Bidirectional Activity of the Endoplasmic Reticulum Ca^{2+}-ATPase of Bovine Adrenal Cortex†

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ABSTRACT: It is generally accepted that the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor play major roles in the complex mechanisms by which agonists increase intracellular Ca^{2+} concentration. In these mechanisms, the endoplasmic reticulum Ca^{2+}-ATPase has been attributed an accessory role of refilling the intracellular Ca^{2+} store. In the present study, the activity of the microsomal Ca^{2+}-ATPase of bovine adrenal cortex was investigated. We show that the Ca^{2+}-pumping activity of the Ca^{2+}-ATPase is related to the ADP/ATP ratio. Our results also show that a brisk increase of the ADP/ATP ratio upon addition of exogenous ADP triggered a rapid release of Ca^{2+} from preloaded microsomes. ADP released Ca^{2+} in a dose-dependent manner with an EC_{50} of 2.98 ± 0.78 mM. ADP-induced Ca^{2+} release was not prevented by heparin, ruling out the participation of the inositol 1,4,5-trisphosphate receptor. ADP-induced Ca^{2+} release could not be attributed to the mere inhibition of the Ca^{2+}-ATPase, since the rate of ADP-induced Ca^{2+} release was 20 times faster than the rate of Ca^{2+} release induced by a maximal concentration of thapsigargin (2 μM). ADP-induced Ca^{2+} release experiments performed in the presence of [^{32}P]PO_4 revealed a concomitant production of [^{32}P]ATP. ADP-induced [^{32}P]ATP production was dose-dependent, with an EC_{50} of 5.50 ± 0.70 mM. ADP-induced [^{32}P]ATP production was prevented by ionomycin (10 μM) and by high concentrations of extramicrosomal Ca^{2+}. These results demonstrate that the microsomal Ca^{2+}-ATPase of adrenal cortex possesses a bidirectional activity that depends on ADP concentrations, the Ca^{2+} gradient across the microsomal membrane, and probably also ATP concentrations. The reverse activity of the endoplasmic reticulum Ca^{2+}-ATPase could therefore play an additional role in cellular Ca^{2+} homeostasis. Indeed, under physiological or pathological conditions, where local ADP concentrations are increasing (and/or ATP concentrations are decreasing), the reverse activity of the Ca^{2+} pump should liberate Ca^{2+} from intracellular stores.

Ca^{2+} is an important second messenger that regulates a whole range of cellular processes (Carafoli, 1987; Berridge, 1993; Tsien & Tsien, 1990). Instead of being synthesized and degraded by enzymatic processes like other second messengers, intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) are finely adjusted by complex mechanisms involving Ca^{2+} channels, Ca^{2+} pumps, and Ca^{2+} exchangers. Each one of these Ca^{2+} carriers has its own regulatory system. Among the most important Ca^{2+} carriers, the inositol 1,4,5-trisphosphate ($InsP_3$)[1] receptor is a Ca^{2+} channel that controls the release of Ca^{2+} from intracellular stores (Berridge, 1993; Streb *et al.*, 1983; Meldolesi *et al.*, 1990). The Ca^{2+} release activity of the $InsP_3$ receptor is under the direct control of a receptor-activated phospholipase C that generates $InsP_3$. The activity of this receptor is also modulated by ATP. Indeed, low concentrations of ATP (10−500 μM) enhance $InsP_3$ binding to its receptor (Spät *et al.*, 1992) and thus potentiate $InsP_3$-induced Ca^{2+} release (Smith *et al.*, 1985; Ferris *et al.*, 1990; Iino, 1991), whereas high concentrations of ATP (>500 μM) competitively inhibit the binding of $InsP_3$ to its receptor (Guillemette *et al.*, 1987; Nunn & Taylor, 1990; Willcocks *et al.*, 1987) and thus interfere with its Ca^{2+} release activity. The intracellular and intraluminal (within the endoplasmic reticulum lumen) Ca^{2+} concentrations are important components of the $InsP_3$-induced Ca^{2+} release regulatory system and have been involved in the complex quantal process by which $InsP_3$ releases Ca^{2+}. Indeed, Ca^{2+} is an important modulator of $InsP_3$ binding and Ca^{2+} release activity [for a review, see Berridge (1993), Marshall and Taylor (1993)]. Thus, Ca^{2+} is able to control its own intracellular concentration by modulating the activity of the phospholipase C and also by modulating $InsP_3$ binding and Ca^{2+} release activity. Cross regulation of the receptor activity by other second messengers has also been suggested. Indeed, phosphorylation of the receptor by cyclic AMP-dependent protein kinase modulates its activity (Supattapone *et al.*, 1988; Burgess *et al.*, 1991; Bird *et al.*, 1993). The $InsP_3$ receptor is also a target for the protein kinase C, Ca^{2+} calmodulin-dependent protein kinase II (Yamamoto *et al.*, 1989; Ferris *et al.*, 1991), and cyclic GMP-dependent protein kinase (Komalavilas & Lincoln, 1994). The phosphorylation sites for protein kinase C, Ca^{2+} calmodulin-dependent protein kinase II, and cyclic AMP-dependent protein kinase are different. The cyclic GMP-dependent protein kinase phosphorylates the same site as the cyclic AMP-dependent protein kinase. Thus, the cyclic AMP-dependent protein kinase and

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[1] Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; CICR, Ca^{2+}-induced Ca^{2+} release; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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the cyclic GMP-dependent protein kinase have their specific regulatory sites on the $InsP_3$ receptor and can then modulate it independently from the PLC-generated $InsP_3$ pathway. Finally the receptor activity is also regulated by intracellular Mg^{2+} (Varney *et al.*, 1990; Volpe *et al.*, 1990), pH (Brass & Joseph, 1985; Guillemette & Segui, 1988; Joseph *et al.*, 1989; Worley *et al.*, 1987), and redox state (Bird *et al.*, 1993; Bootmann *et al.*, 1992; Poitras *et al.*, 1993; Swann, 1991; Hilly *et al.*, 1993).

The ryanodine receptor is another important Ca^{2+} channel responsible for the release of Ca^{2+} from intracellular stores (McPherson & Campbell, 1993). The opening of this Ca^{2+} channel is also modulated by adenine nucleotides, sulhydryl reagents, Mg^{2+}, and Ca^{2+} (Fleischer & Inui, 1989). Elevations of $[Ca^{2+}]_i$ are responsible for the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism by which the ryanodine receptor channel is mostly regulated. A novel second messenger, cyclic ADP-ribose, has been described as the endogenous activator of the CICR channel (Galione, 1993). The ryanodine receptor channel is a substrate for cyclic AMP protein kinase and Ca^{2+} calmodulin-dependent protein kinase II, suggesting a cross regulation of this channel by other second messengers.

Following an elevation of the $[Ca^{2+}]_i$, plasma membrane Ca^{2+}-ATPases (PMCA family) and sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPases (SERCA family) rapidly restore the initial $[Ca^{2+}]_i$ by removing the excess of Ca^{2+} from the cytosol. The SERCA Ca^{2+}-ATPases are also responsible for the refilling of the intracellular Ca^{2+} stores, thus preparing the cell for further stimulations. Ca^{2+} pumping is initiated by the binding of two Ca^{2+} ions to a high-affinity binding site on a cytosolic domain of the pump. Following the binding of one ATP molecule and the phosphorylation of the Ca^{2+}-ATPase, the enzyme undergoes a conformational change leading to the translocation of Ca^{2+} from the cytosolic side to the luminal side of the endoplasmic reticulum. This conformational change is accompanied by a transition from a high- to low-affinity state for Ca^{2+}, thus releasing Ca^{2+} in the lumen. Recombinant DNA studies revealed that SERCA Ca^{2+}-ATPases originate from at least three alternatively spliced genes, producing five different proteins. Proteins from SERCA 1 (splices A and B) and SERCA 2 (splice A) genes are expressed in cardiac and skeletal muscles, while proteins from SERCA 2 (splice B) and SERCA 3 genes are expressed in smooth muscle and nonmuscle tissues and in various tissues, respectively [for a review, see Edes and Kranias (1995), MacLennan *et al.* (1991)]. Despite the high homology between the different gene products (75−85%), differences in their regulatory mechanisms are observed. Ca^{2+}-ATPase activity is modulated by intracellular pH, redox state, temperature, and Ca^{2+} concentrations (MacLennan *et al.*, 1992; Poitras *et al.*, 1993; Ribeiro-Do-Valle *et al.*, 1994). Differences in the sensitivity of the Ca^{2+}-ATPase to vanadate, Ca^{2+}, and pH have been observed between the Ca^{2+}-ATPase isoforms SERCA 1 and SERCA 3 (MacLennan *et al.*, 1992). Ca^{2+}-ATPase activity is also modulated by the low molecular weight protein phospholamban. The dephosphorylated form of phospholamban is an inhibitor of the Ca^{2+}-ATPase. Phosphorylation of phospholamban by PKA disrupts the interaction between phospholamban and the Ca^{2+}-ATPase, thus relieving the inhibition (Edes & Kranias, 1995).

It is generally considered that the ryanodine receptor and the $InsP_3$ receptor play major roles in the complex mechanisms controlling the release of Ca^{2+} from intracellular stores, whereas the Ca^{2+}-ATPase plays only an accessory role of refilling the Ca^{2+} pools. In this study, we looked at the activity of the microsomal Ca^{2+}-ATPase of bovine adrenal cortex and found that this Ca^{2+} pump has a bidirectional activity that depends on the ADP/ATP ratio and the Ca^{2+} gradient across the endoplasmic reticulum membrane. Therefore, this bidirectional activity of the pump could play a significant role in the spatial and temporal changes of $[Ca^{2+}]_i$ in nonmuscular tissues under physiological or pathological conditions.

MATERIALS AND METHODS

Materials. $InsP_3$ (trilithium salt) was obtained from LC Services Corp. (Woburn, MA), and ionomycin and Fura-2 (free acid) were obtained from Calbiochem (La Jolla, CA). $[^{32}P]PO_4$ (acid form; carrier free) and $[\gamma\text{-}^{32}P]ATP$ were from Amersham (Arlington Heights, IL). Anion exchange resin AG 1-X8 was from BIO-RAD (Richmond, CA). All other reagents were from Sigma (St. Louis, MO) or Fisher (Fairlawn, NJ).

Preparation of Microsomes. Bovine adrenal glands were obtained at a nearby slaughterhouse. Bovine adrenal cortices (dissected free of medullary tissue) were homogenized with eight strokes of a Dounce homogenizer (loose pestle) in a medium (medium A) containing 25 mM Tris-HCl buffered at pH 7.2, 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 1 mM dithiothreitol, and 2 mM EGTA. After centrifugation at 500*g* for 15 min, the supernatant was centrifuged at 35000*g* for 20 min. The 35000*g* pellet was resuspended in the same medium without EGTA (medium B) and centrifuged at 35000*g* for 20 min. The pellet was resuspended in medium B supplemented with glycerol (14%, v/v) and sorbitol (1.4%, w/v) at a concentration of 30−40 mg of protein/mL. This preparation was aliquoted and stored at −70 °C until used for ADP-induced Ca^{2+} release and $[^{32}P]$-ATP formation studies.

Ca^{2+} Uptake and Ca^{2+} Release Studies. Bovine adrenal cortex microsomes (8−10 mg of protein) were incubated in a medium containing 20 mM Tris-HCl buffered at pH 7.2, 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 40 mM phosphocreatine, and 20 units/mL creatine kinase in a final volume of 2 mL. No external Ca^{2+} was added since, under our experimental conditions, contaminating Ca^{2+} (around 1 μM) was sufficient to measure Ca^{2+} movements. Ca^{2+} uptake was initiated by the addition of ATP (2 mM) to the bathing medium containing the microsomes. The Ca^{2+}-releasing effects of $InsP_3$ and ADP were measured shortly after ATP-dependent Ca^{2+}-sequestering activity had reached a steady state (about 20 min after the addition of ATP). The free Ca^{2+} concentration of the medium was monitored with Fura-2 (free acid, 1 μM) in a Hitachi F-2000 spectrofluorometer. The excitation wavelength was 340 nm (slit 10 nm), and the emission was recorded at 500 nm (slit 10 nm). Ca^{2+} uptake and release were measured in the presence of oligomycin (10 μg/mL) in order to block mitochondrial ATPase. Incubations were performed at 37 °C. This conventional approach was successfully used to measure $InsP_3$-induced Ca^{2+} release in adrenal cortex microsomes (Guillemette *et al.*, 1987, 1991; Poitras *et al.*, 1993). Each record was calibrated by the addition of a known amount of Ca^{2+} ($CaCl_2$) to the mixture. The actual

free Ca^{2+} concentration of the medium was calculated from the F_{max} and F_{min} values obtained by adding excess Ca^{2+} and EGTA (at pH 8.5), respectively, after treatment with 2 μM ionomycin. The equation used was $[Ca^{2+}] = 224$ nM $(F - F_{min})/(F_{max} - F)$.

[^{32}P]ATP Formation Studies. Bovine adrenal cortex microsomes (4−5 mg of protein) were incubated in the same medium used for Ca^{2+} release studies. This medium was supplemented with [^{32}P]PO_4 (≈2.5 μCi), and the experiments were performed at 37 °C in a final volume of 1 mL in the presence (control conditions) or absence of 10 μM ionomycin (in order to prevent the formation of the Ca^{2+} gradient). Ca^{2+} uptake was initiated by the addition of ATP (2 mM) to the bathing medium containing the microsomes; 20 min after the addition of ATP, ADP was added at different concentrations, and 1 min after the addition of ADP, the incubations were terminated by centrifugation at 15000*g* for 15 min at 4 °C. The supernatants were applied to AG1-X8 columns (1 mL wet bed volume). [^{32}P]PO_4 and [^{32}P]ATP were sequentially eluted by addition of ammonium formate/formic acid mixtures of increasing ionic strength. The radioactivity in the fractions was evaluated by liquid scintillation spectrometry. Net [^{32}P]ATP production was estimated by subtracting the value obtained in the presence of ionomycin from the value obtained in the absence of ionomycin.

RESULTS

When incubated at 37 °C in the presence of 2 mM ATP, bovine adrenal cortex microsomes (8−10 mg of protein) showed high Ca^{2+}-sequestering activity (Figure 1, lower panel). Under these conditions, in the presence of a strong ATP-regenerating system (40 mM phosphocreatine), the ambient Ca^{2+} concentration was decreased from a value *ca.* 1.1 μM to a basal steady state level *ca.* 0.31 μM that could be maintained for at least 2000 s. At steady state, the ADP/ATP ratio is low since most of the ADP derived from the degradation of ATP is rephosphorylated by creatine kinase. Addition of 2 μM ionomycin immediately released all the accumulated Ca^{2+}, indicating the vesicular nature of the Ca^{2+}-sequestering process. When microsomes were incubated in the presence of a weaker ATP-regenerating system (10 mM phosphocreatine) (Figure 1, upper panel), the addition of ATP (2 mM) decreased the ambient Ca^{2+} concentration to a low level that could not be maintained for more than 400 s. The Ca^{2+} leak observed under these conditions could be attributed to the degradation of ATP (thus increasing the ADP/ATP ratio) since further addition of the ATP-regenerating system (phosphocreatine 10 mM) (Figure 1, middle panel) prevented the Ca^{2+} leak and brought back temporarily the Ca^{2+} concentration to its previous low level.

To further emphasize this point, we studied the effects of ADP on microsomal Ca^{2+}-sequestering activity. Figure 2A shows that $InsP_3$ (1 μM) triggered a rapid release of Ca^{2+} from preloaded microsomes. Since $InsP_3$ is rapidly metabolized by a microsomal 5-phosphatase activity, Ca^{2+} was sequestered back into its reservoir, thus decreasing the ambient Ca^{2+} concentration to the basal level. A similar amount of Ca^{2+} was released upon subsequent addition of $InsP_3$, indicating that the system was not desensitized. Figure 2B shows that ADP (2.5 mM) also triggered a rapid release of Ca^{2+} (maximal response obtained within 5−10 s). As observed with $InsP_3$, the increase of ambient Ca^{2+} concentration produced by ADP was transient, suggesting that ADP was phosphorylated by the ATP-regenerating system. The new steady state level of Ca^{2+} was slightly higher than the initial one, indicating an incomplete phosphorylation of ADP, resulting in a slight increase of the ADP/ATP ratio. The release of Ca^{2+} induced by ADP (Figure 2C,D) was not prevented by the $InsP_3$ receptor antagonist heparin (200 μg/mL), ruling out the participating of the $InsP_3$ receptor in the effect of ADP. Similar results were also obtained in the presence of 20 μM ruthenium red, ruling out the participating of the ryanodine sensitive Ca^{2+} channel (unshown results).

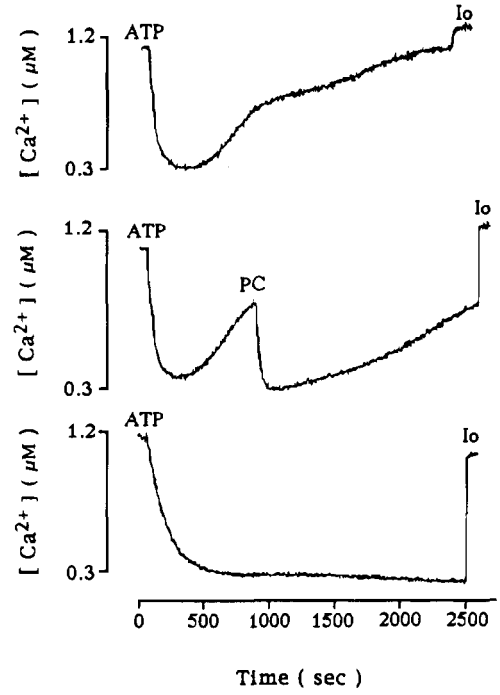

FIGURE 1: ATP-dependent Ca^{2+}-sequestering activity of bovine adrenal cortex microsomes. Microsomes (8−10 mg of protein) were incubated at 37 °C, and the Ca^{2+} uptake activity (monitored with Fura-2 free acid, 1 μM) was initiated with the addition of 2 mM ATP, under the conditions described in Materials and Methods. In the upper and middle panels, the Ca^{2+} uptake activity was monitored in the presence of 10 mM phosphocreatine (ATP-regenerating system). In the middle panel, another 10 mM phosphocreatine was added at 900 s. In the lower panel, the Ca^{2+} uptake activity was monitored in the presence of 40 mM phosphocreatine. ATP, 2 mM ATP; PC, 10 mM phosphocreatine; Io, 2 μM ionomycin. These typical traces were reproduced at least three times with three different microsomal preparations.

The release of Ca^{2+} induced by ADP was dose-dependent (Figure 3). The half-maximal release was obtained with 2.95 ± 0.78 mM ADP (mean ± standard deviation of four experiments), and the maximal effect was obtained with 10 mM ADP. In the inset (Figure 3, upper panel), the same results are expressed as a function of the ADP/ATP ratio. It is shown that a small increase of the apparent ADP/ATP ratio caused only a small variation of the ambient Ca^{2+} concentration, whereas a larger increase of the ADP/ATP ratio caused an important variation of the ambient Ca^{2+} concentration. The rate of Ca^{2+} release (Figure 3, lower panel) induced by a submaximal concentration of ADP (1075 nM/min) was comparable to the rate of Ca^{2+} release induced by a submaximal concentration of $InsP_3$ (774 nM/min) but was 20 times faster than the rate of Ca^{2+} release (55 nM/min) induced by a maximal concentration of thapsigargin (2 μM). These results suggest that ADP-induced Ca^{2+}

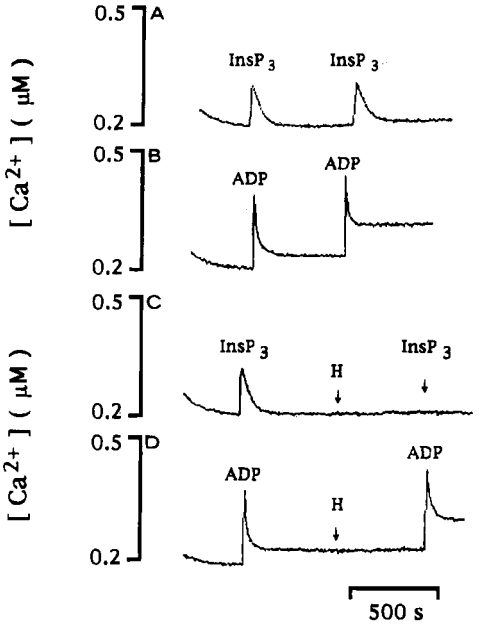

FIGURE 2: $InsP_3$- and ADP-induced Ca^{2+} release activity of preloaded microsomes. Microsomes (8−10 mg of protein) were incubated at 37 °C, and their Ca^{2+} uptake activity (monitored with Fura-2 free acid, 1 μM) was initiated with the addition of 2 mM ATP, under the conditions described in Materials and Methods. $InsP_3$- and ADP-induced Ca^{2+} release activities were evaluated in the absence (panels A and C) or presence (panels B and D) of heparin 15 min after the addition of ATP. $InsP_3$, 1 μM $InsP_3$; ADP, 2.5 mM ADP; H, 200 μg/mL heparin. These typical traces were reproduced at least three times with five different microsomal preparations.

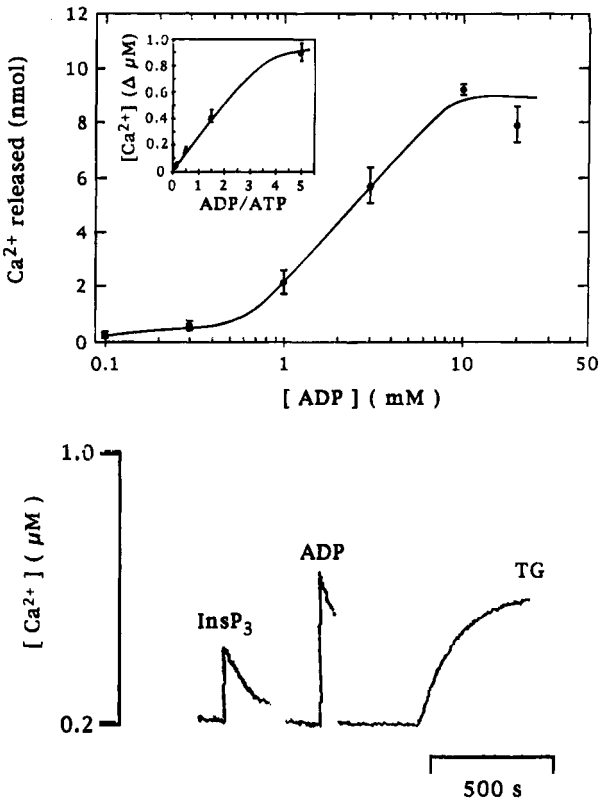

FIGURE 3: Dose−response curve of ADP-induced Ca^{2+} release. Microsomes (8−10 mg of protein) were incubated at 37 °C, and their Ca^{2+} uptake activity was initiated by the addition of 2 mM ATP. Different concentrations of ADP were added 20 min after the initiation of the Ca^{2+}-sequestering process. The amount of Ca^{2+} released was calibrated by addition of a known amount of $CaCl_2$. Upper panel shows the dose−response curve of ADP-induced Ca^{2+} release. The inset of the upper panel shows ambient Ca^{2+} concentration variations after the addition of different concentrations of ADP, as a function of the apparent ADP/ATP ratio. The lower panel shows the rate of Ca^{2+} release induced by 1 μM $InsP_3$ ($InsP_3$), 2.5 mM ADP (ADP), or 2 μM thapsigargin (TG). $InsP_3$, ADP, and TG were added 15 min after the initiation of the ATP-dependent Ca^{2+} uptake activity. Similar results were obtained with four different microsomal preparations.

release could not be due simply to the inhibition of the Ca^{2+}-ATPase.

Like other enzymes, the Ca^{2+}-ATPase may work in a reverse way if proper conditions are established. The reverse activity of the Ca^{2+}-ATPase implies that the Ca^{2+} gradient across the endoplasmic reticulum membrane is a driving force for the formation of ATP from ADP and inorganic phosphate. Figure 4 shows that a significant amount of [^{32}P]-ATP was formed upon addition of 10 mM ADP to preloaded microsomes incubated in the presence of [^{32}P]PO_4 (solid line). Similar results were obtained when the reaction was stopped (with perchloric acid, 5%) shortly (10 s) after ADP addition, indicating that ADP-induced [^{32}P]ATP formation occurs within a similar time interval as ADP-induced Ca^{2+} release. This ADP-induced [^{32}P]ATP formation was inhibited by the Ca^{2+} ionophore ionomycin (10 μM) which dissipates the Ca^{2+} gradient (dashed line). ADP-induced [^{32}P]ATP formation was dose-dependent (Figure 5, upper panel). The half-maximal production of [^{32}P]ATP was obtained with 5.50 ± 0.70 mM ADP (mean ± standard deviation of four experiments), and the maximal effect was observed with 30 mM ADP. As shown in the lower panel of Figure 5, ADP-induced [^{32}P]ATP formation was also prevented by adding excess Ca^{2+} (1.6 mM) in the surrounding medium or under conditions where the microsomes had not been previously loaded with Ca^{2+} (in the absence of ATP).

DISCUSSION

In opposition to the functional roles of the $InsP_3$ receptor and the ryanodine receptor which release Ca^{2+} from intracellular stores, the endoplasmic reticulum Ca^{2+}-ATPase has exclusively been associated with the refilling of the Ca^{2+} stores (Berridge, 1993; Meyer & Stryer, 1991). In the present study, we have demonstrated that the endoplasmic reticulum Ca^{2+}-ATPase exerts a bidirectional activity that is regulated by the Ca^{2+} gradient across the membrane and by the ADP concentration in the surrounding medium. We have shown that the pumping activity (direct activity) of the Ca^{2+}-ATPase was maximal under conditions where high concentrations of ATP and low concentrations of ADP were maintained. Under these conditions, the filling of the Ca^{2+} stores reached a steady state at which the Ca^{2+}-ATPase is expected to maintain a basal activity in order to reuptake the Ca^{2+} that passively leaks from the Ca^{2+} stores. The requirement for a sustained basal activity of the Ca^{2+}-ATPase is better demonstrated in the presence of a weak ATP-regenerating system (upper panel of Figure 1), where the phosphorylation of ADP is too slow or inadequate to maintain the ambient Ca^{2+} concentration at a low level. The most simple explanation for this phenomenon is that the gradual disappearance of ATP slows the Ca^{2+}-ATPase activity to such an extent that it cannot compensate for the passive Ca^{2+} leak from the stores. If ATP was the unique regulator of the Ca^{2+}-ATPase activity, then the addition of

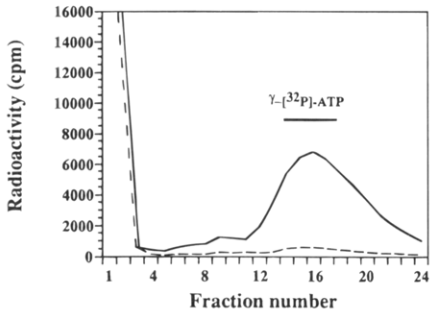

FIGURE 4: Separation of [^{32}P]ATP and [^{32}P]PO_4 by anion exchange chromatography. Microsomes (4−5 mg of protein) were incubated as described in the legend of Figure 3 with [^{32}P]PO_4, and the Ca^{2+} uptake activity was initiated by the addition of 2 mM ATP. ADP (10 mM) was added 20 min after the initiation of the Ca^{2+}-sequestering process, and the incubation was stopped by centrifugation (15000*g* for 15 min at 4 °C) 1 min after the addition of ADP. The supernatants were applied to AG1-X8 columns. [^{32}P]PO_4 and [^{32}P]ATP were sequentially eluted by addition of ammonium formate/formic acid mixtures of increasing ionic strength. Fractions 1−4, 9 mL of 0.3 M ammonium formate/0.1 M formic acid; fractions 5−8, 3 mL of 0.5 M ammonium formate/0.1 M formic acid; fractions 9−24, 2 mL of 1.0 M ammonium formate/0.1 M formic acid. The microsomes were initially incubated in the absence (solid line) or presence (dashed line) of 10 μM ionomycin. The graph shows the coelution of the radioactive material with [γ-^{32}P]ATP (from Amersham).

exogenous ADP would accelerate the formation of ATP (by providing more substrate to the ATP regenerating system) and consequently accelerate the Ca^{2+}-ATPase activity and thus decrease the ambient Ca^{2+} concentration. On the contrary, our results showing that exogenous ADP increased the ambient Ca^{2+} concentration suggest that the ADP concentration and the ADP/ATP ratio are the actual regulators of the Ca^{2+}-ATPase activity. Thus, any experimental condition that decreases the ADP/ATP ratio (high concentration of ATP, strong ATP-regenerating system) will accelerate the Ca^{2+}-ATPase, whereas experimental conditions that increase the ADP/ATP ratio (weak ATP-regenerating system, high concentration of ADP) will slow the Ca^{2+}-ATPase.

We have shown that the rate of ADP-induced Ca^{2+} release was 20 times faster than the rate of thapsigargin-induced Ca^{2+} release. Since the mechanism of action of thapsigargin is known to be the selective inhibition of the endoplasmic reticulum Ca^{2+}-ATPase, the rate of thapsigargin-induced Ca^{2+} release is probably reflecting the passive Ca^{2+} leak from the store. The very high rate of ADP-induced Ca^{2+} release cannot therefore be attributed exclusively to a passive leak resulting from the inhibition of the Ca^{2+}-ATPase. In that case, another mechanism should also be implicated.

Many enzymes, among which the mitochondrial ATP synthase is a good example (Gomez *et al.*, 1986), are known to have a bidirectional activity. Like the ATP synthase (which also has an ATPase activity), the endoplasmic reticulum Ca^{2+}-ATPase should also work in a reverse way under certain conditions. If this enzyme can effectively work in a reverse way, one would expect that ATP synthesis from ADP and inorganic phosphate will occur concurrently with ADP-induced Ca^{2+} release. The energy required to form the γ-phosphate chemical bond of ATP would be provided by the Ca^{2+} gradient across the endoplasmic reticulum membrane. We have shown that the EC_{50} for ADP-induced Ca^{2+} release (2.95 mM) was in the same range as the EC_{50} for ADP-induced $^{32}PO_4$-ATP formation (5.50 mM), and we

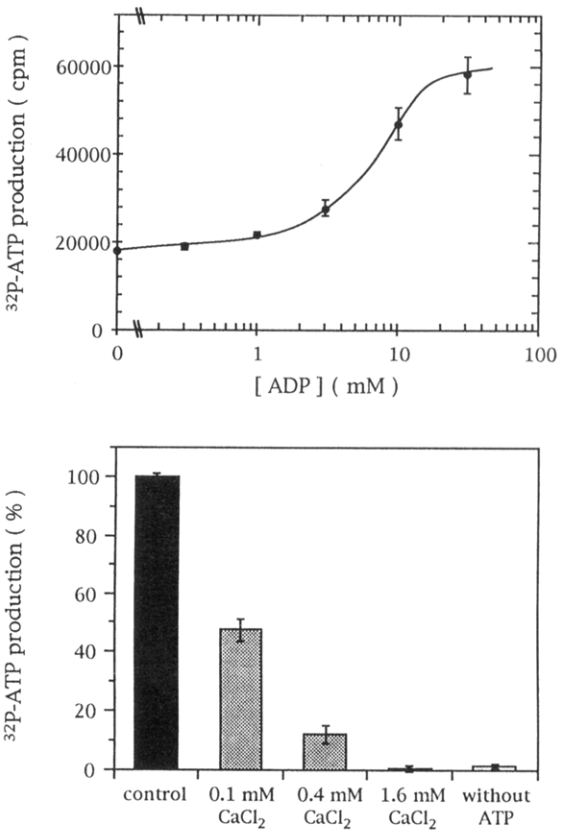

FIGURE 5: Dose−response curve of ADP-induced [^{32}P]ATP production. Microsomes (4−5 mg of protein) were incubated at 37 °C in the presence of [^{32}P]PO_4, and the Ca^{2+} uptake activity was initiated by the addition of 2 mM ATP. Different concentrations of ADP were added 20 min after the initiation of the Ca^{2+}-sequestering process, and the incubation was stopped by centrifugation (15000*g* for 15 min at 4 °C) 1 min after the addition of ADP. The supernatants were applied to AG1-X8 columns, and the separation of [^{32}P]ATP and [^{32}P]PO_4 was performed as described in the legend of Figure 4. Upper panel shows the net production of [^{32}P]ATP induced by different concentrations of ADP. Lower panel shows the net production of [^{32}P]ATP induced by 10 mM ADP in the presence of different concentrations of extraluminal $CaCl_2$ or when no ATP was initially added to activate the sequestration of Ca^{2+}. Similar results were obtained with four different microsomal preparations.

have also observed that the two activities occur simultaneously. These results strongly suggest that the endoplasmic reticulum Ca^{2+}-ATPase possesses a reverse activity that depends on the ADP/ATP ratio in the surrounding medium and on the Ca^{2+} gradient across the endoplasmic reticulum membrane. A threshold concentration around 500 μM ADP was necessary to reverse the activity of the Ca^{2+}-ATPase. This relatively high concentration appears to be much more elevated than the actual free ADP concentration found in living cells (between 20 and 50 μM; Balaban *et al.*, 1986). It is well known that a large proportion of the intracellular ADP is bound to proteins and membranes (Hitzig *et al.*, 1987). This buffering effect of proteins and membranes maintains the free intracellular ADP concentrations at a very low level. In our system, large amounts of microsomes are utilized in order to assure a sufficient Ca^{2+}-sequestering activity that can bring the ambient Ca^{2+} concentration to a level low enough to be evaluated with fluorescent indicators. Under these conditions, an important fraction of added ADP could likely be buffered by the microsomes. It is also important to note that under our conditions, the strong ATP-

regenerating system constitutes another strong ADP buffer. It is thus very likely that the free ADP concentrations in our experiments are much lower than the concentrations that would be reached under nonbuffering conditions.

The reverse activity of the Ca^{2+} pump is also suggested by our data showing that ADP-induced $^{32}PO_4$-ATP formation was totally inhibited under conditions where the Ca^{2+} gradient across the endoplasmic reticulum membrane was abolished (Figure 5, lower panel). Our results are in agreement with those of Makinose (1971) and De Meis (1982) who showed that the sarcoplasmic reticulum Ca^{2+}-ATPase of skeletal muscle possesses a bidirectional activity. As previously mentioned, many (if not most) enzymes are likely to show this property under proper conditions. We observed that a non-negligible amount of [^{32}P]ATP was formed even when no exogenous ADP was added to the preloaded microsomes (Figure 5, upper panel). As soon as an adequate Ca^{2+} gradient is established, a small proportion of the Ca^{2+}-ATPases could thus work in a reverse way even during the pumping process under conditions where the ADP/ATP ratio is rather low. These results suggest that the Ca^{2+}-ATPase could have a bidirectional activity under physiological conditions. Thus, a major proportion of the Ca^{2+}-ATPases would exert a Ca^{2+}-pumping activity when the local ADP/ATP ratio is low (allowing the filling of local compartments of the intracellular Ca^{2+} store), and a major proportion of the Ca^{2+}-ATPases would exert an ATP synthase and a Ca^{2+} release activity when the local ADP/ATP ratio is high (this last activity being also dependent on an adequate Ca^{2+} gradient across the local compartments' membranes of the endoplasmic reticulum).

An important point to address is whether local variations of the ADP and/or ATP concentrations occur under physiological conditions. The precise determination of the free ATP and ADP concentrations in cells is still presenting some difficulties. However, with the noninvasive and nondestructive technique of ^{31}P NMR spectroscopy, actual concentrations of ATP and creatine phosphate (CrP) could be estimated inside muscular cells, but the very low levels of free ADP were under the limit of detection (Hitzig *et al.*, 1987). According to the equilibrium equation of the creatine kinase reaction, and assuming that this reaction was constantly near equilibrium, the actual ADP concentrations were estimated from the CrP/ATP ratio (Veech *et al.*, 1979). While the actual ATP concentrations remained relatively constant in muscular cells during contractile activity, changes (decrease) of the CrP/ATP ratio have been observed, reflecting an increase of the actual free ADP concentration (Hitzig *et al.*, 1987; Bittl & Ingwall, 1986). Since cytoplasmic free ADP content is very low (20−50 μM), a small increase in the ADP content would considerably change the ADP/ATP ratio. These changes of the cytoplasmic ADP concentration and the resulting changes of the ADP/ATP ratio, as small as they may appear in whole cells, probably reflect more important changes of local ADP/ATP ratios within specialized compartments of the cells.

Corkey *et al.* (1988) showed that elevation of the Ca^{2+} concentration occurred when the ADP/ATP ratio was increased (by modifying the strength of the ATP-regenerating system in permeabilized cells) under conditions where no hormonal stimulation was required. Under such conditions, the endoplasmic reticulum Ca^{2+}-ATPase is susceptible to work in a reverse way, and therefore, we believe that the reverse activity of the Ca^{2+}-ATPase is likely to play a significant role in cellular Ca^{2+} homeostasis. Under pathological conditions, following ischemia, the ADP/ATP ratio is likely to increase. Furthermore, under more physiological conditions, after cell stimulation, the release of Ca^{2+} from intracellular stores (via either the $InsP_3$ receptor or the ryanodine receptor) causes a local increase of intracellular Ca^{2+} concentration. This local elevation of Ca^{2+} activates local Ca^{2+}-ATPases, thus enhancing the degradation of ATP and the accumulation of ADP. The local degradation of ATP causes a diffusion of ATP from a nearby region, whereas the newly formed ADP diffuses to a nearby region. These two opposite migrations will significantly change the ADP/ATP ratio at a nearby compartment of the intracellular Ca^{2+} pool and promote the reverse activity of the Ca^{2+}-ATPase. This phenomenon is consistent with the propagation of Ca^{2+} waves.

In conclusion, we have shown that the endoplasmic reticulum Ca^{2+}-ATPase of bovine adrenal cortex possesses a bidirectional activity. Our results suggest the possibility that the Ca^{2+}-ATPase may not be exclusively involved in a "secondary" role of refilling the internal Ca^{2+} stores but may also be involved, under certain conditions, in the release of Ca^{2+} from intracellular stores.

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