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DOES THE HYDROLYSIS OF INOSITOL PHOSPHOLIPIDS LEAD TO THE OPENING OF VOLTAGE OPERATED CA^{2+} CHANNELS IN GUINEA-PIG ILEUM? STUDIES WITH FLUORIDE IONS AND CAFFEINE

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SUMMARY Fluoride ions (1 - 30 mM) stimulate phosphoinositide hydrolysis in guinea-pig ileum longitudinal smooth muscle slices, and this is not inhibited in the presence of indomethacin or nifedipine. This action is associated with a slow contractile response which peaks after approximately five minutes and then declines towards baseline; at this time the contractile response to a maximally effecive concentration of carbachol is also inhibited. Fluoride-induced contractions are inhibited completely in the presence of nifedipine. Similarly, contractions induced by caffeine, which releases Ca^{2+} from intracellular stores, are also inhibited by nifedipine. These data are consistent with a model in which the activation of a G-protein by F^- ions leads to the following sequential events: activation of phospholipase C, release of intracellular Ca^{2+}, opening of voltage operated (i.e. dihydropyridine sensitive) Ca^{2+} channels and contraction. The transient nature of the fluoride contraction and the inhibition of the carbachol contraction may be due to a slow elevation of cAMP levels induced by F^-

Contractions of the guinea-pig ileum longitudinal smooth muscle produced by various agonists e.g. substance P, carbachol or histamine, and by non-receptor mechanisms e.g. high K^+ or low Na^+ buffers, are associated with an increased hydrolysis of phosphoinositides (1-5). The relationship between these two events is uncertain. Contractions induced by sub-maximal concentrations of the above stimuli are very sensitive to Ca^{2+} channel blockers, e.g. nifedipine or Cd^{2+}, and to the removal of extracellular Ca^{2+} (6,7). This suggests that the majority of the Ca^{2+} required for contraction is derived from the extracellular medium. The hydrolysis of phosphatidylinositol 4,5-bisphosphate generates two second messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which stimulate the release of intracellular Ca^{2+} and activate protein kinase C, respectively (8). The role of these two messengers in the sequence of events leading to contraction is not known. The present study now provides evidence that the release of intracellular Ca^{2+}, presumably by the action of inositol 1,4,5-trisphosphate, is involved in the opening of the voltage operated Ca^{2+}

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channels. The activation of phospholipase C appears to involve the activation of a G-protein since inositol phosphates levels are increased in the presence of F^-, a potent modulator of several G-proteins including G_s, G_i and transducin (9-11).

METHODS

Male guinea-pigs (300 - 500 g) were killed by a blow to the head and longitudinal muscle strips of ileum prepared by gently teasing away the muscle layer with cotton wool (2). The muscle strips were either mounted in 3 ml organ baths for isotonic tension recordings or were cross-chopped (350 x 350 um) on a McIlwain tissue chopper for biochemical studies (2). The formation of total [^{3}H]inositol phosphates was carried out as previously described (2); LiCl (10 mM) was included in the incubation buffer in order to block the metabolism of inositol monophosphate to free inositol. Total inositol phosphates represents the fraction eluted from Dowex columns by 6 ml of 800 mM ammonium formate / 0.1 M formic acid after an earlier wash with 16 ml of 60 mM ammonium formate / 5 mM sodium tetraborate to remove inositol and glycerophosphoinositol; the total inositol phosphate fraction therefore contains inositol mono-, bis- and trisphosphates (12). In some experiments the elution of these three inositol phosphates and also inositol tetrakisphosphate from the Dowex columns were analysed separately (12). The formation of cAMP in the cross-chopped slices was measured as described previously (13).

The following reagents were used: carbachol, caffeine, isoprenaline, forskolin, theophylline, indomethacin and nifedipine were from Sigma, Poole, Dorset; the cAMP radioimmunoassay kit and [^{3}H]inositol were from Amersham; Dowex anion exchange resin (200 - 400 mesh) was from Bio-Rad. All other reagent were of analytical grade. [^{3}H]Inositol was cleaned before use by passage through a Dowex column (2).

RESULTS

Formation of inositol phosphates

Sodium fluoride induced a concentration dependent formation of total [^{3}H]inositol phosphates with a maximal effect at 10 mM (% increase above basal after a 30 min incubation with 10 mM F^- was 204 $\pm$ 17 ; n = 14); this action was not altered significantly in the presence of Al^{3+}, indomethacin or nifedipine (Figure 1). No precautions were taken to remove Al^{3+} from the glass and plastic-ware, and so sufficient Al^{3+} may have been present for F^- to activate G-proteins (14). The lack of effects of indomethacin and nifedipine indicate that the stimulatory action of F^- is not due to formation of cyclooxygenase products or entry of extracellular Ca^{2+}, respectively.

Analyses of the individual inositol phosphates revealed that the main increase was in the inositol monophosphate fraction with smaller changes in inositol bis- and trisphosphates and no increase in inositol tetraphosphates after 10 or 30 minutes (data not shown). The levels of inositol monophosphate increased throughout the 30 min incubation. The low levels of the inositol polyphosphates relative to inositol monophosphate resembles similar observations made in histamine or substance P-stimulated guinea-pig ileum longitudinal muscle strips (2,3).

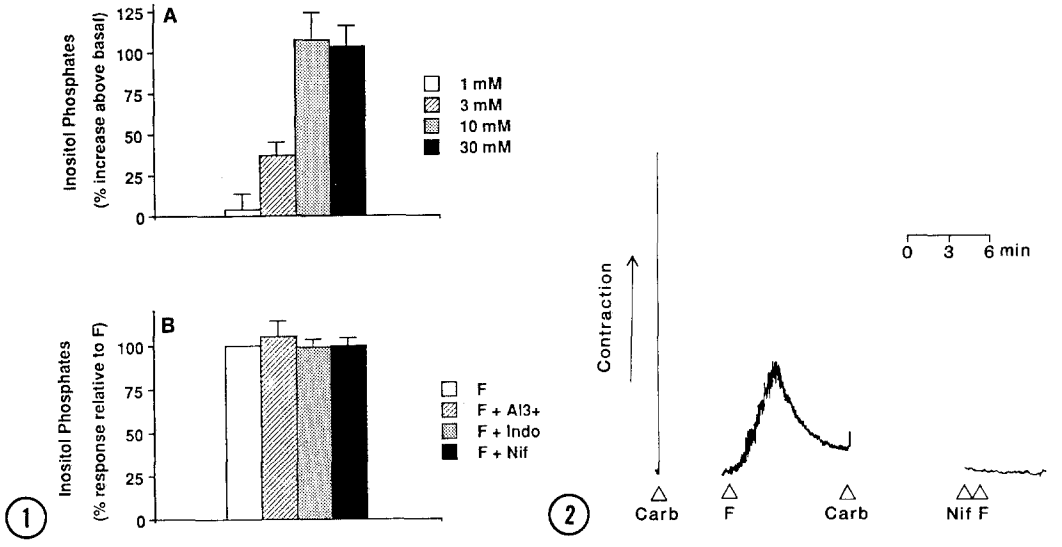

Figure 1A: Concentration response curve for sodium fluoride-induced formation of [^{3}H]inositol phosphates

Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, pre-labelled with [^{3}H]inositol, were challenged with increasing concentrations of sodium fluoride for 30 min. Results are expressed as percentage increase above basal and represent means ± S.E.M. from three experiments performed in triplicate. The mean d.p.m. in the basal fraction was 2189 ± 646 (s.d).

Figure 1B: Effect of various agents on F^- -induced formation of [3H]inositol phosphates

The effect of 100 uM Al^{3+}, 10 uM indomethacin (indo) and 1 uM nifedipine (nif) on F^- (10 mM)-induced formation of inositol phosphates was investigated as described above. Results are expressed as percentage response relative to F^- controls (100 % indicates no affect) and represent the means ± S.E.M. of three experiments performed in triplicate.

Figure 2. Fluoride stimulates a transient contraction of guinea-pig ileum

Shown is a representative trace from three separate experiments illustrating F^- (10 mM)-induced contraction of guinea-pig ileum longitudinal smooth muscle and inhibition of carbachol (Carb)-induced contraction. The contractile response to F^- was inhibited by 1 uM nifedipine (Nif).

Caffeine (10 mM) had no significant effect on the resting levels of inositol phosphates (not shown).

Contractile studies

Sodium fluoride (10 mM) stimulated a slow increase in tension of guinea-pig ileum longitudinal smooth muscle which peaked after approximately 5 min and then declined towards baseline tension (Figure 2). The peak size of the F^- contraction was 35 ± 2.2 (n = 5) % of the response to carbachol (10 uM). In contrast, the response to a just maximally effective concentration of carbachol (1 uM) peaked within 10 sec but was inhibited when given during the decline of the F^- contraction (Figure 2) suggesting that F^- produces a

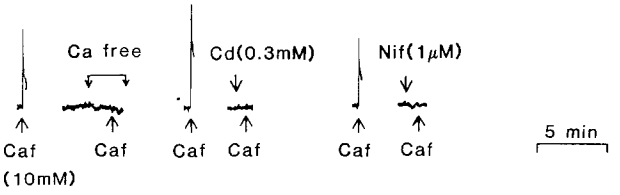

Figure 3. Nifedipine inhibits caffeine-induced contraction of guinea-pig ileum

Shown is a representative trace from three separate experiments illustrating caffeine (10 mM)-induced contraction of guinea-pig ileum longitudinal smooth muscle and inhibition of contraction by the replacement of extracellular Ca^{2+} with 2 mM EGTA or by the presence of nifedipine (Nif) or Cd^{2+}. The response to 10 mM caffeine was 19.0 ± 2.1 % of the response to a maximally effective concentration of carbachol (10 uM).

general relaxation of the smooth muscle. F^- -induced contraction was inhibited completely in the presence of nifedipine (Figure 2) or by Cd^{2+} (not shown).

Caffeine (10 mM) stimulated a rapid contraction of guinea-pig ileum longitudinal smooth muscle with a peak tension of 19.2 ± 2.4 % of the response to a maximally effective concentration of carbachol (10 uM). Caffeine-induced contractions were inhibited by nifedipine, Cd^{2+} or by the removal of extracellular Ca^{2+} (Figure 3).

cAMP measurements

Sodium fluoride (10 mM) induced a time-dependent increase in cAMP levels which had reached statistical significance by 30 min (Table 1). In contrast, isoprenaline and/or forskolin evoked massive increases in the intracellular cAMP concentration by 10 min (Table 1) but had no effect on carbachol-induced formation of total inositol phosphates (Table 2). The latter observation demonstrates that the activation of phospholipase C by F^- is not under regulatory control from cAMP.

Table 1: Fluoride increases cAMP levels in guinea-pig ileum

	pmol cAMP 10 min	pmol cAMP 30 min
Control	0.70 ± 0.1	0.83 ± 0.1
NaF (10 mM)	0.82 ± 0.2	1.51 ± 0.1
Isoprenaline (100 uM) + Forskolin (2 uM)	15.8 ± 2.0	-

Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle were challenged with sodium fluoride (10 mM) or isoprenaline (100 uM) and forskolin (1 uM) in the presence of theophylline (10 mM) for 10 or 30 min. cAMP levels were estimated by radioimmunoassay. Results are shown as means ± S.E.M. of three separate experiments and represent pmol cAMP / 50 ul gently packed tissue slices.

Table 2: Forskolin and/or Isoprenaline do not inhibit carbachol-induced formation of inositol phosphates in guinea-pig ileum

	[3H]inositol phosphates (% basal)
Basal	100 ± 7.8
Carbachol	1009 ± 43.2
Carbachol + Isoprenaline	1016 ± 41.6
Carbachol + Forskolin	1047 ± 69.7
Carbachol + Isoprenaline + Forskolin	997 ± 53.4

Cross-chopped slices of guinea-pig ileum longitudinal smooth muscle, pre-labelled with [^{3}H]inositol, were challenged with carbachol (1 mM) for 30 min in the presence of isoprenaline (100 uM) and/or forskolin (2 uM). Results are shown as means ± S.E.M. of three experiments performed in triplicate.

DISCUSSION

F^- ions have been recently reported to stimulate formation of inositol phosphates in a variety of non-excitable cells including the rat parotid gland (14), human platelets (15) and neutrophils (16). The present study has now shown a similar effect of F^- ions in an electrically excitable tissue, the guinea-pig ileum longitudinal smooth muscle. Several lines of evidence support the idea that this action of F^- occurs through the activation of a G-protein: i) at the concentrations used in these studies F^- has been reported to activate several G-proteins including G_s and G_i (9-11), ii) the formation of inositol phosphates by F^- in guinea-pig ileum is not altered in the presence of cyclooxygenase inhibitors; thus this effect is not mediated through the formation of endoperoxides and thromboxanes which activate phospholipase C (17), iii) the formation of inositol phosphates by F^- in guinea-pig ileum is not altered in the presence of Ca^{2+} channel blockers; thus this effect is not mediated through the influx of Ca^{2+} through voltage operated channels (compare with mechanism of K^+-activation of phospholipase C [5]), iv) caffeine, at a concentration which produces a similar contractile response to F^-, does not induce formation of inositol phosphates; thus the activation of phospholipase C by F^- is not due to the release of intracellular Ca^{2+}. The hypothesis that a G-protein is involved in the activation of phospholipase C in smooth muscle is further supported by the observation that GTP or its stable analogues induce inositol phosphate formation in permeabilised cells e.g. human platelets (18) and also in membrane preparations e.g. porcine coronary artery (19). For a full review of the evidence for the involvement of a G-protein in the activation of phospholipase C see Cockcroft (20).

The activation of phospholipase C by F- in guinea-pig ileum was associated with a weak contractile response suggesting that these events may be linked. It seems unlikely, however, that the onset of contraction involves the activation of protein kinase C by 1,2-diacylglycerol. Salmon et al (21)

have reported that phorbol ester or membrane permeable 1,2-diacylglycerols have no affect on the resting tension or the contraction of the guinea-pig ileum when stimulated by carbachol. In contrast, Baraban et al (22) have shown that the activation of protein kinase C in this tissue by a number of phorbol esters inhibits agonist-induced contractions. The role of inositol 1,4,5-trisphosphate in smooth muscles appears to be involved with the release of intracellular Ca^{2+} from sarcoplasmic reticulum (23-24) and the present study provides evidence that this may be involved indirectly in the onset of contraction. Thus contractions induced by F^-, which stimulates phospholipase C, or by caffeine, which releases intracellular Ca^{2+} (25), are inhibited by the Ca^{2+} channel antagonist nifedipine. The mechanism whereby the release of intracellular Ca^{2+} leads to the onset of depolarisation, which is the trigger for the opening of the voltage operated Ca^{2+} channels, is not known but could involve the activation of a Ca^{2+}-dependent kinase or a change in the opening time of a Ca^{2+}-dependent ion channel.

The decline of the contractile response to F^- is not due to inhibition of phospholipase C activity which continues for at least 30 min. The observation that the contractile response to carbachol is also inhibited following the loss of the fluoride contraction would suggest that F^- induces a non-specific relaxation of the smooth muscle. F^- ions stimulate a slow increase in the formation of cAMP in this tissue, most likely through the activation of G_s (9-11), and cAMP is known to stimulate the relaxation of all smooth muscles (6).

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