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Turnover of bis-diphosphoinositol tetrakisphosphate in a smooth muscle cell line is regulated by β_2-adrenergic receptors through a cAMP-mediated, A-kinase-independent mechanism

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Bis-diphosphoinositol tetrakisphosphate ([PP]$_2$-InsP_4 or 'InsP_8') is a 'high-energy' inositol phosphate; we report that its metabolism is receptor-regulated in DDT$_1$ MF-2 smooth muscle cells. This conclusion arose by pursuing the mechanism by which F^- decreased cellular levels of [PP]$_2$-InsP_4 up to 70%. A similar effect was induced by elevating cyclic nucleotide levels, either with IBMX or by application of either Bt$_2$cAMP (EC_{50} = 14.7 μM), Bt$_2$cGMP (EC_{50} = 7.9 μM) or isoproterenol (EC_{50} = 0.4 nM). Isoproterenol (1 μM) decreased [PP]$_2$-InsP_4 levels 25% by 5 min, and 71% by 60 min. This novel, agonist-mediated regulation of [PP]$_2$-InsP_4 turnover was very specific; isoproterenol did not decrease the cellular levels of either inositol pentakisphosphate, inositol hexakisphosphate or other diphosphorylated inositol polyphosphates. Bradykinin, which activated phospholipase C, did not affect [PP]$_2$-InsP_4 levels. Regulation of [PP]$_2$-InsP_4 turnover by both isoproterenol and cell-permeant cyclic nucleotides was unaffected by inhibitors of protein kinases A and G. The effectiveness of the kinase inhibitors was confirmed by their ability to block phosphorylation of the cAMP response element-binding protein. Our results indicate a new signaling action of cAMP, and furnish an important focus for future research into the roles of diphosphorylated inositol phosphates in signal transduction.

Keywords: β-adrenergic/cAMP/fluoride/inositol phosphates/receptor

Introduction

Organisms from across the phylogenetic spectrum synthesize an assortment of phosphorylated inositol derivatives. Although this family of compounds all share the cyclohexanehexol moiety, they provide the cell with a functionally diverse range of molecules. Their versatility as intracellular signals is particularly remarkable; different sub-groups of inositol-based compounds each have specialized roles in some quite different signal transduction processes (Berridge and Irvine, 1989; Kapeller and Cantley, 1994; Shears, 1997). In this report, we provide a new focus for studies in this field of research, by demonstrating a specific receptor-dependent regulation of the turnover of bis-diphosphoinositol tetrakisphosphate ([PP]$_2$-InsP_4).

[PP]$_2$-InsP_4 is a diphosphorylated polyphosphate, the synthesis and metabolism of which is regulated by two coupled kinase/phosphatase substrate cycles (Figure 1). [PP]$_2$-InsP_4 is formed by an ATP-dependent PP-InsP_5 kinase (Shears *et al.*, 1995); PP-InsP_5 is in turn synthesized from an ATP-dependent InsP_6 kinase (Menniti *et al.*, 1993; Stephens *et al.*, 1993; Shears *et al.*, 1995; Voglmaier *et al.*, 1996). A third diphosphate, PP-InsP_4, is interconverted with InsP_5 in another kinase/phosphatase substrate cycle (Menniti *et al.*, 1993). Interest in this group of compounds has in part arisen from the substantial free energy change associated with the hydrolysis of the β-phosphate in the diphosphate groups (Stephens *et al.*, 1993; Laussmann *et al.*, 1997). It has been suggested that this metabolic turnover may be utilized as a regulatory molecular switch, possibly through the transphosphorylation of one or more proteins (Voglmaier *et al.*, 1996); some exciting preliminary evidence in support of the latter possibility has been obtained (Voglmaier *et al.*, 1994).

We previously have employed F^- as a tool to measure the extent of the metabolic flux through these substrate cycles in intact cells (Menniti *et al.*, 1993; Albert *et al.*, 1997). By inhibiting the β-phosphatases that attack PP-InsP_4 and PP-InsP_5 (Menniti *et al.*, 1993; Shears *et al.*, 1995), F^- acts as a metabolic trap that causes the levels of both PP-InsP_4 and PP-InsP_5 to increase (Menniti *et al.*, 1993). These data indicated that, every hour, 30–50% of the entire cellular pools of InsP_5 and InsP_6 cycle through the diphosphorylated polyphosphates (Menniti *et al.*, 1993; Albert *et al.*, 1997).

There was an intriguing, additional outcome of these experiments with F^-. In earlier studies with the AR4-2J pancreatoma cell line, raising the concentration of F^- from 0.8 to 10 mM caused levels of [PP]$_2$-InsP_4 to decrease (Shears *et al.*, 1995). These results suggest that F^- inhibited the rate of [PP]$_2$-InsP_4 synthesis and/or stimulated the rate of [PP]$_2$-InsP_4 dephosphorylation. However, work with purified enzymes has not yielded any evidence to support these two possibilities: the purified phosphatases that dephosphorylate PP-InsP_5 and [PP]$_2$-InsP_4 are equally sensitive to inhibition by F^- (K_i = 10 μM, unpublished data). Furthermore, F^- did not affect the activity of the purified PP-InsP_5 kinase (C.-H.Huang, S.M.Voglmaier, M.E.Bembenek and S.H.Snyder, unpublished data). Thus, we suggested that F^- has some additional and distinctive effect upon [PP]$_2$-InsP_4 turnover *in vivo* (Shears *et al.*, 1995). We now describe the mechanism by which F^- affects cellular [PP]$_2$-InsP_4 metabolism in the DDT$_1$ MF-2 Syrian hamster vas deferens smooth muscle cell line.

Results and discussion

Diphosphorylated inositol polyphosphates in DDT$_1$ MF-2 cells

HPLC was used to resolve the ^{3}H-labeled inositol phosphates (polarity ≥InsP_2) in DDT$_1$ MF-2 cells (Figure 2);

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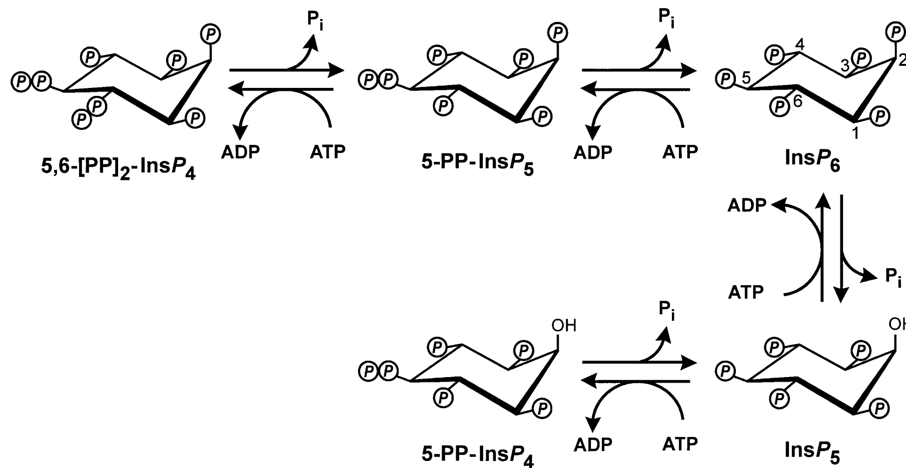

Fig. 1. The metabolism of the diphosphorylated inositol polyphosphates. The diphosphate group of mammalian PP-InsP_5 is shown attached to the 5-carbon (Albert *et al.*, 1997). Based on kinetic data suggesting that the InsP_6 kinase also phosphorylates InsP_5 (Menniti *et al.*, 1993), the diphosphate group of PP-InsP_4 is shown tentatively as also being present on the 5-carbon. In *Dictyostelium*, $[PP]_2$-InsP_4 is the 5,6-isomer (Laussmann *et al.*, 1997); the isomeric structure of the corresponding mammalian material has not yet been confirmed and so its designation in the figure must be considered as tentative.

there were three diphosphorylated compounds (PP-InsP_4, peak v; PP-InsP_5, peak vii; $[PP]_2$-InsP_4, peak viii), all of which have been observed previously in other mammalian cells (Menniti *et al.*, 1993; Stephens *et al.*, 1993; Shears *et al.*, 1995; Albert *et al.*, 1997). Although $[PP]_2$-$[^3H]$InsP_4 was the least abundant of the diphosphates (4787 d.p.m., Figure 2), its levels were of sufficient magnitude to exceed those of all the InsP_4 isomers combined (3906 d.p.m.). $[PP]_2$-InsP_4 was eluted by 1.8–2 M P_i, depending upon the age of the column. No more polar ^{3}H-labeled material was detected in these or any further experiments described in this study, even when $[P_i]$ in the HPLC eluate was increased to 2.6 M.

The effects of F^- upon the turnover of the diphosphorylated inositol polyphosphates

Treatment of intact cells for 30 min with either 0.8 (not shown) or 10 mM NaF (Table I) approximately doubled the levels of PP-$[^3H]$InsP_4 and PP-$[^3H]$InsP_5. This effect is consistent with the previously demonstrated ability of F^- to inhibit the diphosphatase activities that attack PP-InsP_4 and PP-InsP_5 (Menniti *et al.*, 1993; Shears *et al.*, 1995). Thus, F^- acts as a metabolic trap, exposing the ongoing phosphorylation of InsP_5 and InsP_6.

F^- also inhibits $[PP]_2$-InsP_4 diphosphatase activity (Shears *et al.*, 1995), but 30 min treatment of intact cells with 0.8 mM NaF had no net effect upon $[PP]_2$-$[^3H]$InsP_4 levels, even though 2-fold increases in levels of both PP-$[^3H]$InsP_4 and PP-$[^3H]$InsP_5 were observed (data not shown). More striking was the observation that 10 mM F^- caused the levels of $[PP]_2$-$[^3H]$InsP_4 to decrease by 70% (Table I). Levels of $[PP]_2$-$[^3H]$InsP_4 remained at a nadir for 10–30 min (Figure 3), but then started to rise again, so that by 60 min, they were only 25% below those in time-matched control cells. After 2–3 h, $[PP]_2$-$[^3H]$InsP_4 levels exceeded those of control cells (data not shown). In contrast, the levels of both PP-$[^3H]$InsP_4 and PP-$[^3H]$InsP_5 remained elevated throughout the duration of these experiments (Table I, and data not shown). $[PP]_2$-InsP_4 was the only inositol diphosphate to show a decrease in cellular levels following treatment with

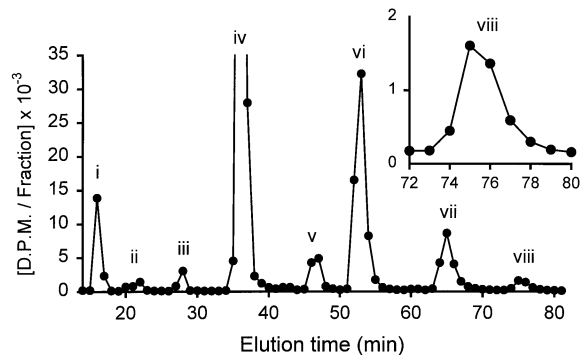

Fig. 2. HPLC analysis of inositol polyphosphates in DDT_1 MF-2 cells. $[^3H]$Inositol-labeled cells were quenched, extracted and analyzed by HPLC as described in Materials and methods. The identity of each peak was ascertained by reference to previous data using this HPLC system (Glennon and Shears, 1993; Shears *et al.*, 1995), and by using standards of $[^3H]$InsP_6, PP-$[^3H]$InsP_5 and $[PP]_2$-$[^3H]$InsP_4. The ^{3}H d.p.m. in each peak were as follows: peak i, InsP_2 = 16 170; peak ii, InsP_3 = 3077; peak iii, InsP_4 = 3906; peak iv, InsP_5 = 170 103; peak v, PP-InsP_4 = 10 502; peak vi, InsP_6 = 59 990; peak vii, PP-InsP_5 = 20 848; peak viii, $[PP]_2$-InsP_4 = 4787. These data are representative of five experiments. The inset shows the chromatography of $[PP]_2$-InsP_4 on an expanded scale.

10 mM F^- (Table I). Since F^- does not directly stimulate $[PP]_2$-InsP_4 phosphatase or inhibit PP-InsP_5 kinase (see Introduction), we considered alternative possible mechanisms that might account for this specific effect of F^-.

Is the effect of F^- on $[PP]_2$-InsP_4 levels caused by a decrease in ATP levels?

At a concentration of 10 mM, F^- is a metabolic poison that depletes cellular ATP levels (see, for example, Okada and Brown, 1988). Indeed, 30 min treatment of DDT_1 MF-2 cells with 10 mM F^- caused ATP levels to drop by 70% (Table I). It is possible that this decrease in ATP supply for the PP-InsP_5 kinase might contribute to the F^-–mediated decline in $[PP]_2$-$[^3H]$InsP_4 levels. On the other hand, cellular [ATP] remained depleted after 60 min F^- treatment (7 ± 2 nmol/mg protein), at a time when levels of $[PP]_2$-$[^3H]$InsP_4 began to recover (see above).

Table I. Cellular levels of inositol polyphosphates and ATP; the effects of various stimulii

Treatment	InsP_4 (% of control)	InsP_5 (% of control)	PP-InsP_4 (% of control)	InsP_6 (% of control)	PP-InsP_5 (% of control)	$[PP]_2$-InsP_4 (% of control)	[ATP] nmol/mg protein
Control	–	–	–	–	–	–	30 ± 7 (9)
NaF	817 ± 84 (7)	92 ± 1 (7)	192 ± 25 (7)	88 ± 3 (7)	200 ± 5 (7)	31 ± 7 (7)	9 ± 5 (9)
Bradykinin (2 min)	294 ± 32 (4)	98 ± 1 (4)	98 ± 2 (3)	105 ± 1 (3)	105 ± 3 (4)	103 ± 6 (4)	n.d.
Bradykinin (30 min)	208 ± 5 (4)	101 ± 1 (4)	96 ± 4 (3)	96 ± 4 (3)	103 ± 2 (4)	92 ± 6 (4)	n.d.
IBMX	104 ± 2 (4)	100 ± 1 (4)	101 ± 18 (3)	106 ± 3 (3)	87 ± 7 (4)	52 ± 8 (4)	30 ± 8 (9)
Bt_2cAMP	91 ± 7 (9)	99 ± 1 (9)	101 ± 6 (7)	104 ± 2 (7)	109 ± 2 (9)	49 ± 5 (9)	36 ± 10 (4)
Bt_2cGMP	88 ± 5 (9)	100 ± 1 (9)	93 ± 7 (8)	102 ± 2 (8)	103 ± 4 (9)	58 ± 8 (9)	33 ± 7 (4)
Isoproterenol	93 ± 4 (9)	100 ± 1 (9)	112 ± 7 (7)	98 ± 1 (7)	113 ± 3 (9)	45 ± 6 (9)	31 ± 7 (4)

[^{3}H]Inositol-labeled cells were treated for either 2 or 30 min with 100 nM bradykinin, and for 30 min with either 10 mM NaF, 100 μM IBMX, 1 mM Bt_2cAMP, 1 mM Bt_2cAMP or 1 μM isoproterenol. Cells were quenched, extracted and analyzed by HPLC as described in Materials and methods. The data represent the average percentage change (with standard errors) compared with time-matched controls. The number of experiments is indicated in parentheses. ATP levels were measured (see Materials and methods) under similar conditions, but in cells that had not been radiolabeled with [^{3}H]inositol; n.d. = not determined.

This increased rate of synthesis of $[PP]_2$-InsP_4 demonstrates the competence of the PP-InsP_5 kinase even at these reduced levels of ATP.

Is the effect of F^- on $[PP]_2$-InsP$_4$ levels mediated through phospholipase C?

Another consequence of adding 10 mM NaF to cells is the formation of an AlF_4^- complex that activates heterotrimeric GTP-binding proteins (Sternweis and Gilman, 1982). This has a number of repercussions, among which is the activation of phospholipase C (PLC) (Blackmore *et al.*, 1985). Indeed, 10 mM F^- stimulated PLC activity in DDT_1 MF-2 cells, as indicated by the increases in levels of [^{3}H]InsP_2, [^{3}H]InsP_3 and [^{3}H]InsP_4 to, respectively, 227 ± 27%, 356 ± 140% and 817 ± 84% of control levels (Table I, and data not shown). We next investigated if there was any effect upon the diphosphorylated inositol phosphates when PLC activity was activated by an appropriate ligand of the cell surface receptors in DDT_1 MF-2 cells, namely, bradykinin (Sipma *et al.*, 1995). At a concentration of 100 nM, this agonist stimulated PLC activity, since cytoplasmic [Ca^{2+}] increased from 58 ± 2 nM (n = 11) to a peak value of 132 ± 8 nM (n = 10), and levels of [^{3}H]InsP_2, [^{3}H]InsP_3 and [^{3}H]InsP_4 increased to, respectively, 128 ± 6%, 128 ± 12% and 294 ± 32% of control levels (Table I, and data not shown). However, bradykinin did not significantly affect the levels of either $[PP]_2$-InsP_4 or the other diphosphorylated inositol phosphates (Table I).

Is the effect of F^- on $[PP]_2$-InsP$_4$ levels mediated through adenylate cyclase?

Basal levels of cAMP in DDT_1 MF-2 cells (2.1 ± 0.5 pmol/mg protein, n = 12) were elevated by a 30 min treatment with 10 mM F^- (to 8.1 ± 0.8 pmol/mg protein, n = 4, $P<0.001$ unpaired t-test), due to AlF_4^--mediated activation of adenylate cyclase (Sternweis and Gilman, 1982). We therefore studied the effects upon $[PP]_2$-InsP_4 levels of alternate procedures to elevate intracellular [cAMP]. The phosphodiesterase inhibitor, IBMX, caused $[PP]_2$-[^{3}H]InsP_4 levels to decrease by about half (Table I). Levels of this polyphosphate also decreased ~50% after application of cell-permeant Bt_2cAMP (Table I). Thus, we conclude that the predominant mechanism by which F^- modifies $[PP]_2$-InsP_4 turnover is through activation of adenylate cyclase.

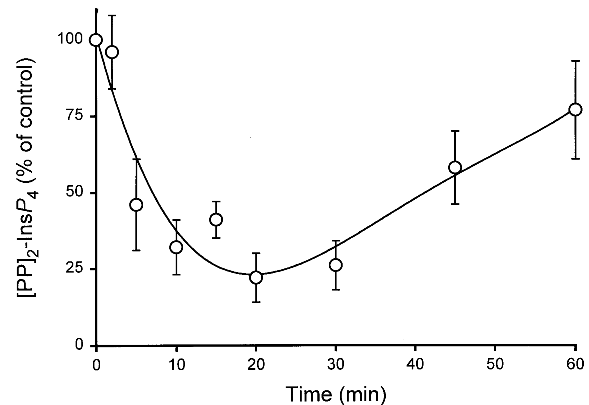

Fig. 3. Time-dependent effects of F^- on cellular levels of $[PP]_2$-[^{3}H]InsP_4. [^{3}H]Inositol-labeled cells were incubated for the indicated times with 10 mM NaF. Cells were quenched, extracted and analyzed by HPLC as described in Materials and methods. The levels of $[PP]_2$-[^{3}H]InsP_4 are shown. Data are means and standard errors from 4–7 experiments, presented as percentages of the values obtained from time-matched control incubations, performed in the absence of NaF.

Three additional aspects of the data in Table I are important to note. First, Bt_2cGMP reduced $[PP]_2$-[^{3}H]InsP_4 levels to almost the same degree as did Bt_2cAMP (Figure 4). In fact, very similar dose–response curves were obtained for the two cyclic nucleotides (EC_{50} for Bt_2cAMP = 14.7 μM; EC_{50} for Bt_2cGMP = 7.9 μM). Second, there is a specificity to the effect of cyclic nucleotides upon $[PP]_2$-InsP_4 turnover. The levels of the other inositol polyphosphates were not affected by elevations in cellular [cAMP] or [cGMP] (Table I). Finally, the mechanisms of action of IBMX, Bt_2cGMP and Bt_2cAMP were not targeted at cellular ATP levels, which these agents did not affect (Table I).

The effect of isoproterenol upon $[PP]_2$-InsP$_4$ turnover and levels of cyclic nucleotides in intact cells

Isoproterenol was also used to elevate cAMP levels up to 16-fold in DDT_1 MF-2 cells (Figure 5, and see Norris *et al.*, 1983). The mean EC_{50} value from seven experiments was 9 nM. In agreement with previous studies using other types of smooth muscle cell, including those of non-vascular origin (Jiang *et al.*, 1992; Murthy and Makhlouf, 1995), levels of cGMP (0.45 ± 0.24 pmol/mg protein,

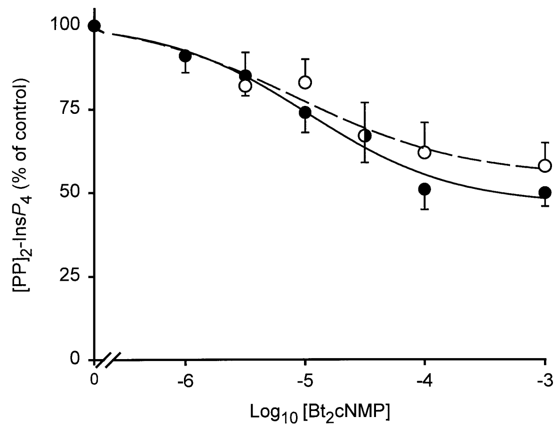

Fig. 4. Dose-dependent effects of Bt_2cAMP and Bt_2cGMP upon cellular levels of $[PP]_2$-[^{3}H]InsP_4. [^{3}H]Inositol-labeled cells were incubated for 30 min with the indicated concentrations of either Bt_2cAMP (●) or Bt_2cGMP (○). Cells were quenched, extracted and analyzed by HPLC as described in Materials and methods. Data are presented as percentages of time-matched controls from 4–5 experiments; vertical bars indicate the standard errors.

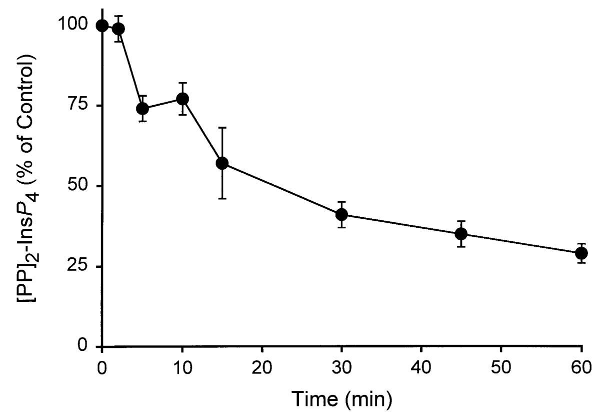

Fig. 6. The time course for the effects of isoproterenol upon cellular levels of $[PP]_2$-[^{3}H]InsP_4. [^{3}H]Inositol-labeled cells were incubated for the indicated times with either vehicle or 1 μM isoproterenol. Cells were quenched, extracted and analyzed by HPLC as described in Materials and methods. Shown are the mean levels of $[PP]_2$-[^{3}H]InsP_4 from four experiments, all calculated as percentages relative to time-matched controls.

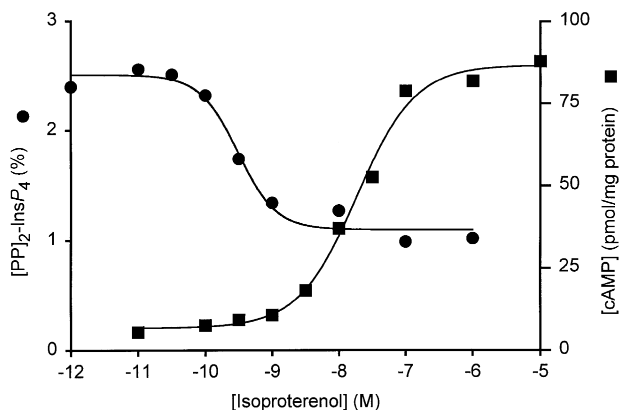

Fig. 5. Dose-dependent regulation of cellular levels of cAMP and $[PP]_2$-[^{3}H]InsP_4 by isoproterenol. [^{3}H]Inositol-labeled cells were incubated for 30 min with the indicated concentration of isoproterenol. Cells were quenched and extracted, and $[PP]_2$-[^{3}H]InsP_4 levels (●) were analyzed by HPLC (see Materials and methods). Samples were prepared in parallel from non-radiolabeled cells which were analyzed for cAMP content (■) (see Materials and methods). The amounts of $[PP]_2$-InsP_4 were quantified as a percentage of total radioactivity in [InsP_5 +PP-InsP_4 + InsP_6 + PP-InsP_5 + $[PP]_2$-InsP_4]. Data are from a representative experiment, typical of four $[PP]_2$-[^{3}H]InsP_4 assays and seven cAMP assays.

n = 4) were unaffected by 2–30 min incubation with 1 μM isoproterenol. PLC was not activated (Table I).

Isoproterenol also caused a dose-dependent decrease in $[PP]_2$-[^{3}H]InsP_4 levels (Figure 5; the mean EC_{50} value was 0.4 nM, n = 4). This agonist-mediated effect was highly specific; incubation with isoproterenol did not lead to a drop in the levels of any other inositol polyphosphate (Table I). A time course experiment (Figure 6) shows that 1 μM isoproterenol caused the levels of $[PP]_2$-[^{3}H]InsP_4 to decrease 25% by 5 min. The reduction continued throughout a 60 min treatment, at which point the 71% decrease was the same degree of effect as that which was brought about by treatment with 10 mM F^- (Table I). Consistent with data showing that isoproterenol acts through β_2-adrenergic receptors in DDT_1 MF-2 cells (Norris *et al.*, 1983), the agonist's action upon $[PP]_2$-[^{3}H]InsP_4 turnover was unaffected when α-adrenergic receptors were blocked with 10 μM phentolamine (data not shown). The novelty of these observations is worth emphasizing; previously, the metabolism of the diphosphorylated inositol polyphosphates was not known to be agonist-regulated (Menniti *et al.*, 1993; Shears *et al.*, 1995).

cAMP regulates $[PP]_2$-InsP_4 turnover independently of A-kinase

We next investigated if A-kinase participated in the response of $[PP]_2$-InsP_4 turnover to treatment with Bt_2cAMP and isoproterenol. Three cell-permeant A-kinase antagonists were used: H-89 (Chijiwa *et al.*, 1990), HA1077 (Asano *et al.*, 1989) and R_pCPT-cAMPS (Roseboom and Klein, 1995). In these experiments, two precautions were taken to optimize the opportunity for the antagonist to effectively block A-kinase. First, we used relatively high concentrations of the antagonists. Second, we chose slightly submaximal concentrations of agonists (3 nM isoproterenol and 300 μM Bt_2cAMP) which induced 80% of the maximum possible depletion in $[PP]_2$-InsP_4 levels. We also independently checked the efficacy of the A-kinase antagonists by measuring the degree of phosphorylation by A-kinase of cAMP response element-binding protein (CREB), using an anti-phospho-CREB antibody (Ginty *et al.*, 1993). Figure 7 shows the prominent increase in the phospho-CREB signal in extracts from cells treated with 3 nM isoproterenol (lane 3) compared with control cells (lane 1). All three cell-permeant antagonists of A-kinase reduced the phospho-CREB signal to levels that were either at or below basal levels (Figure 7, lanes 4–6). These data confirm the effectiveness of the antagonist protocols.

We found that the effect upon $[PP]_2$-InsP_4 metabolism of both isoproterenol and Bt_2cAMP was unaffected by any of the three A-kinase antagonists (Figure 8). The antagonists also had no effect by themselves (Figure 8). These data indicate that cAMP regulates $[PP]_2$-InsP_4 turnover independently of A-kinase. We considered that one possible explanation for this mode of cAMP action

might be the cross-activation of G-kinase (Jiang *et al.*, 1992; Murthy and Makhlouf, 1995). However, this seems unlikely for several reasons. First, cAMP is such a weak activator of the G-kinase (Gamm *et al.*, 1995) that, in smooth muscle cells at least, it requires 10–100 μM isoproterenol before significant cross-activation can be detected (Jiang *et al.*, 1992; Murthy and Makhlouf, 1995). These concentrations are considerably higher than the EC_{50} value of 0.4 nM for the agonist's effects on $[PP]_2$-InsP_4 metabolism (see above). Second, the A-kinase antagonist, HA1077, is equally effective against G-kinase (Asano *et al.*, 1989), yet HA1077 also did not antagonize cyclic nucleotide-mediated changes in $[PP]_2$-InsP_4 metabolism (Figure 8). Third, we used another G-kinase antagonist, R_p-cGMPS (Butt *et al.*, 1994), and it also failed to reverse the decrease in $[PP]_2$-InsP_4 levels brought about by either isoproterenol or by Bt_2cAMP (Figure 8).

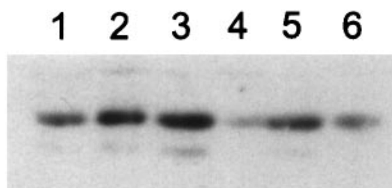

Fig. 7. CREB phosphorylation as an assay for the efficacy of action of okadaic acid and cell-permeant antagonists of A-kinase. DDT_1 MF-2 cells were pre-treated for 15 min with either vehicle (samples 1 and 3) or with 100 μM R_pCPT-cAMPS (sample 4), 1 μM H-89 (sample 5) or 30 μM HA1077 (sample 6). Then 3 nM isoproterenol was added to samples 3–6 for 30 min. Sample 2 was only treated with 1 μM okadaic acid for 30 min. All samples were quenched, resolved by SDS–PAGE and probed for phosphorylated CREB, as described in Materials and methods. The figure represents one of three independent experiments with similar results.

Finally, the conclusion that G-kinase does not participate in this response is reinforced further by the observation that R_p-cGMPS failed to reverse the effect of Bt_2cGMP upon $[PP]_2$-InsP_4 turnover (Figure 8). In other words, it seems that both cGMP and cAMP regulate the metabolism of $[PP]_2$-InsP_4 independently of activating their respective kinases.

We further excluded the participation of protein phosphorylation processes in the regulation of $[PP]_2$-InsP_4 turnover by blocking protein phosphatases with okadaic acid (Cohen, 1989). Treatment of intact cells with okadaic acid increased the phospho-CREB signal (Figure 8, lane 2) over that in control cells (Figure 8, lane 1) but did not affect the cellular levels of $[PP]_2$-$[^3H]$InsP_4 (data not shown).

One well-recognized kinase-independent effect of cyclic nucleotides is to open Ca^{2+}-permeable cation channels in the plasma membrane (Yau, 1994). Although these ion channels were thought initially to be restricted to sensory cells, they are now known to be more widely distributed (Yau, 1994; Ding *et al.*, 1997). We found that cyclic nucleotide-mediated depletion in $[PP]_2$-InsP_4 levels was not associated with any measurable increase in the rate of Ca^{2+} entry, as cytoplasmic $[Ca^{2+}]$ was unaffected by either 1 mM IBMX, 3 mM Bt_2cAMP, 3 mM Bt_2cGMP or 1 μM isoproterenol (data not shown). Moreover, we also incubated cells for 30 min in the absence of extracellular Ca^{2+} so that no Ca^{2+} entry could occur. In these incubations, $[PP]_2$-$[^3H]$InsP_4 levels were unaffected (95 ± 9% of control, $n = 4$), and 1 μM isoproterenol still caused $[PP]_2$-$[^3H]$InsP_4 levels to decrease (to 51 ± 7% of control, $n = 3$) to the same extent as in medium with the normal extracellular $[Ca^{2+}]$ (to 45 ± 6% of control, see Table I).

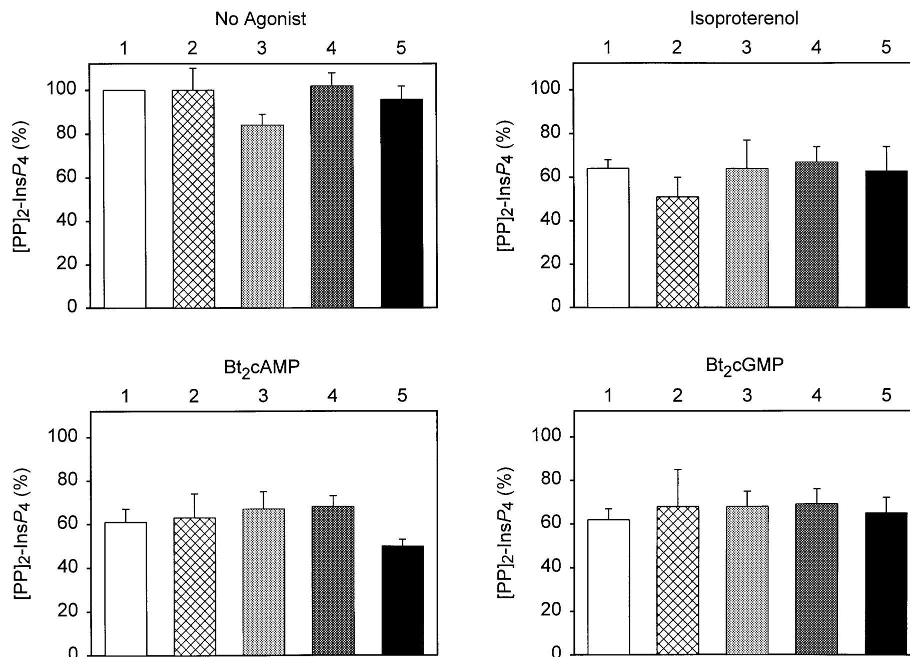

Fig. 8. The effects of antagonists of A-kinase and G-kinase upon the depletion of $[PP]_2$-$[^3H]$InsP_4 levels induced by isoproterenol and cell-permeant cyclic nucleotides. Cells were radiolabeled with $[^3H]$inositol as described in Materials and methods. Cells were incubated for 15 min with either vehicle (histogram 1, $n = 8$–12) or one of the following list of antagonists: 100 μM R_pCPT-cAMPS (histogram 2, $n = 3$); 1 μM H-89 (histogram 3, $n = 3$–4); 30 μM HA1077 (histogram 4, $n = 4$–5); 1 mM R_p-cGMPS (histogram 5, $n = 3$). Similar results were obtained if this pre-incubation time with inhibitors was extended to 1 h. The cells were next treated for 30 min with either vehicle or one of the following agonists: 3 nM isoproterenol, 300 μM Bt_2cAMP or 300 μM Bt_2cGMP. Cells were quenched, extracted and analyzed by HPLC as described in Materials and methods. Levels of $[PP]_2$-InsP_4 are presented as percentage values compared with time-matched controls, with standard errors as indicated.

General conclusions

Among several new conclusions to arise from this study, the most important is that the metabolism of $[PP]_2$-InsP_4 is regulated by the β_2-adrenergic agonist, isoproterenol. Our data therefore provide the first evidence that there is a close relationship between $[PP]_2$-InsP_4 levels and signal transduction events. This effect of isoproterenol was mediated through cAMP but, significantly, the latter acted independently of the activation of A-kinase, or the cross-activation of G-kinase. The receptor-regulated diminution in $[PP]_2$-InsP_4 levels could arise as a consequence of a decrease in the rate of PP-InsP_5 phosphorylation, and/or an increase in the rate of $[PP]_2$-InsP_4 dephosphorylation. Distinguishing between these two possibilities will be an important future direction as we unravel the molecular events that underlie this new regulatory process. It is an exciting possibility that an increased rate of utilization of $[PP]_2$-InsP_4 could reflect up-regulation of its proposed action as a molecular switch and/or a donor for trans-phosphorylation reactions (Voglmaier *et al.*, 1994, 1996).

The model system for the current study was the non-vascular, smooth muscle cell line, DDT_1 MF-2. In these cells, F^- affects $[PP]_2$-InsP_4 metabolism through an elevation in cAMP levels. Since an F^--mediated reduction in $[PP]_2$-InsP_4 levels has also been observed in AR4-2J pancreatoma cells (Shears *et al.*, 1995), we can therefore anticipate a wider occurrence of these effects of cAMP upon $[PP]_2$-InsP_4 turnover. Moreover, our demonstration that Bt_2cGMP is as effective a stimulus as Bt_2cAMP (Figure 4) suggests that physiologically relevant elevations in cellular [cGMP] [such as those induced by nitric oxide (Garthwaite and Boulton, 1995)] will also prove to regulate $[PP]_2$-InsP_4 metabolism.

The diphosphorylated polyphosphates have already attracted attention in view of their rapid interconversion in coupled substrate cycles (Figure 1), and because of the considerable free energy change upon the hydrolysis of the β-phosphate of the diphosphate groups. It is surely the case that the cell reaps some considerable benefit from the energy it expends in order to sustain the steady-state levels of these metabolically active compounds. Now that we know this turnover is receptor-regulated, the need for further research into the biological significance of this class of compounds is stronger than ever.

Materials and methods

Cell culture, [^{3}H]inositol labeling and HPLC of [^{3}H]inositol phosphate

DDT_1 MF-2 Syrian hamster vas deferens smooth muscle cells (provided by Dr D.Gill, University of Maryland School of Medicine, Baltimore, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 'high-glucose' (i.e. 25 mM), 2 mM glutamine and 5% fetal calf serum at 37°C in 5% CO_2/95% humidified air. Cells were harvested and plated at a density of ~200 000 cells/well (16 mm diameter, 24-well muliplates) in the DMEM-based culture medium described above, supplemented with 50 µCi/ml [^{3}H]inositol. Medium was replaced 3 and 5 days after plating. On the 7th day, cell monolayers were washed (2×250 µl) and then incubated (250 µl) in [^{3}H]inositol-free HEPES-buffered Krebs solution (115 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 11 mM glucose, 1.36 mM $CaCl_2$, 25 mM HEPES, pH 7.4 with NaOH). Cells were maintained at 37°C for 3 h prior to the beginning of any experiments. All experimental protocols were time-matched with control incubations.

Experiments were quenched by rapid aspiration of the Krebs solution, followed by addition of 250 µl of ice-cold 0.6 M perchloric acid (PCA) containing 0.2 mM InsP_6, and neutralized by the addition of 70 µl of 1 M K_2CO_3 containing 5 mM Na_2EDTA. After being kept at 4°C for 30 min, the perchlorate precipitate was removed by centrifugation (10 000 *g*, 2 min). The supernatants were finally diluted with 3 vols of 1 mM Na_2EDTA. Similar data were also obtained when cells were quenched with a Triton-based buffer, followed by deproteinization using NENSORB columns (Shears *et al.*, 1995).

Samples were stored at –20°C prior to being loaded onto a 4.6×125 mm Partisphere 5 µm SAX HPLC column. Inositol phosphates were eluted at 1 ml/min by the following gradient generated by mixing buffer A (1 mM Na_2EDTA) and buffer B [buffer A plus 1.3 M $(NH_4)_2HPO_4$, pH 3.85 with H_3PO_4; total $[P_i]$ = 2.6 M]: 0–10 min, 0% B; 10–25 min, 0–35% B; 25–105 min, 35–100% B; 105–115 min, 100% B. Fractions of 1 ml were collected, mixed with 3 vols of Monoflow 4 scintillant, and radioactivity was determined using liquid scintillation spectrometry.

Analysis of CREB phosphorylation by Western blotting

Cells were prepared in 24-well multiplates as described above, except that [^{3}H]inositol was omitted from the culture medium. Following agonist stimulation, experiments were quenched in hot Laemmli sample buffer, and cell extracts were drawn through a 27 gauge needle to reduce viscosity. Samples were run on a 10% polyacrylamide gel, and electro-blotted onto a nitrocellulose membrane. After washing with phosphate-buffered saline (PBS), the membrane was incubated with an anti-phospho-CREB rabbit polyclonal antibody according to the manufacturer's protocol. Antibody–antigen complexes were detected using a horseradish peroxidase-coupled anti-rabbit IgG.

Cellular [Ca^{2+}] measurements

DDT_1 MF-2 cells were grow on glass coverslips, washed with Krebs buffer, and equilibrated for 4 h prior to loading with 3 µM Fura-2/AM for 30 min at room temperature in the dark. Cells were then washed with Krebs buffer and fluorescence was monitored using a PTI dual wavelength spectrofluorimeter system, with excitation at 340 and 380 nm, and emission intensity was measured at 505 nm. The average free cytoplasmic [Ca^{2+}] was then calculated (Grynkiewicz *et al.*, 1986).

Other assays

The appropriate kits (see Materials) and methodologies recommended by the supplier were used to assay for cAMP, cGMP and ATP (in PCA-quenched, K_2CO_3-neutralized cell extracts, see above). Protein concentration was determined as described by Bradford (1976). EC_{50} values were derived using Prism (GraphPad, San Diego, CA).

Materials

[^{3}H]Inositol (10–25 Ci/mmol; 10 mCi/ml; in sterile water), NENSORB columns, and standards of [^{3}H]InsP_6, PP-[^{3}H]InsP_5 and $[PP]_2$-[^{3}H]InsP_4 were purchased from NEN Life Science Products (Boston, MA). R_p-cGMPS and HA1077 were purchased from RBI (Natick, MA), R_pCPT-cAMPS was purchased from Biolog (La Jolla, CA). Bt_2cAMP and Triton X-100 were purchased from Boehringer-Mannheim (Indianapolis, IN). Bradykinin, H-89, Bt_2cGMP, okadaic acid and the ATP assay kit were purchased from Calbiochem-Novabiochem Int. (La Jolla, CA). cAMP and cGMP assay kits were purchased from Amersham Corp. (Arlington Heights, IL). Anti-phospho-CREB rabbit polyclonal antibody was supplied by Upstate Biotechnology, Waltham, MA. Fetal calf serum was purchased from Gibco (Grand Island, NY). HPLC columns were supplied by Krackler Scientific (Durham, NC). Monoflow 4 scintillant was purchased from National Diagnostics (Manville, NJ). InsP_6 was purchased from Aldrich (Milwaukee, WI). Fura-2/AM was purchased from Molecular Probes, Eugene OR. Other reagents were purchased from either Sigma or Fluka.

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