Ca^{2+} -dependent and Ca^{2+} -independent degradation of phosphatidylinositol in rabbit vas deferens

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The effects of Ca^{2+} and acetylcholine on the degradation and synthesis of phosphatidylinositol in rabbit vas deferens was studied *in vitro* by a pulse-chase technique and by measuring the content of the phospholipid in the tissue. Ca^{2+} -dependent degradation of phosphatidylinositol was found in slices and homogenates prelabelled with *myo*-[2- ^3H]inositol. The phosphatidylinositol content of the slices also decreased by a Ca^{2+} -dependent mechanism. On the other hand, removal of intracellular Ca^{2+} with the ionophore A23187 and EGTA increased the amount of phosphatidylinositol. These results indicate that the intracellular Ca^{2+} concentration has an important role in regulating the phosphatidylinositol content of the tissue. Increasing the extracellular K^+ concentration, which causes an increase in plasma-membrane Ca^{2+} permeability, did not enhance phosphatidylinositol breakdown nor decrease its tissue content. However, phosphatidylinositol synthesis was clearly inhibited. After stimulation of the smooth muscle with acetylcholine, degradation of phosphatidylinositol was enhanced. Furthermore, the content of phosphatidylinositol in the tissue also decreased. These phenomena were evident even in the absence of Ca^{2+} . The acetylcholine-induced degradation of phosphatidylinositol was blocked by the muscarinic antagonist atropine, but not by the nicotinic antagonist (+)-tubocurarine. The acetylcholine-induced decrease in the phosphatidylinositol content of the tissue led to the compensatory synthesis of phosphatidylinositol. Synthesis was separated from degradation in the same tissue. Compensatory synthesis was inhibited by acetylcholine. The degradation of phosphatidylinositol induced by acetylcholine was not inhibited by 8-bromoguanosine 3':5'-cyclic monophosphate, indicating that the degradative process was not mediated by an increase in the cyclic nucleotide.

It is known that when various tissues and cells are exposed to physiological (e.g. hormones and neurotransmitters) or mitogenic stimulation phosphatidylinositol turnover is enhanced (Michell, 1975). This enhancement has been attributed to a stimulation in phosphatidylinositol degradation, concomitant with a secondary increase in the resynthesis of the phospholipid. It has been postulated that the degradation of phosphatidylinositol is associated with the opening of a Ca^{2+} gate, resulting in an increase in intracellular $[\text{Ca}^{2+}]$, as Ca^{2+} is not necessary for the enhanced phosphatidylinositol turnover but physiological responses require the ion (Jafferji & Michell, 1976*b,c*). However, the exact

relationship between Ca^{2+} and phosphatidylinositol breakdown has not been established.

Smooth muscle, such as rat vas deferens (Canessa de Scarnati & Lapetina, 1974) and the longitudinal muscle of guinea-pig ileum (Jafferji & Michell, 1976*a*), shows α -adrenergic and muscarinic cholinergic-induced enhancements in phosphatidylinositol turnover and increases in Ca^{2+} influx. Although the concentration of the agonist required for this response is much higher than that required for muscle contraction, the resulting increase in cytosolic $[\text{Ca}^{2+}]$ does affect the contraction of the muscle (Michell *et al.*, 1976).

In most reports, phosphatidylinositol turnover is

expressed in terms of specific radioactivity of phosphatidylinositol labelled *in vitro* with [^{32}P]P_i. However, this technique does not distinguish enhancement of degradation from that of synthesis. In addition, it has been shown in the insect salivary gland that an increase in intracellular [Ca^{2+}] can inhibit the synthesis of phosphatidylinositol (Berridge & Fain, 1979). This finding, if applicable to other tissues, further complicates the interpretation of results on the effects of hormones.

In the present report the degradation and synthesis of phosphatidylinositol in the vas deferens of the rabbit was studied with emphasis on the effect of Ca^{2+} and the action of acetylcholine. Phosphatidylinositol was monitored by the pulse-chase technique using *myo*-inositol and by the measuring of the phospholipid content in the tissue.

Materials and methods

Materials

myo-[2- ^3H]Inositol (sp. radioactivity 18 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. The Ca^{2+} ionophore A23187 was a gift from Eli Lilly Co., Indianapolis, IN, U.S.A. Acetylcholine, (+)-tubocurarine chloride, atropine and 8-bromoguanosine 3':5'-cyclic monophosphate (8-bromo cyclic GMP) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Methods

Preparation of tissues and pulse-chase experiments. Vasa deferentia were quickly excised from rabbits weighing 2.5–3.5 kg and placed in ice-cold Krebs–Ringer bicarbonate (KRB) buffer, pH 7.4, containing 10 mM-D-glucose. The tissues were trimmed to remove foreign tissues and the ducts were perfused with the same buffer to wash out the seminal content. The tissue was cut into transverse slices 0.5 to 1.0 mm in width. Portions (about 50 mg wet wt.) were placed in capped vials that contained 3 ml of medium. The medium consisted of KRB or Ca^{2+} -free KRB buffer containing 10 mM-D-glucose and 1 μCi of *myo*-[2- ^3H]inositol. The vials were flushed with O_2/CO_2 (19:1) and capped. The incubation was carried out for 30 min at 37°C. After the incubation, the slices were rinsed with ice-cold medium without labelled precursor, and were re-incubated for 60 min in 3 ml of fresh medium containing KRB or Ca^{2+} -free KRB buffer. When the medium was changed, the vials were gassed again. After the re-incubation, the medium was removed by aspiration and another 3 ml of fresh medium containing KRB or Ca^{2+} -free KRB buffer, 10 mM-D-glucose, unlabelled 2 mM-*myo*-inositol, and additional compounds, as indicated, was added. The mixture was incubated again for 30 min at 37°C under the same atmosphere, unless specified other-

wise. After the third incubation, the mixture was chilled immediately to 0°C and the medium was removed by aspiration. The slices were washed three times with 3 ml of ice-cold 5% trichloroacetic acid, blotted on filter paper, and then homogenized in a glass homogenizer to which 2 ml of chloroform/methanol (2:1, v/v) and about 20 mg of sand were added. The homogenate was transferred to a test tube and the homogenizer was washed four times with 1 ml of the chloroform/methanol. To the combined homogenate $\frac{1}{2}$ vol. of 0.5 M-KCl was added and mixed. The mixture was separated into two phases by centrifugation at 270 g for 5 min. The lower phase was washed three times by phase separation. A portion of the lower phase was taken to dryness in a scintillation vial, to which 5 ml of scintillation fluid was then added. Radioactivity was measured in a liquid-scintillation counter. It was found that at least 96% of the radioactivity in the lower phase was associated with phosphatidylinositol by co-chromatography with authentic phosphatidylinositol as described below. Another portion of the lower phase was taken to dryness in a test tube, digested with HClO_4 and the phosphorus content was determined by the method of Fiske & Subbarow (1925). The specific radioactivity was expressed in terms of the radioactivity in the lipid fraction (lower phase) per μg of total lipid phosphorus.

For pulse-chase experiments with labelled homogenate, about 400 mg of the slices was labelled similarly in 20 ml of the medium containing 10 μCi of *myo*-[2- ^3H]inositol and suspended in 0.25 M-sucrose/20 mM-Tris/HCl (pH 7.4) and homogenized with a Polytron homogenizer. The homogenate was centrifuged at 100 000 g for 60 min. The resulting precipitate was washed with the same medium and centrifuged again. The washed residue was re-suspended in 10 vol. of the same medium. The incubation mixture consisted of 0.1 ml of this suspension and 0.1 ml of the sucrose buffer containing additional compounds as indicated. The mixture was incubated at 37°C for a specified period. After the incubation, the mixture was extracted with 4 ml of chloroform/methanol (2:1, v/v). The extract was washed as described above and counted for radioactivity.

Determination of phosphatidylinositol content. About 70–80 mg of vas-deferens slices was incubated in 5 ml of KRB or Ca^{2+} -free KRB buffer containing additional compounds as indicated. After the incubation, the slices were homogenized and the lipid fraction was prepared as described above. The lipid fraction was dried *in vacuo* and applied to formaldehyde-impregnated paper and chromatographed by the method of Kai & Hawthorne (1966). The lipid spots on the paper were detected by immersing the paper in 0.05 M- H_2SO_4 containing

0.002% (w/v) Nile Blue. The spots of phosphatidylinositol and other phospholipids were cut out and digested in HClO_4 . The phosphorus content of the digest was determined. It was ascertained that the amount of total phospholipid phosphorus was essentially constant during the incubation. The amount of phosphatidylinositol was expressed in terms of the percentage of phosphorus in the phosphatidylinositol spot relative to the amount of phosphorus in the total phospholipids.

Results

In pulse-chase experiments, vas-deferens slices labelled with *myo*-[2- ^3H]inositol were first incubated in *myo*-inositol-free medium for 60 min and then incubated in the presence of unlabelled *myo*-inositol plus other compounds. In preliminary experiments, when the chase was performed directly after prelabelling, incorporation of radioactivity into phosphatidylinositol continued even after the addition of the unlabelled *myo*-inositol. This incorporation indicated that the cells retained a large pool of labelled *myo*-inositol that could not be effectively diluted by extracellularly added unlabelled *myo*-inositol. Therefore, an incubation with *myo*-inositol-free medium was instituted before the chase to minimize the pool size. By this technique the subsequent chase was made more effective and further incorporation was negligible.

Fig. 1. Effect of Ca^{2+} on the degradation of phosphatidylinositol in vas-deferens slices

The pulse-chase experiment of vas-deferens slices prelabelled with *myo*-[2- ^3H]inositol was carried out as described in the text in Ca^{2+} -free KRB buffer containing 1 mM-*myo*-inositol, 10 μM -ionophore A23187, and 0.5 mM-EGTA (O), 0.01 mM- CaCl_2 (●), 0.1 mM- CaCl_2 (Δ) or 1 mM- CaCl_2 (▲). Pre-loaded slices contained 2320 ± 140 d.p.m. of [^3H]phosphatidylinositol/ μg of total lipid phosphorus. Values are means $\pm$ S.E.M. for triplicate determinations.

When the chase of the labelled vas-deferens slices was carried out in the presence of the ionophore A23187 and various concentrations of CaCl_2 , the specific radioactivity of phosphatidylinositol decreased in a manner dependent on the concentration of CaCl_2 (Fig. 1). Likewise, when the labelled slices were homogenized, washed, and incubated with various concentrations of CaCl_2 , a Ca^{2+} -dependent decrease of the label in the phospholipid was found (Fig. 2).

The phosphatidylinositol content of the vas-deferens slices was determined after incubation of the slices for 30 min at 37°C in a medium containing KRB buffer (control), KRB buffer plus 10 μM -ionophore A23187, or Ca^{2+} -free KRB buffer plus 10 μM -ionophore A23187 and 500 μM -EGTA. Without incubation, the phosphatidylinositol content of the slices was 66.7 ± 1.8 ng of phosphatidylinositol phosphorus/ μg of total lipid phosphorus (mean $\pm$ S.D.). After incubation in the buffer alone, the phosphatidylinositol phosphorus content did not significantly change, being 64.5 ± 0.5 . However, when the slices were incubated in the Ca^{2+} -containing buffer plus the ionophore A23187 (10 μM), which would admit extracellular Ca^{2+} and result in an increased concentration of intracellular Ca^{2+} , the phosphatidylinositol content of the slices decreased to 50.0 ± 1.8 , a value significantly different

Fig. 2. Effect of Ca^{2+} on the degradation of phosphatidylinositol in vas-deferens homogenate

The pulse-chase experiment on vas-deferens homogenates prelabelled with *myo*-[2- ^3H]inositol was carried out as described in the text in 0.25 M-sucrose/30 mM-Tris/HCl buffer (pH 7.4) containing 1 mM-*myo*-inositol (●). Added to the incubation mixture was 10 mM-EGTA (O), 0.01 mM- CaCl_2 (Δ), 0.1 mM- CaCl_2 (▲), 1 mM- CaCl_2 (□) or 5 mM- CaCl_2 (■). The incubation mixture contained 11595 ± 727 d.p.m. of [^3H]phosphatidylinositol/100 μl of membrane suspension at zero time. Values are means $\pm$ S.E.M. for triplicate determinations.

($P < 0.01$) from the control. In contrast, when intracellular Ca^{2+} was removed, by incubating the slices in Ca^{2+} -free KRB buffer containing both $10\text{ }\mu\text{M}$ -ionophore A23187 and 0.5 mM -EGTA, the phosphatidylinositol content of the slices was increased to 75.0 ± 1.7 , a value significantly different ($P < 0.01$) from the control. Thus, this finding expands the observation of Billah & Michell (1979) that the phosphatidylinositol content of Ca^{2+} -depleted rat hepatocytes appeared to be higher than that of Ca^{2+} -replete cells. These results may be the consequence of the desuppression of CDP-diacylglycerol-inositol 3-phosphatidyltransferase activity, since this enzyme is very sensitive to Ca^{2+} . The potent inhibitory effect of Ca^{2+} on CDP-diacylglycerol-inositol 3-phosphatidyltransferase in the vas deferens has been reported previously (K. Egawa, T. Takenawa & B. Sacktor, unpublished work); the percentage inhibition of the enzyme in a cell-free system was $79.7 \pm 4.3\%$ with $10\text{ }\mu\text{M}$ - CaCl_2 .

Since it is known that high concentrations of KCl in the incubation medium cause enhancement of Ca^{2+} influx by depolarizing the cell membrane and increase phosphatidylinositol turnover in guinea-pig ileum smooth muscle (Jafferji & Michell, 1976c), the effects of 95 mM -KCl on the incorporation of *myo*-[2- ^3H]inositol into phosphatidylinositol, the phosphatidylinositol content and the degradation of the phospholipid in slices of the vas deferens was determined (Table 1). It was found that the incorporation of the label was significantly decreased, whereas neither the phosphatidylinositol content in the slice nor its degradation was measurably altered. These results seem to show that the presumed increase in the intracellular [Ca^{2+}] induced by the high concentration of KCl was sufficient for the inhibition of phosphatidylinositol synthesis, which is very sensitive to Ca^{2+} , but not enough to affect Ca^{2+} -dependent degradation.

The effect of acetylcholine on the degradation of phosphatidylinositol in the vas deferens was examined. In preliminary labelling experiments, it was found that acetylcholine did not cause a significant increase in specific radioactivity of phosphatidylinositol after labelling with *myo*-[2- ^3H]inositol. At very high concentrations of acetylcholine (0.5 mM or more), the rate of labelling decreased to some extent without appreciably affecting the specific radioactivity. On the other hand, degradation of phosphatidylinositol induced by acetylcholine was demonstrated by pulse-chase experiments (Fig. 3). The acetylcholine-dependent decrease of the specific radioactivity of phosphatidylinositol was evident in both the presence and absence of Ca^{2+} in the medium. Moreover, degradation was detected within 1 min after the addition of the neurotransmitter. It should be noted that the enhanced breakdown of phosphatidylinositol in the rat vas deferens by adrenaline was observed previously (Jones *et al.*, 1979).

The relationship between various concentrations of acetylcholine and the induced decrease in specific radioactivity on phosphatidylinositol is shown in Fig. 4. Significant effects were found with $10\text{--}1\text{ }\mu\text{M}$ -acetylcholine. The dependence of the degradation on the concentration was approximately similar whether or not Ca^{2+} was present in the incubation medium.

A decrease in the phosphatidylinositol content of the vas-deferens slices was found after the slices were incubated with acetylcholine (Table 2). This effect was seen in both the presence and absence of Ca^{2+} in the media. The degradation induced by acetylcholine was antagonized by atropine but not by (+)-tubocurarine (Table 3). Therefore, it can be concluded that the decrease is mediated by muscarinic and not by nicotinic acetylcholine receptors.

As noted above, acetylcholine did not effect a

Table 1. *Effect of KCl on the incorporation of myo-[2- ^3H]inositol into phosphatidylinositol (PI), the phosphatidylinositol content of the vas-deferens slices and the degradation of the phospholipid*

Determination of the incorporation of *myo*-inositol was as described in the text. The slices were incubated at 37°C for 30 min in the indicated medium containing $1\text{ }\mu\text{Ci}$ of *myo*-[2- ^3H]inositol. In the pulse-chase experiment the slices preloaded with *myo*-[2- ^3H]inositol as described in the text were incubated for 10 min at 37°C in KRB buffer containing 1 mM -*myo*-inositol. After the incubation, the medium was changed to fresh buffer containing 1 mM -*myo*-inositol, with and without 95 mM -KCl, and re-incubated for 20 min at 37°C . Preloaded slices contained $2375 \pm 190\text{ d.p.m.}$ of [^3H]phosphatidylinositol/ μg of total lipid phosphorus, and this value is designated as having a relative specific radioactivity of the lipid fraction of 100. Values are means $\pm$ s.e.m. for triplicate determinations.

Incubation medium	$10^{-2} \times$ Incorporated <i>myo</i> -[2- ^3H]inositol (d.p.m. in PI/ μg of total lipid phosphorus)	Phosphatidylinositol content (ng of PI phosphorus/ μg of total lipid phosphorus)	Relative specific radioactivity of the lipid fraction
KRB buffer (control)	5558 ± 78	72.2 ± 1.0	99 ± 3
KRB buffer + 95 mM -KCl	$4732 \pm 250^*$	72.3 ± 4.0	102 ± 2

* Significantly different from control ($P < 0.05$).

statistically significant increase in the incorporation of *myo*-[2-³H]inositol into phosphatidylinositol of the *vas-deferens* slices. However, when the slices were incubated with acetylcholine, which resulted in

a decrease in the phosphatidylinositol content of the slice, and then re-incubated with *myo*-[2-³H]inositol in the absence of acetylcholine, a significant increase in the incorporation compared with the

Fig. 3. Effect of acetylcholine, in the presence and absence of Ca²⁺, on the degradation of phosphatidylinositol in *vas-deferens* slices

Labelling of the slices with *myo*-[2-³H]inositol and the incubations as described below were carried out in KRB buffer (○, ●) or Ca²⁺-free KRB buffer containing 10 μM-ionophore A23187 and 0.5 mM-EGTA (Δ, ▲). The slices labelled in KRB buffer contained 3453 ± 208 d.p.m. of [³H]phosphatidylinositol/μg of total lipid phosphorus and those labelled in the absence of Ca²⁺ had 4780 ± 145 d.p.m. The radioactive slices were then re-incubated at 37°C in KRB buffer containing 1 mM-*myo*-inositol (○) or 1 mM-*myo*-inositol plus 50 μM-acetylcholine (●), or Ca²⁺-free KRB buffer containing 1 mM-*myo*-inositol, 10 μM-ionophore A23187, and 0.5 mM-EGTA, without (Δ) or with (▲) 50 μM-acetylcholine. Values are means ± S.E.M. for triplicate determinations.

Fig. 4. The effect of the concentration of acetylcholine, in the presence and absence of Ca²⁺, on the degradation of phosphatidylinositol in *vas-deferens* slices

Labelling of the slices with *myo*-[2-³H]inositol and the incubations were carried out in KRB buffer (●) or Ca²⁺-free KRB buffer containing 10 μM-ionophore A23187 and 0.5 mM-EGTA (○). The former slices contained 2920 ± 210 d.p.m. of [³H]phosphatidylinositol/μg of total lipid phosphorus and the latter slices contained 3550 ± 248 d.p.m. The slices were incubated at 37°C for 10 min in the same media containing 1 mM-*myo*-inositol. The slices were then re-incubated at 37°C for 20 min in their respective media with various concentrations of acetylcholine. The asterisk indicates the relative specific radioactivity before the addition of acetylcholine. Values are means ± S.E.M. for triplicate determinations.

Table 2. Effect of acetylcholine on the phosphatidylinositol (PI) content of *vas-deferens* slices
Incubations were carried out at 37°C for 30 min as described in the Materials and methods section. Eserine (50 μM) was added to all incubation media.

Incubation medium	Phosphatidylinositol content (ng of PI phosphorus/μg of total lipid phosphorus)
Without incubation	64.1 ± 1.0
KRB buffer (control)	64.1 ± 0.3*
KRB buffer + acetylcholine (50 μM)	56.8 ± 0.4†
KRB buffer + acetylcholine (500 μM)	52.6 ± 3.3‡
Ca ²⁺ -free KRB buffer + ionophore A23187 (10 μM) + EGTA (500 μM) (control)	73.2 ± 1.4‡
Ca ²⁺ -free KRB buffer + ionophore A23187 (10 μM) + EGTA (500 μM) + acetylcholine (50 μM)	48.1 ± 6.0§
Ca ²⁺ -free KRB buffer + ionophore A23187 (10 μM) + EGTA (500 μM) + acetylcholine (500 μM)	54.4 ± 0.7§

* Significantly different (*P* < 0.01) from †.
‡ Significantly different (*P* < 0.01) from §.

Table 3. *Effects of atropine and (+)-tubocurarine on the acetylcholine-induced decrease in phosphatidylinositol (PI) content of vas-deferens slices*

Incubations were carried out as described in the legend to Table 2. All incubation media contained KRB buffer and eserine (50 μ M).

Additions	Phosphatidylinositol content (ng of PI phosphorus/ μ g of total lipid phosphorus)
No additions	75.3 $\pm$ 2.5
Acetylcholine (50 μ M)	67.8 $\pm$ 3.2 [†]
Atropine (5 μ M)	83.5 $\pm$ 3.7
Acetylcholine (50 μ M) + atropine (5 μ M)	81.3 $\pm$ 3.0*
(+)-Tubocurarine (5 μ M)	85.9 $\pm$ 3.7
Acetylcholine (50 μ M) + (+)-tubocurarine (5 μ M)	67.4 $\pm$ 3.9

* Significantly different from [†] ($P < 0.05$).

Table 4. *Compensatory synthesis of phosphatidylinositol after acetylcholine-induced decrease of the phosphatidylinositol (PI) content of vas-deferens slices*

Slices were preincubated at 37°C for 30 min in the first medium, rinsed three times with 3 ml of ice-cold KRB buffer, and then incubated at 37°C for 30 min in the labelling medium containing 1 μ Ci of *myo*-[2-³H]inositol. When added the concentrations of the following substances were: eserine, 50 μ M; acetylcholine, 50 μ M; atropine, 5 μ M; (+)-tubocurarine, 5 μ M.

Preincubation medium	Labelling medium	10 ⁻² $\times$ Incorporated <i>myo</i> -[2- ³ H]inositol (d.p.m. in PI/ μ g of total lipid phosphorus)
KRB buffer	KRB buffer	1522 $\pm$ 195 [†]
KRB buffer + acetylcholine + eserine	KRB buffer	1940 $\pm$ 175*
KRB buffer + acetylcholine + eserine	KRB buffer + acetylcholine + eserine	1537 $\pm$ 117 [‡]
KRB buffer + acetylcholine + eserine	KRB buffer + atropine	2325 $\pm$ 180 [‡]
KRB buffer + acetylcholine + eserine	KRB buffer + (+)-tubocurarine	1987 $\pm$ 132

* Significantly different from [†] ($P < 0.05$).

[‡] Significantly different from * ($P < 0.05$).

Table 5. *Effect of 8-bromo cyclic GMP on the acetylcholine-induced degradation of phosphatidylinositol in vas-deferens slices*

The slices were labelled with *myo*-[2-³H]inositol in KRB buffer at 37°C for 30 min. The radioactive slices were incubated additionally at 37°C for 60 min in Ca²⁺-free KRB buffer containing 10 μ M-ionophore A23187 and 500 μ M-EGTA. The slices were then incubated at 37°C for 10 min in the indicated experimental medium, after which acetylcholine was added and the mixture was re-incubated at 37°C for 30 min. When added, the concentration of eserine was 50 μ M, acetylcholine was 50 μ M and 8-bromo cyclic GMP was 50 μ M.

Experimental medium	10 ⁻² $\times$ Incorporated <i>myo</i> -[2- ³ H]inositol (d.p.m. in PI/ μ g of total lipid phosphorus)
Before chase	3995 $\pm$ 70
Ca ²⁺ -free KRB buffer	4165 $\pm$ 160 [†]
Ca ²⁺ -free KRB buffer + 8-bromo cyclic GMP	4037 $\pm$ 275
Ca ²⁺ -free KRB buffer + eserine + acetylcholine	3082 $\pm$ 240* [‡]
Ca ²⁺ -free KRB buffer + eserine + acetylcholine + 8-bromo cyclic GMP	2997 $\pm$ 70* [§]

* Significantly different from [†] ($P < 0.01$).

[‡] Not significantly different from [§] ($P > 0.05$).

control was observed (Table 4). Presumably, this enhancement in incorporation into the phospholipid reflected a mechanism to compensate for the decreased phosphatidylinositol content. If, on the

other hand, acetylcholine was also added to the labelling medium, the compensatory incorporation was completely blocked. This inhibition in the recovery reaction might be explained by the action

of acetylcholine in inducing a high intracellular concentration of Ca^{2+} , which would inhibit the activity of CDP-diacylglycerol-inositol 3-phosphatidyltransferase. Table 4 also shows that compensatory incorporation of *myo*-[2- ^3H]inositol was even more pronounced when atropine was added to the labelling medium to inhibit the action of residual acetylcholine. Again, (+)-tubocurarine did not have any effect. These results indicate that the action of acetylcholine on the incorporation of *myo*-inositol into phosphatidylinositol is triggered by the degradation of phosphatidylinositol.

Since it is known that acetylcholine increases the [cyclic GMP] of smooth muscle (Lee *et al.*, 1972; Katsuki & Murad, 1977), it was possible that the acetylcholine-induced degradation was mediated by an increase in intracellular [cyclic GMP]. It was reported that 8-bromo cyclic GMP added externally relaxed the acetylcholine-propagated contraction of the vas deferens (Schultz *et al.*, 1977). However, as shown in Table 5, 8-bromo cyclic GMP did not affect the degradation of phosphatidylinositol. Furthermore, the acetylcholine-induced decrease was not abrogated. These results suggest that the acetylcholine-dependent degradation of phosphatidylinositol is not a result of the increase in [cyclic GMP].

Discussion

The results in the present report show that in rabbit vas deferens there is a Ca^{2+} -dependent inhibition in the incorporation of *myo*-inositol into phosphatidylinositol, a Ca^{2+} -dependent degradation of phosphatidylinositol and a decrease in the tissue content of phosphatidylinositol when the intracellular $[\text{Ca}^{2+}]$ is increased. The inhibition of phosphatidylinositol synthesis may be related to the reactions effecting the formation of the phospholipid. The final reaction in phosphatidylinositol synthesis is catalysed by CDP-diacylglycerol-inositol 3-phosphatidyltransferase (Takenawa & Egawa, 1977) or by the inositol exchange enzyme (Paulus & Kennedy, 1960; Takenawa *et al.*, 1977). The former enzyme catalyses the reaction:

The latter catalyses the exchange between free *myo*-inositol and phosphatidylinositol-bound *myo*-inositol. Ca^{2+} inhibits the activity of both enzymes, with the CDP-diacylglycerol-inositol 3-phosphatidyltransferase of smooth muscle being especially sensitive at low $[\text{Ca}^{2+}]$ (Egawa *et al.*, 1980).

The decrease in tissue phosphatidylinositol found when the $[\text{Ca}^{2+}]$ was experimentally increased can be attributed to the inhibition by Ca^{2+} of phos-

phatidylinositol synthesis as well as the enhancement by Ca^{2+} of phosphatidylinositol degradation. For example, a high concentration of KCl, which increases intracellular $[\text{Ca}^{2+}]$, inhibits synthesis. Acetylcholine, which also causes an increase in intracellular $[\text{Ca}^{2+}]$ (Chang & Triggle, 1973), enhances degradation. Moreover, a Ca^{2+} -dependent enzyme that cleaves phosphatidylinositol by a phospholipase C-like mechanism has been reported in various tissues (Dawson, 1959; Kemp *et al.*, 1961; Allan & Michell, 1974; Irvine & Dawson, 1978); it is predominantly cytosolic in location. More recently, a Ca^{2+} -independent enzyme has been reported that degrades phosphatidylinositol also by phospholipase C-like action, but it is limited to the lysosome (Irvine *et al.*, 1978). Whether this latter enzyme is activated by cell stimulation with acetylcholine has yet to be examined. Thus, the two mechanisms for Ca^{2+} -dependent decreases in phosphatidylinositol content are found in the same tissue.

The increase in phosphatidylinositol turnover in different tissues by various stimuli has been described (Michell, 1975). In the present study with the vas deferens cholinergic stimulation induces both the degradation of phosphatidylinositol and the compensatory synthesis of phosphatidylinositol. The two processes were clearly separated. This separation suggests that the increased labelling of phosphatidylinositol in various stimulated tissues is not the direct effect of the stimulus but a secondary effect of the stimulus. The compensatory synthesis of the phospholipid is itself inhibited by acetylcholine, possibly due to the increase in intracellular $[\text{Ca}^{2+}]$ in the presence of the neurotransmitter.

The finding that phosphatidylinositol synthesis is inhibited in the rabbit vas deferens when intracellular $[\text{Ca}^{2+}]$ is raised is similar to the situation as found in the blowfly salivary gland (Berridge & Fain, 1979). This type of inhibition has not been reported previously in other mammalian tissues. Indeed, in some cases an increase in ^{32}P labelling into phosphatidylinositol of the rat vas deferens during 2 h of incubation has been reported (Canessa de Scarnati & Lapetina, 1974). In the present study *myo*-[2- ^3H]inositol incorporation is decreased for at least the first 0.5 h and as long as 1 h (results not shown). Several possible explanations of the difference may be suggested. (1) The incorporation of *myo*-inositol may not correlate precisely with the incorporation of phosphorus (Tolbert *et al.*, 1980). (2) In most reports, the increase in phosphatidylinositol turnover is expressed in terms of specific radioactivity. But an increase in specific radioactivity does not necessarily reflect an increase in synthesis. If there is a decrease of synthesis to a relatively minor extent and an enhancement of degradation (decrease in content) to a relatively larger extent, there will result an increase in specific

radioactivity despite an inhibition of synthesis, at least in the initial phase. (3) In some tissues the application of stimulus may result in an increase in specific radioactivity as well as an enhancement of phosphatidylinositol synthesis. In this situation, degradation of phosphatidylinositol and its resynthesis are tightly coupled. The coupling may be loose in the rabbit vas deferens. One possible explanation of this looseness of coupling is as follows. Phosphatidylinositol synthesis in the intact rabbit vas deferens has been found to be partially inhibited by endogenous Ca^{2+} (Egawa *et al.*, 1980). This inhibition is almost complete in rabbit aorta. Removal of Ca^{2+} by the ionophore A23187 and EGTA results in the enhancement of phosphatidylinositol synthesis (Egawa *et al.*, 1980). This suggests that the rate of phosphatidylinositol synthesis is dependent, at least in part, on the endogenous $[\text{Ca}^{2+}]$. If one assumes that in the rabbit vas deferens the $[\text{Ca}^{2+}]$ is rather high, then any additional influx of Ca^{2+} , due to acetylcholine stimulation, will be sufficient to inhibit phosphatidylinositol synthesis. On the other hand, in other tissues, e.g. rat liver, the endogenous $[\text{Ca}^{2+}]$ may be relatively low. Then, even after influx of Ca^{2+} due to stimulation, the $[\text{Ca}^{2+}]$ does not reach the concentration at which phosphatidylinositol synthesis is inhibited.

It has been suggested that the increase in [cyclic GMP] that accompanies smooth-muscle contraction may be part of a negative-feedback mechanism to induce Ca^{2+} influx or to increase Ca^{2+} efflux (Schultz *et al.*, 1977). Another possibility was proposed by Diamond & Janis (1978) that cyclic GMP may not mediate the relaxant effect, since some relaxants act on the vas deferens without altering [cyclic GMP]. However, since Ca^{2+} inhibits activated guanylate cyclase in smooth muscle (Takenawa & Sacktor, 1979) and 8-bromo cyclic GMP decreases the contraction of smooth muscle (Nawrath, 1977), it has also been hypothesized that cyclic GMP and Ca^{2+} may participate in a reciprocal negative-feedback mechanism (Takenawa & Sacktor, 1979). The present findings show that 8-bromo cyclic GMP does not inhibit the degradation of phosphatidylinositol and fails to inhibit the acetylcholine-induced degradation of the phospholipid at a concentration that should block the tonic response of acetylcholine. These results argue additionally that the acetylcholine-induced degradation of phosphatidylinositol is not mediated by cyclic GMP.

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