosphate fraction (Sasaguri & Watson 1988). Results are expressed relative to basal levels of inositol phosphates and are shown in the text as mean $\pm$ s.e.mean. Statistical comparisons were made by a Mann-Whitney U-test.

Preparation of divalent cation: EGTA buffers

A number of Ca^{2+} :EGTA buffers (pH 7.4) were prepared containing different free calcium concentrations. Various quantities of CaCl_2 were added to a solution containing (mM): HEPES/Tris buffer 10, NaCl 135, KCl 4, glucose 12, KH_2PO_4 1, MgCl_2 2, EGTA 0.5. The total Ca^{2+} concentrations required to give desired free Ca^{2+} concentrations were calculated from the affinity constants determined by Fabiato & Fabiato (1979). It was assumed that the Ca^{2+} concentration in water is 10 μ M as this is the approximate concentration present after distillation.

The accuracy of the 10 nM, 100 nM, and 1 μ M free Ca^{2+} buffers was verified with the calcium binding fluorescent probe Indo-1 which has a K_d for Ca^{2+} of 250 nM at pH 7.4 (Grynkiewicz *et al.*, 1985). Fluorescence intensities were measured at 410 nm and actual free Ca^{2+} values were calculated from the equation:

$$\text{Free } \text{Ca}^{2+} = K_d[(F-F_{\min})/(F_{\max}-F_{\min})]$$

Materials

Scorpion toxin (*Leiurus quinquestriatus*), veratradine, nifedipine, atropine and carbachol were from Sigma. ω -Conotoxin and substance P were from Peninsula Laboratories. [3 H]-inositol (17.9 Ci mmol $^{-1}$) was from Amersham and Indo-1 from Calbiochem. All other reagents were of analytical grade.

Results

Scorpion toxin stimulates formation of inositol phosphates

Scorpion toxin and veratradine cause persistent activation of Na^+ channels in neurones by blocking channel inactivation (Catterall, 1987). Persistent Na^+ channel activation causes depolarization of the membrane and may therefore give rise to the activation of phospholipase C via voltage-sensitive Ca^{2+} entry. In the guinea-pig ileum longitudinal smooth muscle

Figure 1 Concentration-response curve for formation of inositol phosphates by scorpion toxin. Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, pre-labelled with [3 H]-inositol, were incubated with scorpion toxin in the absence (□) or presence (■) of 1 μ M tetrodotoxin for 30 min. Results are shown as mean % increase over basal values from four experiments performed in quadruplicate; s.e.mean shown by vertical bars.

Na^+ channels are located on neurones, but not smooth muscle cells (Gershon, 1967).

Scorpion toxin stimulated a concentration-dependent formation of inositol phosphates (Figure 1). Similar results were seen with veratradine (not shown). At a near maximally effective concentration, scorpion toxin (12 μ g ml $^{-1}$) induced a $309 \pm 13\%$ increase of inositol phosphates above basal. Tetrodotoxin (1 μ M) inhibited this response to scorpion toxin (Figure 1) confirming that it is initiated through the opening of Na^+ channels. On the other hand, atropine had no significant effect on the response to 12 μ g ml $^{-1}$ scorpion toxin (response in the presence of atropine = $295 \pm 18\%$). Scorpion toxin (12 μ g ml $^{-1}$) induced a similar formation of inositol phosphates in collagenase dispersed cells ($307 \pm 29\%$) to that seen in cross-chopped slices.

Responses to K^+ and scorpion toxin are not additive

We have previously shown that K^+ stimulates a concentration-dependent formation of inositol phosphates in guinea-pig ileum longitudinal smooth muscle and that this is not altered significantly in the presence of atropine or tetrodotoxin (Sasaguri & Watson, 1988). At a near maximally effective concentration the response to K^+ is similar to that seen with scorpion toxin or veratradine and, when given in combination, the responses to all three agents show little additivity (Table 1). The small increases observed are probably due to the use of nearly maximal rather than supramaximal concentrations of each stimulant.

Table 1 Formation of inositol phosphates by K^+ , scorpion toxin and veratradine is not additive

	Inositol phosphates (% basal)
K^+ (112 mM)	248 ± 6
Scorpion toxin (12 μ g ml $^{-1}$)	260 ± 14
Veratradine (100 μ M)	236 ± 10
K^+ + scorpion toxin	273 ± 13
K^+ + veratradine	280 ± 12
Scorpion toxin + veratradine	284 ± 12

Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, prelabelled with [3 H]-inositol, were challenged with K^+ , scorpion toxin and veratradine either alone or in combination. Results are shown as mean $\pm$ s.e.mean of six experiments and represent % basal values of inositol phosphates (= 100%).

Figure 2 Concentration-response curve for formation of inositol phosphates (InsPs) by Ca^{2+} in the presence of nifedipine or ω -conotoxin. Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, pre-labelled with [3 H]-inositol, were incubated with various concentrations of Ca^{2+} for 30 min. The Ca^{2+} concentration was controlled by use of Ca^{2+} /EGTA buffer (see methods). Results are shown as mean % increases over basal values ($Ca^{2+} = 10$ nM) from one experiment performed in quadruplicate; two other experiments gave similar results; s.e. mean shown by vertical bars. (a) (□) Control; (■) ω -conotoxin (100 nM). (b) (□) Control; (■) nifedipine (1 μ M). The difference in maximal responses seen in (a) and (b) reflects inter-experimental variation.

Accumulation of inositol phosphates as a function of extracellular Ca^{2+} concentration

EGTA (3 mM), sufficient to reduce the extracellular Ca^{2+} concentration from 2 mM to 300 nM, was found to inhibit significantly the responses to K^+ (112 mM) and carbachol (1 mM). These responses recovered completely following the addition of Ca^{2+} (3 mM) to the extracellular medium (results not shown). This demonstrates that the inhibitory effect of EGTA is due to the removal of Ca^{2+} and that EGTA can be used to buffer the Ca^{2+} concentration.

Surprisingly, increasing the extracellular Ca^{2+} concentration from 10 nM to 2 mM induced a rise in inositol phosphates from $100 \pm 8.4\%$ to $438 \pm 14\%$ (100% is the response at 10 nM Ca^{2+}). Thus a rise in extracellular Ca^{2+} alone is able to elicit a response. A full dose-response curve for this is shown in Figure 2.

The response to elevated potassium was abolished completely in the presence of 10 nM Ca^{2+} ($94 \pm 4\%$ compared to the control value of $100 \pm 6\%$). The response to K^+ recovered partially when the Ca^{2+} concentration was raised from 100 nM to 100 μ M ($500 \pm 31\%$ compared to control $325 \pm 41\%$). Thereafter there was a sharp increase in formation of inositol phosphates by K^+ to $1246 \pm 67\%$ compared with $501 \pm 25\%$ at 2 mM Ca^{2+} . A full dose-response curve to K^+ is shown in Figure 3a. Similar results were seen with scorpion toxin (see Figure 3b). There was no significant formation of inositol phosphates by scorpion toxin at 10 nM extracellular Ca^{2+} and a marked increase in response as extracellular Ca^{2+} was raised from 100 μ M to 2 mM.

Figure 3 Concentration-response curves for formation of inositol phosphates (InsPs) by K^+ and scorpion toxin over a range of extracellular Ca^{2+} concentrations. Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, pre-labelled with [3 H]-inositol, were incubated with various concentrations of Ca^{2+} for 30 min in the presence of K^+ (112 mM) or scorpion toxin (12 μ g/ml). The Ca^{2+} concentration was controlled by use of Ca^{2+} /EGTA buffer (see methods). Results are shown as mean % increases over basal values ($Ca^{2+} = 10$ nM) from one experiment performed in quadruplicate; s.e. mean shown by vertical bars. Two other experiments gave similar results. (a) (□) Control; (■) K^+ . (b) (□) Control; (■) scorpion toxin.

In contrast, both carbachol (1 mM) and substance P (1 μ M) gave a significant response at 10 nM extracellular Ca^{2+} ($426 \pm 40\%$ basal and $269 \pm 29\%$ basal, respectively). The responses were potentiated as extracellular Ca^{2+} was increased to 2 mM; the carbachol response showing a much greater sensitivity to potentiation by Ca^{2+} (Figure 4).

Effect of Ca^{2+} channel blockers on accumulation of inositol phosphates

To investigate the role of Ca^{2+} entry through voltage-operated channels in the formation of inositol phosphates, ω -conotoxin, which blocks N-type Ca^{2+} channels (Tsien *et al.*, 1988), and nifedipine, which blocks L-type Ca^{2+} channels (Tsien *et al.*, 1988), were used.

Neither ω -conotoxin (100 nM) nor nifedipine (10 μ M) had any influence on the accumulation of inositol phosphates by increasing extracellular Ca^{2+} from 10 nM to 2 mM (Figure 2). Thus Ca^{2+} channels are not involved in extracellular Ca^{2+} stimulated formation of inositol phosphates. Similarly, neither agent had any effect on the responses to carbachol or substance P either on their own (not shown) or in combination (Table 2).

When given on their own ω -conotoxin (100 nM) or nifedipine (10 μ M) significantly reduced the response to K^+ by 40–50% (Table 2). When given in combination the effect of these two Ca^{2+} channel blockers was additive, the response to K^+ being blocked by more than 80% (Table 2). Similarly, ω -conotoxin (100 nM) partially inhibited the response to scorpion toxin but, in contrast to K^+ , the response to scorpion toxin was not altered in the presence of nifedipine (Table 2).

Figure 4 Concentration-response curves for formation of inositol phosphates (InsPs) by carbachol and substance P over a range of extracellular Ca^{2+} concentrations. Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, pre-labelled with [^3H]-inositol, were incubated with various concentrations of Ca^{2+} for 30 min in the presence of carbachol (1 mM) or substance P (1 μ M). The Ca^{2+} concentration was controlled by use of Ca^{2+} /EGTA buffer (see methods). Results are shown as mean % increases over basal values (Ca^{2+} = 10 nM) from one experiment performed in quadruplicate; s.e.mean shown by vertical bars. One other experiment gave similar results. (a) (□) Control; (■) carbachol. (b) (□) Control; (■) substance P (N.B. carbachol induces a much larger stimulation than substance P as shown by the scale differences).

Effect of divalent cations on the accumulation of inositol phosphates

The possibility that there may exist a divalent cation receptor linked to the inositol phospholipid pathway as reported for bovine parathyroid cells (Schoback *et al.*, 1988) was investigated. None of the chloride salts of Cd^{2+} (300 μ M), Ba^{2+} (2 mM), Co^{2+} (2 mM) or Ni^{2+} (2 mM) induced a significant increase in the level of inositol phosphates relative to 10 nM Ca^{2+} with the exception of Ni^{2+} which gave a small response (Table 3). In addition, none of the divalent cations mimicked the ability of Ca^{2+} to potentiate carbachol-stimulated accumulation of inositol phosphates (Table 3).

Table 3 Various divalent cations are unable to mimic Ca^{2+} -induced formation of inositol phosphates or potentiation of the response to carbachol

	[^3H]-inositol phosphates (% of basal)
Control	100 $\pm$ 4
Ca^{2+} 2 mM	473 $\pm$ 43
Cd^{2+} 300 μ M	90 $\pm$ 7
Ba^{2+} 2 mM	96 $\pm$ 9
Co^{2+} 2 mM	104 $\pm$ 6
Ni^{2+} 2 mM	163 $\pm$ 10
Carbachol (1 mM)	378 $\pm$ 21
Carbachol + Ca^{2+} 2 mM	3733 $\pm$ 124
Carbachol + Cd^{2+} 300 μ M	378 $\pm$ 43
Carbachol + Ba^{2+} 2 mM	280 $\pm$ 7
Carbachol + Ni^{2+} 2 mM	467 $\pm$ 74

Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, prelabelled with [^3H]-inositol, were challenged with various divalent cations in the absence or presence of carbachol (1 mM) in buffer in which the Ca^{2+} concentration was 10 nM unless stated otherwise. Results are shown as mean $\pm$ s.e.mean of a single experiment (repeated with essentially similar results) and represent % basal level of inositol phosphates at 10 nM Ca^{2+} .

Discussion

Mechanism of K^+ stimulated inositol phospholipid hydrolysis

These results provide further evidence that the response to K^+ is mediated by entry of extracellular Ca^{2+} . The K^+ response is abolished when extracellular Ca^{2+} is lowered to levels which abolish the Ca^{2+} gradient across the plasma membrane, and is greatly reduced by the inclusion of the Ca^{2+} channel blockers nifedipine and ω -conotoxin.

The response to K^+ appears to occur predominantly in neuronal tissue. The dihydropyridine-insensitive component of the K^+ response is inhibited by ω -conotoxin which blocks N-type channels present in neuronal cells but absent in smooth muscle (Tsien *et al.*, 1988). Further, the nifedipine-sensitive component of the response to K^+ is unlikely to occur in smooth muscle cells. The formation of inositol phosphates by carbachol is insensitive to concentrations of nifedipine which block its contractile ability (therefore suggesting that nifedipine is blocking the increase of intracellular Ca^{2+} which normally enters through voltage operated channels and the responses to K^+ and carbachol are slightly greater than additive indicating that they occur in separate cells (Sasaguri & Watson, 1988). Further, the responses to K^+ and scorpion toxin (which stimulates formation of inositol phosphates in neuronal tissue—see below) are not additive. Taken together, these data suggest that the response to K^+ occurs by Ca^{2+} entry through L-type and N-type channels located in neuronal cells.

Table 2 Effect of Ca^{2+} channel blockers on formation of inositol phosphates

	Control	+ ω -Conotoxin (100 nM)	+ Nifedipine (10 μ M)	+ ω -Conotoxin & nifedipine
K^+ (112 mM)	284 $\pm$ 17	206 $\pm$ 14*	210 $\pm$ 10*	135 $\pm$ 8*
Scorpion toxin (12 μ g ml $^{-1}$)	295 $\pm$ 18	238 $\pm$ 13*	276 $\pm$ 12	228 $\pm$ 16*
Carbachol (1 mM)	1012 $\pm$ 122	—	—	1074 $\pm$ 48
Substance P (1 μ M)	252 $\pm$ 28	—	—	261 $\pm$ 24

Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, prelabelled with [^3H]-inositol, were challenged for 30 min with various agents in the presence or absence of ω -conotoxin and/or nifedipine. Tissues were preincubated with ω -conotoxin for 15 min before the addition of other compounds. Results are shown as mean $\pm$ s.e.mean of three to five experiments and represent % basal levels of inositol phosphates (= 100%).

The possibility that the K^+ response occurs predominantly in neurones suggests that phospholipase C is activated by increases in cytosolic Ca^{2+} in neurones but not smooth muscle cells. This may reflect the existence of different species of phospholipase C in the two cell types. The existence of multiple species of phospholipase C is well established and it is clear that some, but not all, species are activated by the mobilisation of Ca^{2+} over its physiological range (Cockcroft, 1987).

Mechanism of scorpion toxin-stimulated inositol phospholipid hydrolysis

The formation of inositol phosphates by scorpion toxin is initiated by the opening of the Na^+ channel since it is inhibited in the presence of tetrodotoxin. The molecular basis of the response appears to involve the entry of extracellular Ca^{2+} as it is abolished when the Ca^{2+} gradient across the membrane is removed. As with K^+ , a significant proportion of this Ca^{2+} entry occurs through ω -conotoxin-sensitive channels which open as a consequence of membrane depolarization. The basis of the ω -conotoxin insensitive component of the response to scorpion toxin is uncertain; however, it does not appear to be related to the entry of Ca^{2+} through L-type Ca^{2+} channels since it is not blocked by nifedipine. Scorpion toxin must therefore induce the entry of extracellular Ca^{2+} through an additional, uncharacterized pathway e.g. Na^+/Ca^{2+} exchange.

Several lines of evidence indicate that the activation of phospholipase C by scorpion toxin takes place in neuronal tissue. Na^+ channels are located on neurones and are absent from smooth muscle cells in this preparation (Gershon, 1967). Further, a similar response to scorpion toxin is observed in collagenase-dispersed cells even though these lack the close synaptic contacts found in slice preparations and thus would be expected to minimize the influence of neurotransmitter release. Finally, the response to scorpion toxin is not altered in the presence of atropine which inhibits the response to the major excitatory transmitter in the ileum, acetylcholine.

References

- BEST, L. & BOLTON, T.B. (1986). Depolarisation of guinea-pig visceral smooth muscle causes hydrolysis of inositol phospholipids. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **333**, 78–82.
- BOLTON, T.B. (1972). The depolarizing action of acetylcholine or carbachol in intestinal smooth muscle. *J. Physiol.*, **220**, 647–671.
- CATTERALL, W.A. (1987). Molecular pharmacology of voltage-sensitive sodium channels. *Trends Pharmacol. Sci.*, **8**, 57–65.
- COCKCROFT, S. (1987). Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, Gp. *Trends Biochem. Sci.*, **12**, 75–78.
- FABIATO, A. & FABIATO, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)*, **75**, 463–501.
- GERSHON, M.D. (1967). Effects of tetrodotoxin on innervated smooth muscle preparations. *Br. J. Pharmacol. Chemother.*, **29**, 259–279.
- 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.
- JAFFERJI, S.S. & MICHELL, R.H. (1976a). Investigation of the relationship between cell-surface calcium ion gating and phosphatidylinositol turnover by comparison of the effects of elevated extracellular potassium ion concentration on ileum smooth muscle and pancreas. *Biochem. J.*, **160**, 397–399.
- JAFFERJI, S.S. & MICHELL, R.H. (1976b). Effects of calcium antagonistic drugs on the stimulation by carbamoylcholine and histamine of phosphatidylinositol turnover in longitudinal smooth muscle of guinea-pig ileum. *Biochem. J.*, **160**, 163–169.
- LAI, J., SASAGURI, T. & WATSON, S.P. (1989). K^+ stimulation of the phosphoinositide pathway in guinea-pig ileum is predominantly neuronal in origin and mediated by Ca^{2+} entry. *Br. J. Pharmacol.*, **97**, 450P.
- SASAGURI, T. & WATSON, S.P. (1988). Lowering of the extracellular Na^+ concentration enhances high K^+ -induced formation of inositol phosphates in the guinea-pig ileum. *Biochem. J.*, **252**, 883–888.
- SHOBACK, D.M., MEMBRENO, A. & MCGHEE, J.G. (1988). High calcium and other divalent cations increase inositol trisphosphate in bovine parathyroid cells. *Endocrinology*, **123**, 382–389.
- TSIEN, R.W., LIPSCOMBE, D., MADISON, D.V., BLEY, K.R. & FOX, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.*, **11**, 431–438.
- WATSON, S.P. & DOWNES, C.P. (1983). Substance P induced hydrolysis of inositol phospholipids in guinea-pig ileum and rat hypothalamus. *Eur. J. Pharmacol.*, **93**, 245–253.

Role of extracellular Ca^{2+} in receptor-stimulated inositol phospholipid hydrolysis

Carbachol and substance P evoke a significant stimulation of inositol phospholipid hydrolysis even when the extracellular Ca^{2+} concentration is reduced below the normal cytosolic level. This is consistent with the activation of phospholipase C via a G-protein rather than through the entry of extracellular Ca^{2+} . The responses to carbachol and substance P, however, are potentiated when the extracellular Ca^{2+} concentration is raised from 10 nM to 2 mM. The mechanism of this effect is unknown. Conceivably, Ca^{2+} could influence the activity of phospholipase C without gaining entry to the cell by an action on the receptors for substance P or carbachol, or by binding to an extracellular domain of phospholipase C or its G-protein.

Extracellular Ca^{2+} -stimulated inositol phospholipid hydrolysis

Raising the extracellular concentration of Ca^{2+} from 10 nM to 2 mM induces formation of inositol phosphates. This response is insensitive to ω -conotoxin and nifedipine suggesting that it is not due to the entry of Ca^{2+} through voltage-operated channels although the possibility of entry through non-voltage gated channels cannot be ruled out. A receptor for Ca^{2+} and other divalent cations linked to the activation of the phosphoinositide pathway has been described in bovine parathyroid cells (Shoback *et al.*, 1988). If such a receptor exists in guinea-pig ileum it must be highly specific for Ca^{2+} since other divalent cations are unable to mimic the effect of Ca^{2+} .

This work was supported by the Medical Research Council. S.P.W. is a Royal Society University Research Fellow. The experiments with scorpion toxin were the result of a stimulating discussion with Fabian Gusovsky.

(Received March 28, 1989
Revised August 17, 1989
Accepted August 24, 1989)