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Calcium- and Calmodulin-Promoted Phosphorylation of Membrane Proteins during Senescence in Apples[1,2]

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Sodium dodecylsulfate-polyacrylamide gel electrophoresis of microsomal membrane proteins from post-climacteric apples at an early and an advanced stage of senescence showed only slight qualitative changes in the protein pattern. Though there was a 30% reduction in the total microsomal protein content in apples at an advanced stage of senescence, a polypeptide with 18,000 molecular weight increased in quantity during senescence. In vitro phosphorylation of several proteins was promoted by calcium in membranes from apples at an early stage of senescence. Phosphorylation of proteins with molecular weights of 95,000, 91,000, 53,000 and 50,000 was promoted by calcium and calmodulin. Phosphorylation of these proteins increased with increasing calcium concentration. Proteins with molecular weights of 53,000 and 50,000 showed marked promotion of phosphorylation over the calcium-promoted level when the amount of calmodulin in the assay mixture was increased. Calcium- and calmodulin-promoted phosphorylation of membrane proteins showed considerable decrease when the apples were at an advanced stage of senescence. Moreover, increasing the concentrations of calcium and calmodulin in the assay mixture did not have any promoting effect on the phosphorylation of these proteins. Phosphoprotein phosphatase activity as measured by the loss of label from phosphorylated proteins following chase with cold ATP, did not differ to a great extent in membrane preparations from normal and senesced apples. Hydrolysis of ATP by senesced apple membrane preparation, however, was found to be relatively higher. The significance of these observations in relation to senescence is discussed.

Key words: Calmodulin — *Malus domestica* — Membrane — Protein phosphorylation — Senescence.

Among the inorganic elements calcium has been found to play a major role in the growth and development of plants (Leopold et al. 1974, Hanson 1983). Calcium has been found to inhibit abscission (Poovaiah and Leopold 1973a) and synergistically act with cytokinins in delaying senescence of corn leaf discs (Poovaiah and Leopold 1973b). The biochemical effects of calcium are observed to be mediated through a calcium-binding protein of ubiquitous occur-

Abbreviations: DTT, dithiothreitol; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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rence called calmodulin (Cheung 1970, Kakiuchi and Yamazaki 1970). NAD kinase (Muto and Miyachi 1977), calcium ATPase (Dieter and Marmé 1981) and isofloridoside phosphate synthase (Kauss 1983) are some of the enzyme systems known to be promoted by calcium and calmodulin in plants. Because of its wide ranging effects calcium has been considered as a second messenger in plants (Marmé 1982).

Covalent modification of enzymes between active and inactive forms is a key process involved in metabolic regulation. Covalent modifications caused by reversible phosphorylation of enzymes catalyzed by protein kinases and phosphatases are well studied (Schulman et al. 1980, Trewavas 1976). The physiological roles of protein kinases are well known in animal systems (Greengard 1978). Calcium- and calmodulin-promoted protein phosphorylation has been reported in many plant systems (Hetherington and Trewavas 1982, Polya and Davies 1982, Salimath and Marmé 1983, Veluthambi and Poovaiah 1984a, b). However, very few reports exist on the physiological roles of protein phosphorylation in plants (Refeno et al. 1982, Kauss 1983, Zocchi et al. 1983).

Earlier studies in our laboratory showed that calcium treatment delayed the senescent breakdown of post-climacteric Golden Delicious apples (Poovaiah and Shekhar 1978). During investigations on membrane properties of apples we observed that microsomal membranes from normal apples responded to in vitro addition of calcium by showing breaks in the fluorescence polarization profile of membrane-embedded diphenyl hexatriene. However, the same treatment using membranes from apples at an advanced stage of senescence did not induce such breaks (Paliyath et al. 1982). Increase in microviscosity of membranes has been noted with senescence (McKersie et al. 1978). Such changes could alter the microenvironment of proteins thus affecting their conformation and function. Inhibition of calmodulin activity by endogenous compounds was found to increase during senescence in apples (Paliyath and Poovaiah 1984). Therefore, calcium- and calmodulin-promoted phosphorylation of membrane proteins which is one of the possible indications of the functional status of the membranes was studied in normal and senesced apples.

Materials and Methods

Plant material and chemicals—Apple fruits (*Malus domestica* Borkh. cv. Golden Delicious) were collected in their climacteric phase and stored at 2°C. Before the experiment, a set of apples was transferred to −20°C and stored frozen (these apples are referred to as normal apples or apples at an early stage of senescence). At the same time another set of apples was transferred to room temperature to accelerate senescence processes and stored for 45 days until wrinkles appeared on the surface due to dehydration. These apples are referred to as senesced apples or apples at an advanced stage of senescence. These apples were also stored frozen at −20°C.

[γ-^{32}P]ATP was obtained from New England Nuclear, Boston, Massachusetts. All supplies for electrophoresis were purchased from BioRad Laboratories, Richmond, California. Calmodulin (bovine brain), HEPES, EDTA, EGTA, DTT and PMSF were obtained from Sigma Chemical Co., St. Louis, Missouri.

Preparation of the membrane fraction—The pulp tissue was ground with the extraction buffer containing 50 mM HEPES pH 7, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 5 mM EDTA, 0.2 mM EGTA and 0.25 M sucrose [1.5 ml·(g fr wt)$^{-1}$] using a mortar and pestle. The extract was squeezed through 4 layers of cheese cloth and centrifuged at 8,000$\times g$ for 10 min. The supernatant from this step was again centrifuged at 105,000$\times g$ for 60 min. The pellet was resuspended in a buffer containing 50 mM HEPES, pH 7, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.25 M sucrose and 1 mM DTT and centrifuged at 105,000$\times g$ for 40 min. Resuspension and centrifugation was repeated. The pellet obtained in the final step was resuspended in an appropriate volume of resuspension buffer using an all glass homogenizer which gave a fine particulate suspension. Protein estimation was conducted by the Coomassie dye binding assay (Bradford 1976).

Phosphorylation of proteins—Protein phosphorylation was conducted according to the methods of Schulman and Greengard (1978). The assay buffer consisted of 50 mM HEPES, pH 7, 5 mM $MgCl_2$ and 0.2 mM EGTA. The final volume of the assay mixture was 100 μl. Three hundred microgram membrane protein was added per assay. Triton X-100 (0.1%, w/v) was added 15 min before starting the assay. Appropriate amounts of calcium and calmodulin were added wherever necessary. The assay mixture was preincubated at 30°C for 1 min and 0.25 nmol of [γ-^{32}P]ATP (30–40 Ci·mmol^{-1}) was added. Phosphorylation was allowed to occur for 1 min at 30°C and the reaction was terminated by the addition of an equal volume of electrophoresis sample buffer (0.12 M Tris-HCl, pH 6.8, 4% SDS, 10% mercaptoethanol and 20% glycerol) and heating the preparation for 5 min in a boiling water bath. The tubes were centrifuged in an Eppendorf centrifuge and the supernatant obtained was used for electrophoresis.

Gel electrophoresis—Sodium dodecylsulfate-polyacrylamide gel electrophoresis of the proteins was performed according to the method by Laemmli (1970). The separation gel consisted of an 8 to 16% (w/v) acrylamide linear gradient and the stacking gel contained 4% (w/v) acrylamide. Aliquots containing 45 μg protein were loaded on 1.5 mm thick slab gels and run at 20 mA current per slab gel till the proteins reached the separation gel and then at 30 mA per slab gel. The gels were stained in 0.25% (w/v) Coomassie brilliant blue, 50% (v/v) methanol and 7.5% (v/v) acetic acid for 4 h. The gels were destained in 30% (v/v) methanol, 7.5% (v/v) acetic acid and incubated in the same destaining fluid containing 3% (v/v) glycerol overnight. The gels were dried using a slab gel drier and exposed to Kodak X-Omat AR film for an appropriate period (2–3 days) for autoradiography. The autoradiograms were scanned using a Quick Quant II scanner.

Measurement of phosphoprotein phosphatase activity and ATP hydrolysis—Relative differences in phosphoprotein phosphatase activity in membrane preparations from normal and senesced apples were determined as follows: Protein phosphorylation was conducted as described earlier in presence of 1 mM calcium and 0.6 μM calmodulin. After 1 min of incubation with labelled ATP, the incorporated radioactivity was chased with 100 fold excess (25 nmol) of unlabelled ATP. The reaction was stopped by adding 200 μl of 50% TCA at various time intervals. Chloroform : methanol (1 : 1, 1 ml) was added to the reaction mixture and filtered using Whatman GF/C glass microfiber filters. The filters were washed with 90% methanol (25 ml) and the radioactivity on the filter determined by counting in a Packard Tricarb liquid scintillation counter. The rate of decrease in the radioactivity from the proteins as a function of time would be a measure of phosphoprotein phosphatase activity. In order to obtain nearly equal amounts of phosphorylation higher amounts of protein were used in the case of senesced apples (200 μg per assay for normal apple membranes, 300 μg per assay for senesced apple membranes).

ATP hydrolysis was measured by estimating the amount of free ATP in the assay mixture with time, as well as by estimating ATP incorporated into the proteins. The amount of ATP that cannot be accounted for would give a relative measure of ATP hydrolyzed. In this experiment it was assumed that the disappearance of label from ATP due to phosphoprotein phosphatase activity on phosphorylated proteins was nearly equal in normal and senesced apples. The assay mixture contained 50 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.6 μM calmodulin, 500,000 cpm of [γ-^{32}P]ATP, 5 μM unlabelled ATP and 0.1% Triton X-100 (v/v) in a total volume of 500 μl. The reaction was allowed to occur at 30°C for various time intervals and was stopped by heating in a boiling water bath. The tubes were cooled and centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was collected and an aliquot (25 μl) was fractionated using a Waters HPLC system equipped with a C_{18} μ-Bondapak column (BioRad, 25 cm × 4 mm). KH_2PO_4 (10 mM, pH 2.5) was used as the solvent and a flow rate of 1.5 ml/min was used.

Absorbance was monitored at 260 nm. Fraction corresponding to ATP was collected and counted in a Packard Tricarb liquid scintillation counter. The pellet obtained after centrifugation was resuspended in 1 ml chloroform : methanol (1 : 1) and processed as described earlier and counted.

Results

Changes in the pattern of membrane proteins from normal and senesced apples—There was a 30% reduction in the membrane protein content (37.5 μg·(g fr wt)$^{-1}$ in normal apples to 25 μg·(g fr wt)$^{-1}$ in senesced apples). The qualitative pattern of the proteins remained nearly the same (Fig. 1). However, proteins with molecular weights of 65,000, 59,000, 56,000 and 39,000 are reduced in membranes from senesced apples. A protein with a molecular weight of 18,000 showed marked increase in membranes of senesced apples as compared to that from normal apples.

Calcium- and calmodulin-promoted phosphorylation of proteins in membranes from normal apples—Minimal phosphorylation of proteins was observed in the absence of added calcium except in non-specific (calcium-independent) phosphorylation (66 kD). Addition of calcium resulted in the promotion of phosphorylation of several proteins with molecular weights of 81,000, 44,000, 40,000, 35,000 and 18,000 (Fig. 2). Addition of exogenous calmodulin promoted the phos-

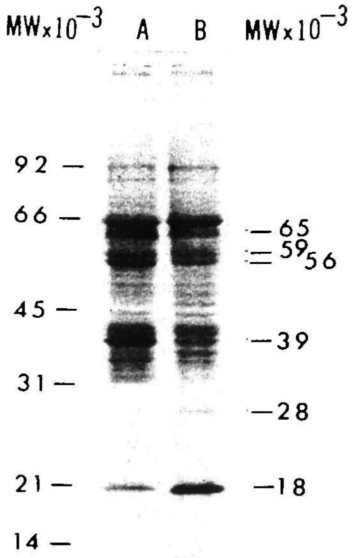

Fig. 1

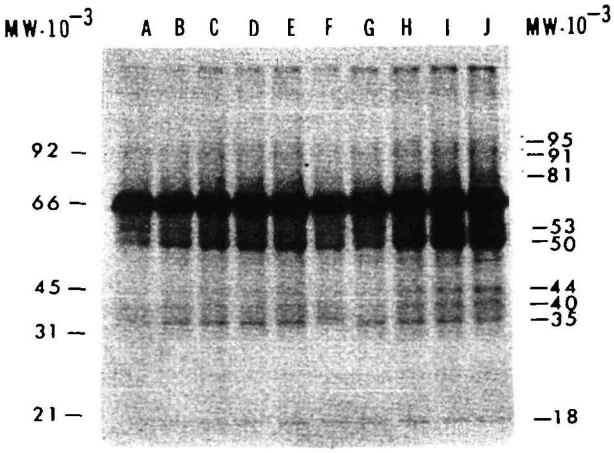

Fig. 2

Fig. 1 Separation of membrane proteins on SDS-polyacrylamide gel. 45 μg protein from normal apples (lane A) and senesced apples (lane B) was loaded. The molecular weights of standard proteins are indicated on the left side of figure. The proteins that have shown quantitative changes are indicated on the right side of the figure.

Fig. 2 Effect of calcium and calmodulin on the phosphorylation of microsomal membrane proteins from normal apples. Lane A, −Ca; lanes B to E, calcium concentrations of 250 μM, 500 μM, 1 mM and 2 mM, respectively; lane F, +calmodulin (2.5 μg) in the absence of calcium; lanes G to J, +Ca (1 mM) and increasing calmodulin concentrations from 0.5 μg, 1 μg, 2.5 μg and 5 μg (0.06 μM to 0.6 μM), respectively. The molecular weights of standard proteins are shown on the left side of the figure. The molecular weights of phosphorylated proteins (promoted by calcium or calcium and calmodulin) are indicated on the right.

phorylation of these proteins. Calmodulin-promoted phosphorylation was marked in proteins with molecular weights of 95,000, 91,000, 53,000 and 50,000.

Effect of increasing the calcium and calmodulin concentrations in the assay mixture on protein phosphorylation can be clearly seen in Fig. 2. Most of the proteins, the phosphorylation of which was promoted by calcium, showed increased phosphorylation with increasing calcium concentration (from 250 μM to 2 mM). However, the saturation level of phosphorylation for different proteins could differ and therefore concentration dependence could clearly be observed only in some proteins. The assay mixture already contains 0.2 mM EGTA. Higher concentrations of calcium were required to attain saturation of protein phosphorylation probably because of low free calcium concentration in the assay mixture resulting from chelation by EGTA, non-specific binding to membranes and uptake of calcium into microsomal vesicles. Increasing the calmodulin concentration from 0.5 μg (0.06 μM) to 5 μg (0.6 μM) showed increased phosphorylation of those proteins, the phosphorylation of which was promoted by calcium. As pointed out earlier the effect was most clearly observed in the phosphorylation of proteins with molecular weights of 53,000 and 50,000. Calmodulin alone (in the absence of added calcium) did not promote phosphorylation over the $-$Ca control (Fig. 2, lane F).

Calcium- and calmodulin-promoted protein phosphorylation in membranes from senesced apples—When phosphorylation was conducted using membrane preparations from senesced apples under identical conditions as used for membranes from normal apples (Fig. 2), a marked reduction in the calcium- and calmodulin-promoted phosphorylation of proteins was observed (Fig. 3). Increasing the calcium concentration from 0.5 to 2 mM or increasing the calmodulin concentration (0.5 μg to 5 μg) in the presence of 1 mM calcium did not show any increase in protein phosphorylation as obtained when membranes from normal apples were used. In contrast, the calcium-independent phosphorylation of the protein with mol wt of 66,000 in membranes from senesced apples did not show a decrease.

Changes in calcium- and calmodulin-promoted protein phosphorylation during senescence—To obtain a quantitative estimation of the promotion of protein phosphorylation by increasing concentrations of calcium and calmodulin and their decrease during senescence, the lanes shown in Fig. 2 and 3 were scanned. The peak areas for the protein with mol wt of 50,000 were calculated and plotted against calcium and calmodulin concentrations. The results are shown in Fig. 4. Saturation of protein phosphorylation was attained at 500 μM calcium concentration. Phosphorylation of this protein increased in presence of 1 mM Ca^{++} and calmodulin concentrations ranging from 0.5 μg (0.06 μM) to 5 μg (0.6 μM). However, other proteins were not as responsive

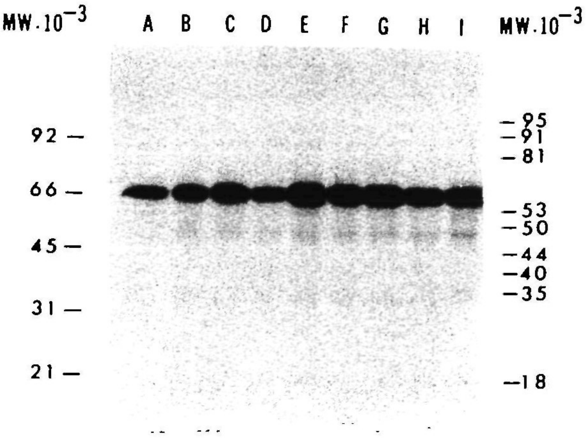

Fig. 3 Calcium- and calmodulin-promoted phosphorylation of membrane proteins from senesced apples. Lane A, $-$Ca; lanes B, C, D, +Ca (500 μM, 1 mM, 2 mM, respectively); lane E $-$Ca +calmodulin (2.5 μg); lanes F, G, H, I, +Ca (1 mM) +calmodulin (0.5 μg, 1 μg, 2.5 μg and 5 μg, respectively). For control see lanes G–J in Fig. 2). The molecular weights of standard proteins are shown on the left side of the figure. The molecular weights of phosphorylated proteins (promoted by calcium or calcium and calmodulin) are indicated on the right.

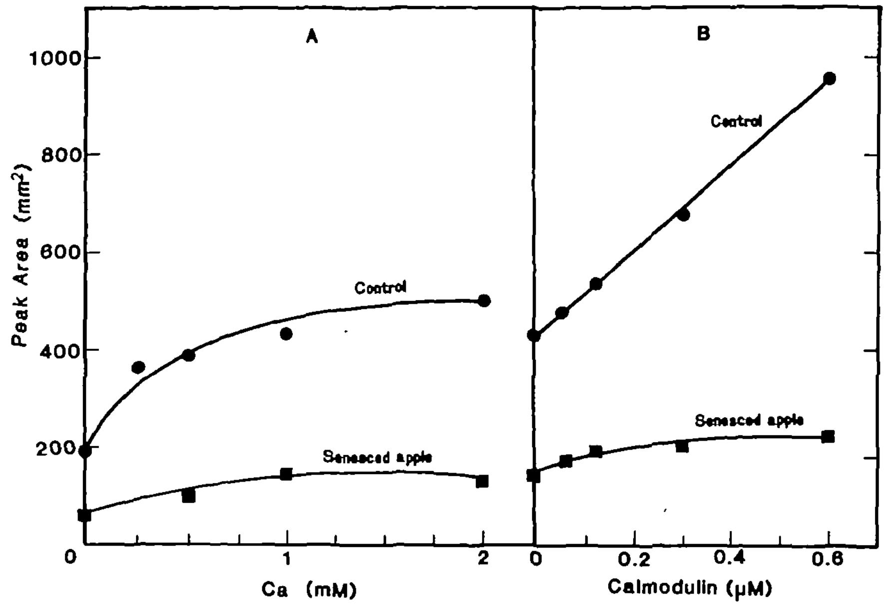

Fig. 4 Effect of increasing the concentrations of calcium (A) and calmodulin (B) on phosphorylation of the protein with mol wt of 50,000 in normal and senesced apples. Calcium concentration was varied from 250 μM to 2 mM and calmodulin concentration from 0.06 μM to 0.6 μM. Calmodulin-promoted phosphorylation was performed in the presence of 1 mM calcium.

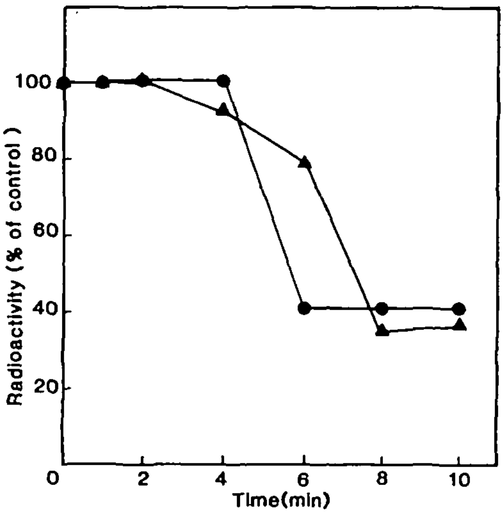

Fig. 5

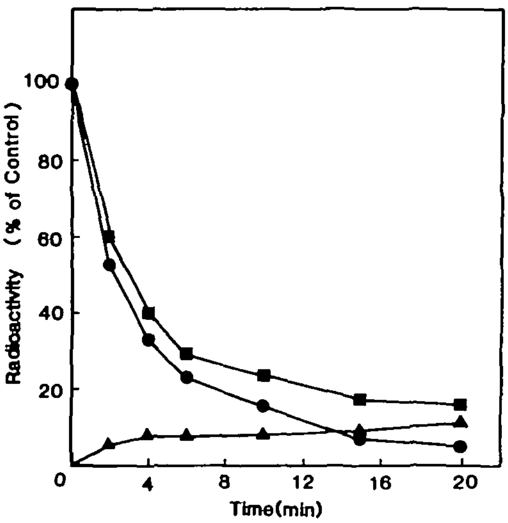

Fig. 6

Fig. 5 Phosphoprotein phosphatase activity in membrane preparations from normal (-●-) and senesced apples (-▲-). Protein phosphorylation obtained before the addition of cold ATP is considered as 100%. The total incorporation (100%) in normal apple membranes was 396,000 cpm·mg protein^{-1}·min^{-1} and 400,000 cpm·mg protein^{-1}·min^{-1} in senesced apple membranes.

Fig. 6 ATP hydrolysis in membrane preparations from normal apples. (-▲-) incorporation into membrane proteins; (-●-) free ATP in the assay mixture; (-■-) total accountable ATP. ATP hydrolysis at time "t" expressed as percent of supplied ATP = 100 − percent total ATP remaining at time "t". 100% = 500,000 cpm.

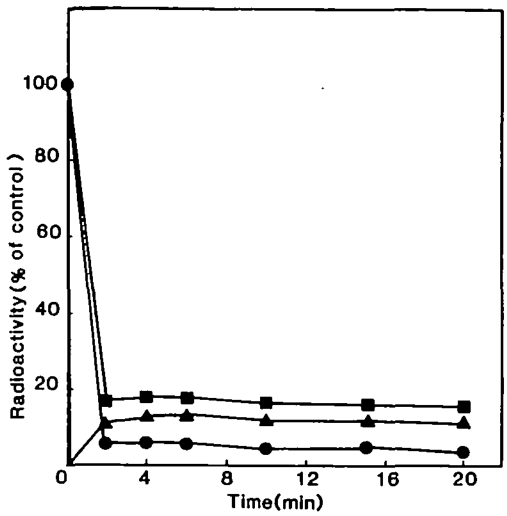

Fig. 7 ATP hydrolysis in membrane preparations from senesced apples. (-▲-) Incorporation into membrane proteins; (-●-) free ATP in the assay mixture; (-■-) total accountable ATP. Other parameters are the same as in Fig. 6.

as this protein to increasing calmodulin concentration and reached saturation at low levels of calmodulin.

Phosphoprotein phosphatase activity in membranes—Since Ca- and calmodulin-promoted protein phosphorylation was found to decrease during senescence, it appeared possible that phosphoprotein phosphatase activity might be higher in membrane preparation from senesced apples. Phosphoprotein phosphatase activity in normal and senesced apple membranes is shown in Fig. 5. Two to four minutes after addition of unlabelled ATP a decrease in protein labelling could be noted in both preparations. Protein-bound radioactivity continued to decrease and appeared to reach a steady value after 6 to 8 minutes. This amounted to 35 to 40% of the incorporation of label obtained at zero time (before the addition of unlabelled ATP). These results suggested that phosphoprotein phosphatase activity is not higher in senesced apples than in normal apples and the decrease in Ca- and calmodulin-promoted protein phosphorylation obtained in senesced apple membranes may not be the result of a higher phosphoprotein phosphatase activity.

ATP hydrolysis in membrane preparation—The activities of hydrolytic enzymes are known to increase during senescence. Therefore it was possible that an excessive ATP hydrolase activity in senesced apples could deplete the supplied ATP, thus limiting the availability of ATP for phosphorylation. Therefore the extent of ATP hydrolysis by membrane preparations from normal and senesced apples was investigated. The results are shown in Fig. 6 and 7. In membranes from normal apples ATP hydrolysis increased with time and reached saturation after 10 min (Fig. 6). Nearly 40% of the supplied ATP was hydrolyzed within the first two min. From Fig. 5 it was seen that phosphoprotein phosphatase activity could hardly be detected within this time period. Within the normal time period of phosphorylation assays (1 min) 20% of ATP was hydrolyzed (Fig. 6). ATP hydrolysis was considerably higher in senesced apples. Nearly 40% of the supplied ATP is hydrolyzed within the first minute (Fig. 7) and after the loss of 80% of the label, it did not decrease further.

Discussion

The promotion of Ca^{++} and calmodulin-dependent protein kinases by Ca^{++} and calmodulin provides a mechanism by which calcium can regulate diverse biochemical processes. The occur-rence of Ca^{++} and calmodulin-dependent protein kinases in membranes from a wide variety of tissues such as coleoptiles, hypocotyls, cell suspensions and fruits suggests that such a phosphorylation system could be of general importance as a biochemical mechanism of action of calcium. In addition to protein kinases these membranes also contain a wide array of substrates whose functions ultimately determine the physiological status of the cell, tissue or the plant. These observations offer a promising approach for studying the biochemical mechanism of action of calcium as a second messenger in plant systems.

Earlier studies in our laboratory showed that the fluorescence polarization of membrane embedded diphenyl hexatriene increased in membranes from senesced apples as compared to normal cold-stored apples (Paliyath et al. 1984). This indicated an increase in microviscosity of apple membranes with senescence. Moreover, membrane preparations from senesced apples showed a different response to added calcium (Paliyath et al. 1982). These results indicated a change in membrane property during senescence. Since there was no significant difference in the phospholipid content between senesced and normal apple membranes we presumed that there could be a change in the protein pattern or in their functions. The results showed that with senescence there was a decrease in the membrane protein content as observed in other senescing systems (Marinos 1962, Thimann 1980). The decrease was marked in some proteins. An interesting observation was the increase in a low molecular weight protein (18 kD) in membranes from senesced apples. Synthesis of new proteins have also been observed in ripening tomatoes (Rattanapanone et al. 1978). The physiological significance of the 18 kD protein to senescence in apples requires further study.

Another interesting feature from our study was the decrease in calcium- and calmodulin-promoted protein phosphorylation in membranes from senesced apples. Though equal amounts of membrane proteins were used from both normal and senesced apples, the phosphorylation of proteins promoted by Ca^{++} and calmodulin, was considerably reduced in membrane proteins from senesced apples. Phosphoprotein phosphatase activity in membrane preparations from normal and senesced apples did not differ much which suggested that a relatively high phosphoprotein phosphatase activity in senesced apple membranes could not be a major factor that resulted in the decrease in Ca- and calmodulin-promoted protein phosphorylation. Another factor that could reduce phosphorylation is the availability of ATP itself. An increase in ATP hydrolysis during senescence could result in higher amounts of ATP hydrolysis in senesced apple membrane preparations. ATP hydrolysis was found to be higher in senesced apples. However, it becomes a significant factor only during long-term experiments. Within the time of phosphorylation studies employed (1 min) there is only a 20% increase in ATP hydrolysis over the value obtained for normal apples (40% in senesced apples, 20% in normal apples; assuming a linear decrease up to 2 min). This might partly be due to a higher protein content used in the assay (300 μg for senesced apples, 200 μg for normal apples). Even under advanced conditions of senescence, calcium-independent phosphorylation in membranes of senesced apples was not affected. Therefore, under the assay conditions employed, an excessive ATP hydrolysis might not have resulted in decreased Ca- and calmodulin-promoted phosphorylation.

Thus it appears that the decrease in membrane protein phosphorylation during senescence could be due to: 1) selective degradation of protein kinases and substrates or, 2) inhibition of enzyme activities by unfavorable microenvironment. These aspects are currently being studied in our laboratory. The inhibition of calcium- and calmodulin-promoted protein phosphorylation could alter the biochemical processes in the cell and could be a key feature in the onset and progress of senescence.

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