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INTERCONVERSION OF INOSITOL (1,4,5)-TRISPHOSPHATE TO INOSITOL (1,3,4,5)-TETRAKISPHOSPHATE AND (1,3,4)-TRISPHOSPHATE IN PERMEABILIZED ADRENAL GLOMERULOSA CELLS IS CALCIUM-SENSITIVE AND ATP-DEPENDENT

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SUMMARY : The metabolism of [^{3}H]inositol (1,4,5)-trisphosphate was followed in permeabilized bovine adrenal glomerulosa cells. At low Ca^{++} concentration (pCa = 7.2), more than 90% of [^{3}H]inositol (1,4,5)-trisphosphate had disappeared within 2 min, while two other metabolites, [^{3}H]inositol (1,3,4)-trisphosphate and [^{3}H]inositol (1,3,4,5)-tetrakisphosphate appeared progressively. At higher Ca^{++} concentrations (pCa = 5.7 and 4.8), the formation of these two metabolites was markedly increased, but completely abolished if the medium was ATP-depleted. The peak levels for the generation of [^{3}H]inositol (1,3,4,5)-tetrakisphosphate (1 min) preceded those of [^{3}H]inositol (1,3,4)-trisphosphate and were closely correlated. These results suggest that, in adrenal glomerulosa cells, the isomer inositol (1,3,4)-trisphosphate is generated from inositol (1,4,5)-trisphosphate via a calcium-sensitive and ATP-dependent phosphorylation/dephosphorylation pathway involving the formation of inositol (1,3,4,5)-tetrakisphosphate.

The octapeptide hormone, angiotensin II, stimulates inositol trisphosphate formation (1) and raises cytosolic free calcium concentration (2) in isolated adrenal glomerulosa cells, which produce aldosterone in response to the hormone. $Ins(1,4,5)P_3$ [1], a product of phosphatidylinositol 4,5-bisphosphate hydrolysis by a receptor-activated phospholipase C, has been shown to play a key role in cellular responses to stimuli involving Ca^{++} mobilization

[1] The abbreviations used are: $Ins(1,4,5)P_3$: inositol (1,4,5)-trisphosphate, $Ins(1,3,4)P_3$: inositol (1,3,4)-trisphosphate, $Ins(1,3,4,5)P_4$: inositol (1,3,4,5)-tetrakisphosphate, $Ins(1,4)P_2$: inositol (1,4)-bisphosphate, EGTA: ethylene glycol bis (beta-aminoethyl ether) N,N,N',N'-tetraacetic acid, Hepes: 4-(2-hydroxyethyl) 1-piperazine ethane sulfonic acid, BSA: bovine serum albumin.

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from intracellular Ca^{++} pools (3). The presence in stimulated tissues of another isomer of inositol trisphosphate, Ins(1,3,4)P_3, has been recently demonstrated (4), but the role of this metabolite has not yet been established. The kinetics of generation of the two isomers are different (5) and it has been proposed that formation of Ins(1,3,4)P_3 could occur via 5-dephosphorylation of Ins(1,3,4,5)P_4 (6). A phospholipidic precursor for this higher phosphorylated form of inositol has not been found (6), but a specific Ins(1,4,5)P_3 3-kinase activity has been recently characterized in a variety of animal tissues (7). This suggests that an alternative metabolic pathway for the degradation of Ins(1,4,5)P_3 could exist, involving a 3-phosphorylation followed by a 5-dephosphorylation. The aim of this study was to verify the presence of this pathway in adrenal glomerulosa cells permeabilized by high voltage electric field discharge, and to test its Ca^{++} sensitivity and its requirement for ATP.

MATERIALS AND METHODS

Collagenase type I, Hepes, Mg-ATP, BSA, succinate, rotenone, oligomycin, phosphocreatine, creatine kinase were obtained from Sigma. Medium 199 was purchased from Seromed and EGTA from Fluka. [^{3}H]Ins(1,4,5)P_3 was purchased from New England Nuclear and Ins(1,4,5)P_3 was a generous gift of Dr R. Irvine, Cambridge, U.K.
Isolated bovine adrenal glomerulosa cells were prepared by collagenase digestion and mechanical dispersion as previously described (2). After two washes with medium 199, the isolated cells were resuspended in a buffer containing 20 mM Hepes, 137 mM NaCl, 3 mM KCl, 5.6 mM D-glucose and 0.05% BSA, at pH 7.2 and incubated for 15 min at 37°C in the presence of 1 mM EGTA.
The cells were then washed twice in the same buffer (without EGTA) and once in a medium containing 250 mM sucrose and 5 mM Hepes, at pH 7.2; they were resuspended in this non-ionic buffer at a concentration of 6×10^6 cell/50 µl (2.5 mg protein) and then permeabilized by repeated exposition to a high voltage electric field (3 kV/cm) created between two electrodes, as described by Knight and Baker (8). The effectiveness of permeabilization was assessed with Trypan blue: about 85% of the cells stained with the dye, while this ratio amounted to less than 10% in control untreated cells. An aliquot (50 µl) of this cell preparation was mixed with 350 µl of one of the four media described below (media 1 to 4), and incubated for 5 min at 37°C.
All four media contained an "intracellular cationic background": 5 mM NaCl, 115 mM KCl, 5 mM $NaHCO_3$, 1 mM KH_2PO_4, 1 mM $MgCl_2$ and 5 mM succinate, 0.1 µM rotenone, 1 µM oligomycin, 0.05% BSA, 20 mM Hepes, at pH 7.2. Media 1 to 3 were supplemented with an

ATP-regenerating system (2 mM Mg-ATP, 10 mM phosphocreatine and 8 U/ml creatine kinase), while medium 4 contained 10 mM D-glucose and 2 U/ml hexokinase in order to remove the ATP present in the medium. $[Ca^{++}]$ was buffered in these media with 1 mM EGTA and various amounts of $CaCl_2$, and then measured with a Ca^{++}-selective minielectrode prepared and calibrated as previously described by Prentki et al. (9). The pCa values ($-\log[Ca^{++}]$), as determined by this method, were respectively 7.2 (medium 1), 5.7 (medium 2) and 4.8 (media 3 and 4).

Four microliters of a solution containing $Ins(1,4,5)P_3$ (100 µM) and $[^3H]Ins(1,4,5)P_3$ (9 µCi/ml) were added to the incubation media; aliquots (50 µl) were removed at the indicated times and poured into 1 ml of an ice-cold 10% trichloroacetic acid solution. The samples were then centrifuged for 10 min at 2500 x g and extracted with 3 x 5 volumes of ether. Finally, polyphosphoinositols were separated by HPLC, according to the method described by Irvine et al. (7) and the radioactivity was determined in the fractions where $Ins(1,4,5)P_3$, $Ins(1,3,4)P_3$ and $Ins(1,3,4,5)P_4$ were expected.

RESULTS AND DISCUSSION

When permeabilized adrenal glomerulosa cells were resuspended in media in which $[Ca^{++}]$ was buffered at various values (pCa = 7.2, 5.7 or 4.8), more than 90% of the added $[^3H]Ins(1,4,5)P_3$ disappeared within 2 min (Fig.1) and this disappearance was not Ca^{++}-dependent. At low $[Ca^{++}]$ (pCa = 7.2), two metabolites were generated: $Ins(1,3,4,5)P_4$, which increased transiently, reaching a maximum within 1 min (Fig. 2, left), and $Ins(1,3,4)P_3$, which rose rapidly in 2 min and tended to a maximum at 5 min (Fig. 2, right). At higher $[Ca^{++}]$ (pCa = 5.7 or 4.8), the formation of these two products was increased by about three-fold, with the same kinetics. When the medium was ATP-depleted in the presence of hexokinase, the formation of these compounds was completely abolished. The fact that the formation of $Ins(1,3,4)P_3$ was prevented in the absence of ATP suggests that $Ins(1,3,4,5)P_4$ could be a metabolic intermediate between the two $InsP_3$ isomers; the transient increase of $Ins(1,3,4,5)P_4$, which precedes $Ins(1,3,4)P_3$ accumulation, is in agreement with this hypothesis. This view is reinforced by the observation that a close relationship exists between the levels of $Ins(1,3,4,5)P_4$ at 1 min and the levels of $Ins(1,3,4)P_3$ detected at 2 min (Fig.3).

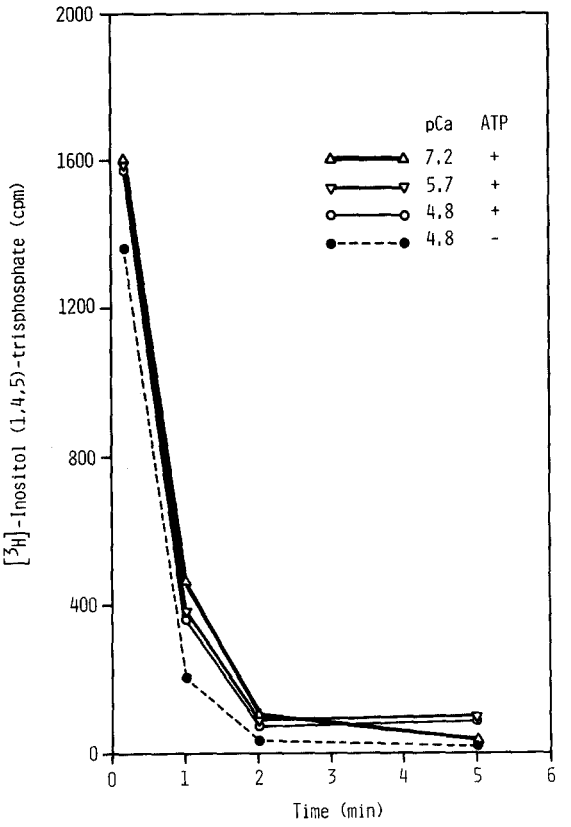

Figure 1: The degradation of Ins(1,4,5)P_3 in permeabilized adrenal glomerulosa cells. Permeabilized cells were incubated in buffers at the indicated [Ca^{++}], with or without ATP, as described in Methods. At time 0, [^{3}H]Ins(1,4,5)P_3 was added to the media and aliquots were taken after 10 s, 1, 2, or 5 min. The kinetics of disappearance of the peak corresponding to [^{3}H]Ins(1,4,5)P_3 was determined after separation by HPLC. Each point represents the mean value from two separate experiments.

At high [Ca^{++}], 30% of the added radioactivity had passed within 5 min from Ins(1,4,5)P_3 to Ins(1,3,4)P_3. This result clearly confirms that a metabolic pathway exists between these two isomers in adrenal glomerulosa cells and the requirement for ATP suggests that isomerization involves the formation of a higher phosphorylated intermediate, as proposed by Irvine et al. (7) (Fig.4). The low radioactivity levels detected in the fraction corresponding to Ins(1,3,4,5)P_4 could be explained by a higher activity of the phosphatase, as compared to that of the kinase. The kinase alone appears to be Ca^{++}-sensitive in the physiological range, as

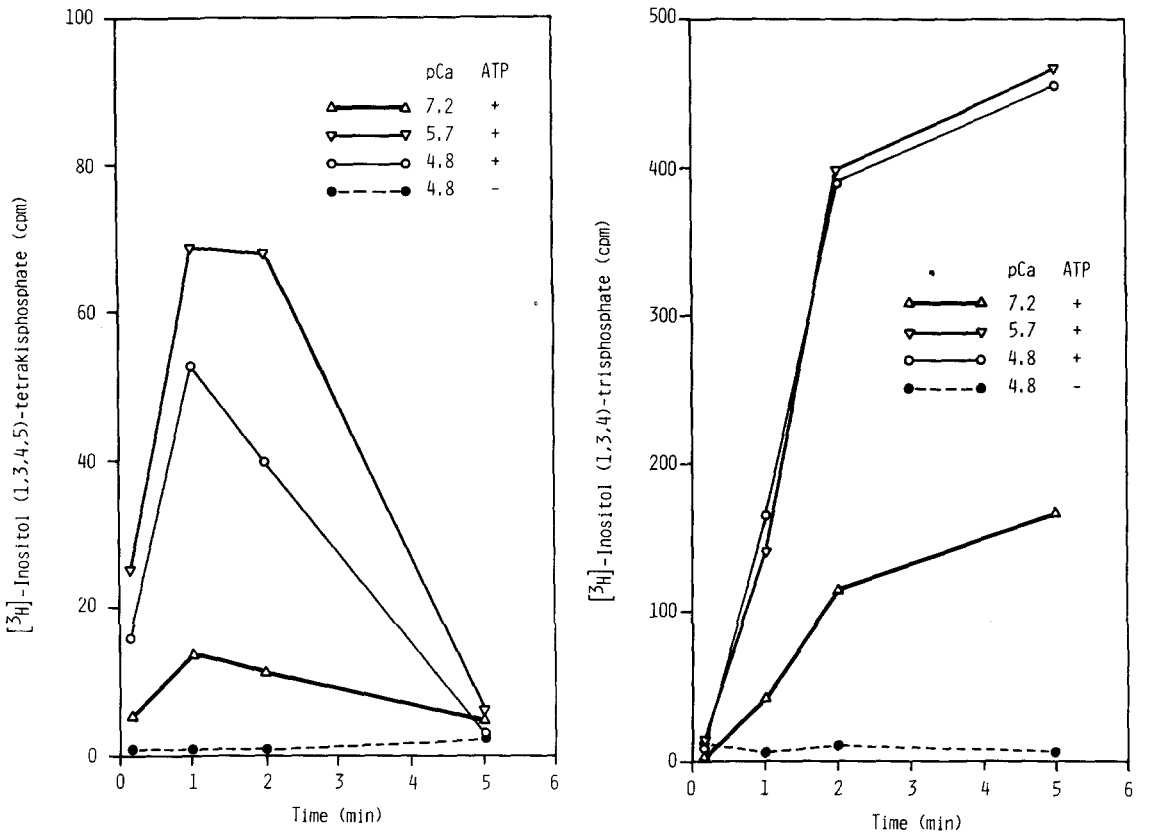

Figure 2: The generation of Ins(1,3,4,5)P_4 (left) and of Ins(1,3,4)P_3 (right) in permeabilized adrenal glomerulosa cells, as a function of extracellular [Ca^{++}]. The time course of appearance of the peak corresponding to Ins(1,3,4,5)P_4 and to Ins(1,3,4)P_3 was determined as described in the legend of Fig.1. Each point is the mean value from two separate experiments.

suggested by the close linear correlation we observed between Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 levels, independently of the Ca^{++} concentration. While contrasting with the recent report of Irvine et al. (7), the Ca^{++} sensitivity of this alternative pathway has also been recently observed by others in RINm5F cells (10) and HL60 cells (11) and could bear an important physiological significance (Fig. 4): at low cytosolic [Ca^{++}], most of the formed Ins(1,4,5)P_3 is dephosphorylated to yield Ins(1,4)P_2 (not measured in our study) (3), but an elevation of cytosolic [Ca^{++}] (consecutive to angiotensin II stimulation and mediated by Ins(1,4,5)P_3), will lead to a selective activation of the alternative pathway. The switch to

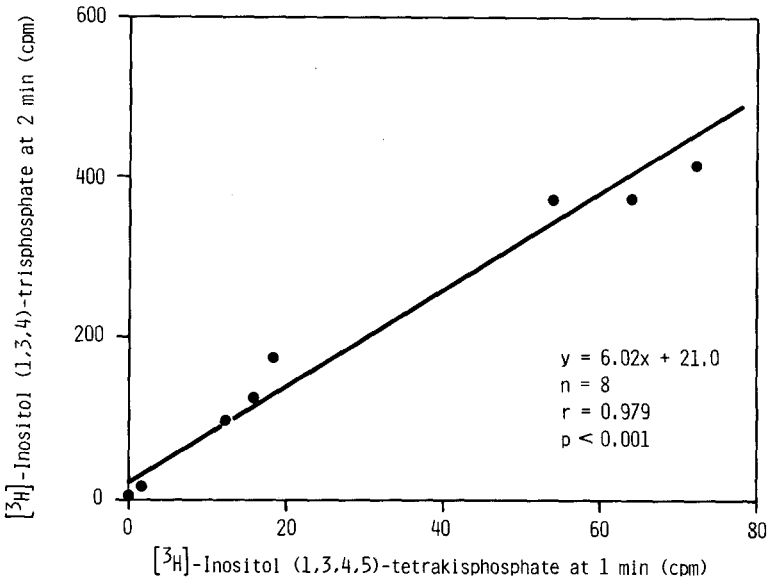

Figure 3: The correlation between the levels of Ins(1,3,4,5)P_4 at 1 min and the levels of Ins(1,3,4)P_3 at 2 min after the addition of Ins(1,4,5)P_3 to permeabilized adrenal glomerulosa cells. The linear regression analysis was performed on the data of Figure 2, obtained at various extracellular [Ca^{2+}].

this pathway does not seem to accelerate the degradation of Ins(1,4,5)P_3 (see Fig.1) but rather permits the formation of another potential second messenger (6,7), whose role remains to be defined.

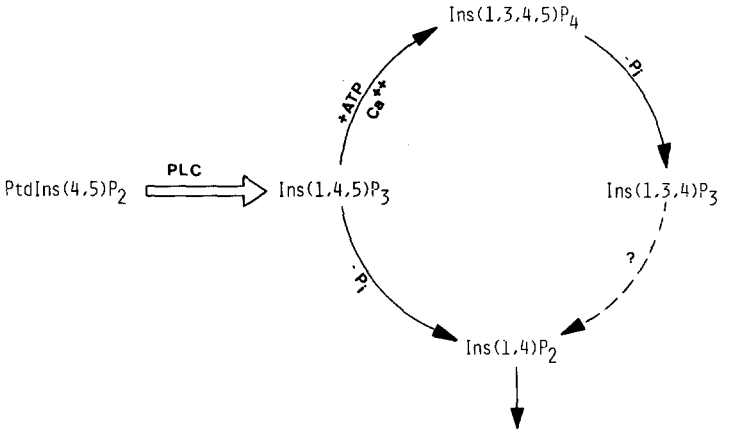

Figure 4: The possible metabolic pathways for higher phosphorylated forms of inositol in adrenal glomerulosa cells. PtdIns(4,5)P_2 = phosphatidylinositol (4,5)-bisphosphate, PLC = receptor-activated phospholipase C; the dashed arrow represents a still speculative pathway. See discussion for explanation.

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