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ATP-sensitive K^+ transport in rat pancreatic zymogen granule

membranes and activation by protein kinase A.

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ABSTRACT: Pancreatic secretory granules contain electrolyte
transport pathways for Cl^- and K^+ that may contribute to exocyto-
tic membrane fusion or net fluid secretion following membrane
fusion. Recently the granule K^+ transport pathway was shown to
exhibit characteristics of the ATP-sensitive K^+ channels which
include inhibition by ATP. K^+ transport by the zymogen granule
membrane was measured indirectly following K^+ dependent osmotic
swelling and ionophore-induced lysis of the granule while incu-
bated in KCl and sucrose solutions, pH 7.0, at 37°C. This lysis
rate was K^+ dependent and ATP inhibited. The results also show
that this granule K^+ transport pathway can be activated by cAMP-
dependent protein kinase phosphorylation. ATP reduced the K^+
dependent rate in a dose dependent manner. Subsequent incubation
of apical plasma membranes with 15 units/ml of the catalytic
subunit of protein kinase A restored K^+ transport in fusion
experiments. Although protein kinase C induced K^+ transport was

not elevated over control levels, the results of protein kinase A stimulated K^+ transport support the assertion of ATP-sensitive K^+ transport by secretory granules. This suggests a mechanism for the regulation of secretory granules consistent with the known signaling mechanisms controlling stimulus-secretion coupling in pancreatic acinar cells.

Introduction

Exocytosis is the process by which transport vesicles, leaving the trans Golgi network, fuse with the apical membrane and release their contents into the extracellular environment. Fusion requires that the transport vesicles migrate to and recognize the target membrane before docking. However, regulated exocytotic fusion does not occur until it is triggered by an extracellular signal.

Extracellular signals, such as hormones or neurotransmitters, are required to stimulate secretion. Since most of these signaling molecules are hydrophillic, they are unable to cross the plasma membrane. Instead, they bind to cell-surface receptors which in turn generate the intracellular signals required for secretion.

The majority of intracellular signaling proteins are either protein kinases or G-proteins. The net result is typically a phosphorylation cascade mediated by two main classes of protein kinases. These are the serine/threonine kinases and the tyrosine kinases. The activation of these protein kinases allows them to phosphorylate specific sets of proteins in a target cell. Phosphorylation acts as a molecular switch to alter the structure of these proteins which in turn promote secretion. The target cell contains three main families of cell-surface receptors which convert extracellular signals into a cellular response. Such receptors are the ion-channel linked receptors which are gated to open or close in response to a bound ligand; G-protein linked

receptors, which modulate the activity of membrane bound enzymes or ion channels via G proteins; and enzyme-linked receptors, which can act directly as enzymes or protein kinases to phosphorylate specific proteins in the target cell.

The intracellular signaling processes in pancreatic acinar cells relies on the regulation of cytosolic Ca^{2+} and cyclic AMP. Cytosolic Ca^{2+} is regulated by G-protein linked receptors coupled to the inositol-phospolipid signaling pathway.

The binding of a signaling molecule to a G-protein linked receptor in the plasma membrane activates phospholipase C. This enzyme cleaves PI-bisphosphate (PIP_2) to generate inositol trisphosphate and diacylglycerol. Diacylglycerol remains in the plasma membrane and activates protein kinase C. However, the inositol trisphosphate (IP_3) is a small water soluble molecule that leaves the plasma membrane and rapidly diffuses through the cytosol. It releases Ca^{2+} from the endoplasmic reticulum by binding to IP_3-gated Ca^{2+}-release channels in the endoplasmic reticulum membrane resulting in an increase in cytosolic Ca^{2+}. The concentration of free Ca^{2+} in the cytosol is low at rest, whereas its concentration in the endoplasmic reticulum is high. Consequently, there is a large gradient tending to drive Ca^{2+} into the cytosol from the endoplasmic reticulum when a signal temporarily opens the Ca^{2+} channels.

Another important second-messenger in the regulation of stimulus-secretion coupling is cAMP. As in the previous pathway, its control relies on an extracellular ligand which binds to a receptor-G protein. However, in this pathway, the G-protein

activates adenylyl cyclase, a plasma membrane enzyme. Adenylyl
cyclase is responsible for producing cyclic AMP from ATP which
then activates protein kinase A to phosphorylate specific target
proteins on serine or threonine residues. Phosphorylation of
these proteins can create the proper stimulus to open ion chan-
nels.

An additional regulatory mechanism involves the rab 3 G-pro-
tein. This protein may be responsible for regulating ATP-depend-
ent K^+ channels. Pancreatic secretory granule membranes contain
rab proteins which are monomeric GTP binding proteins(Lambert et
al., 1990; Padfield and Jamieson, 1991; Schnefel et al., 1992).
Once the secretory granule binds to the target membrane at its
specific site, the rab 3 protein hydrolyzes GTP and locks the
vesicle on to the target membrane. Rab bound GDP is then re-
leased into the cytosol and fusion occurs. When the rab 3 pro-
tein has bound GTP, the K^+ channel is closed. When the rab 3
protein has bound GDP, the K^+ channel opens. The combined ef-
forts of protein kinases and G-proteins may have an important
regulatory effect linked to the secretory K^+ channel. Regulation
of ion transport during membrane fusion of the zymogen granule
(pancreatic secretory granule) to the apical membrane is neces-
sary to prevent premature granule swelling and leakage of the
contents into the cytoplasm before exocytosis.

It is believed that the regulation of zymogen granule swell-
ing occurs through electrolyte transport channels in the zymogen
granule. These channels include a Cl^- channel (DeLisle and Hopf-
er, 1986; Gasser et al., 1988a, 1990; Fuller et al., 1989a;

Goddard et al., 1988; Arvan et al., 1984; Pazoles and Pollard, 1978) and an ATP-sensitive K^+ channel (Gasser et al., 1988b; Gasser and Holda, 1993a; Thevenod et al., 1992). The activation of the Cl^- and K^+ channels can promote net salt and fluid secretion. Activation of these channels during exocytosis and secretion must be tightly coordinated to prevent premature granule swelling and subsequent lysis of the granule. As already mentioned, the granule K^+ channel is ATP sensitive whereas the Cl^- channel (Fuller et al., 1989a) is activated by protein kinase A. Based on the known processes of osmotic regulation in animal cells via Na^+-K^+ ATPase, it is probable that activation of just the K^+ channel needs to be controlled in order to maintain electroneutrality and coincide with membrane fusion to prevent premature granule swelling.

Not only do ATP-sensitive K^+ channels have a regulatory role in pancreatic acinar cells, but they also play an important role in regulating insulin secretion in pancreatic B-cells. Similar ATP-sensitive K^+ channels metabolically regulate blood flow in vascular tissue (Nelson and Quayle, 1995). Other ATP-sensitive K^+ channels have been found in smooth muscle (Zhang et al., 1994), heart (Huang et al., 1994; Miyoshi and Nakaya, 1993), and neuronal tissue (Wang and Lipsius, 1995).

The interest in the K_{ATP} channel in this study involves its possible participation in the fusion of the zymogen granule with the apical membrane. The efficiency of fusion should increase when the zymogen granule swells. Swelling causes the membrane to stretch as the ion channels are open. Opening of the K^+ and Cl^-

channels promotes net salt and fluid influx into the granule lumen. Under resting conditions, the amount of ATP present in the cell keeps these channels closed. The mechanism that promotes the opening of these channels allows K^+ and Cl^- to enter the zymogen granule and this solute influx promotes the influx of water osmotically. Stimulus secretion coupling in pancreatic acinar cells also involves an increase in the cytosolic concentration of cyclic AMP and Ca^{2+}. Cyclic AMP activates protein kinase A to phosphorylate the Cl^- channel thus converting the zymogen granule into an active state with an enhanced capacity to fuse with the apical plasma membrane (Gasser et al., 1993). Actual fusion would then occur through G-protein activation of the K_{ATP} channel resulting from contact with the apical plasma membrane. If the Cl^- channel is open before fusion, through regulation by cAMP-dependent protein kinase A, what regulates the K^+ channel?

Voltage-dependent K^+ channels adopt three functional conformations: closed, open, or inactive. It is estimated that the rate of transition between these states are regulated by protein kinase C (PKC) and protein kinase A (PKA) (Saad et al., 1994). It is generally recognized that protein kinases function by phosphorylating intracellular protein substrates, which may include ion channels (Wang and Lipsius, 1995). In fact, protein kinase C has been determined to activate K_{ATP} channels. A study done by Muller et al. (1992) shows that non-esterified fatty acids, such as arachidonic acid, activate protein kinase C and also activate ATP-dependent K^+ channels. It was also determined

that fatty acids, such as palmitic acid or stearic acid, do not activate protein kinase C and therefore do not activate ATP-dependent K^+ channels. Activation of these same ATP-dependent K^+ channels also involves protein kinase A. In Zhang et al. (1994), activation of K^+ channels occurs by calcitonin gene-related peptide (CGRP) in gallbladder smooth muscle. Calcitonin gene-related peptide activated adenylyl cyclase resulting in an elevation of cyclic AMP and stimulation of protein kinase A.

In a study done by DeWeille et al. (1989) of rat insulinoma cells, the K^+ channel is activated by the hormone somatostatin. Somatostatin activation of K_{ATP} channels occurs through a G-protein, since intracellular GTP is required for hyperpolarization of the channel. The author also determines that both somatostatin and the phorbal ester 4B-phorbal 12-myristate 13-acetate (PMA) lead to K_{ATP} channel activation. However, it was also noted that this K^+ channel activity was inhibited by the sulfonylurea glibenclamide. In other insulin secreting cells, such as those of the HIT cell line, inhibitors of K_{ATP} channel activity included phospholipase A_2 (PLA$_2$) (Eddlestone, 1995).

Materials and Methods

Isolation of secretory granules. Secretory granules of the pancreas were isolated from 150-200g male Sprague-Dawley rats as described in Gasser et al. (1988). The pancreas was placed in homogenization buffer, on ice, with the addition of (in mM) 250 sucrose, 40 3-(N-morpholino) propanesulfonic acid (MOPS; titrated with NaOH to pH 7.0), 0.1 $MgSO_4$, 0.1 ethylene glycol-bis(B-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA; free calcium 10^{-7} M), and 0.1 phenylmethyl sulfonyl fluoride (added just before use), as well as 1 mg/ml fatty-acid free bovine serum albumin. The pancreas was briefly minced, homogenized with a glass-Teflon tissue grinder, and lastly disrupted by nitrogen cavitation at 750 psi. The vast majority of the cells were broken by this procedure, however the secretory granules remained intact. The homogenate was supplemented with 100% Percoll stock giving a final concentration of 45% by volume while maintaining the original homogenization buffer constituents and concentrations. Centrifugation at 20,000g for 20 min in a Sorvall SV-288 vertical rotor formed a density gradient. A pipet was used to collect the dense secretory granules which formed a distinctive band toward the bottom of the gradient. Granule preparations were washed free of Percoll by resuspension in a solution of (in mM) 300 sucrose, 5 MOPS (pH 7.0), 0.1 EGTA, and 0.1 $MgSO_4$ in order to avoid Percoll precipitation in high ATP environments (>2.0 mM). The suspension was then centrifuged at 1,000g for 15 min., resulting in a granule pellet and a Percoll suspension. The granules were typically enriched about 4.5-fold with a 30% yield.

Separation of the basolateral and apical membranes were successfully achieved due to the tendency of nitrogen cavitation to lyse epithelial cells at the tight junction. This junctional matrix is located at the interface between the basolateral and apical domains of the plasma membrane. Plasma membranes were removed from a low density fraction in the Percoll gradient. Prior to differential centrifugation, the low density fraction of the apical membrane was diluted with homogenization buffer (1:20) and isolated by divalent cation (Mg^{2+}) precipitation (Eveloff, et al., 1980).

Evaluation of K^+ transport. The K^+ transport of the secretory granules was measured using the technique described in Gasser et al. (1988). By using salt solutions and ionophores, membrane permeabilities have been defined in many organelles from the osmotic influx of fluid and the observed kinetics of the subsequent swelling and lysis of the organelle. By permeabilizing the membrane with an ionophore that is specific for one of the major ions in solution, a driving force is created for the transport of the counter-ion through an endogenous membrane pathway. The granules accumulate solute which causes water to enter osmotically. The granules then swell and lyse. This is measured as a decrease in optical density (OD). The rate of ionophore-induced lysis is dependent upon the K^+ transport through the endogenous granule pathway.

The granules were suspended in a solution of (in mM) 150 KCl, 0.2 EGTA, 0.2 $MgSO_4$, 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and titrated to pH 7.0 with KOH.

The OD of the granule suspension in this solution ranged from 0.2-0.3 at 540 nm. This OD is proportionate to the number of secretory granules in suspension; intact granules constitute at least 90% of the optical signal. K^+ transport was identified by permeabilizing the granule membrane to Cl^- through the use of ionophore combinations. The ionophores used were tripropyltin (TPT; 10 ug/ml), which is a chloride-hydroxyl exchanger (Ovchin-nikov, V.A. et al., 1974), and the protonophore 3,3',4'5-tetrachloro-salicylanilide (TCS; 10 ug/ml). The combination of TPT/TCS gives the equivalent of a Cl^- conductance on the granule membrane (Cl^- influx; H^+ and OH^- efflux). Consequently, net solute influx (KCl), osmotic fluid accumulation, and granule lysis become dependent on, or rate limited by, the K^+ transport via the granule membrane's endogenous pathway. The time required for the OD to decrease by 50% (half-life) is recorded as the transport rate constant (inverse of the half-life; h^{-1}).

Ionophores were added to samples from stock solutions in 100% ethanol after which the ethanol concentration did not exceed 1% in the granule suspension. Granule behavior was not in-fluenced by this level of ethanol (Gasser, K.W., et al., 1988). Protein kinase A (Sigma chemical company-product no. P-2645 from bovine heart) was prepared by solubilizing the lyophilized powder with KCl light scatter solution (150 mM KCl, 20 mM HEPES, 0.2 mM EGTA, 0.2 mM $MgSO_4$, titrated to pH 7.0) and stored at -80°C in aloquots of 50 units each. Protein kinase C (Calbiochem-Nova-biochem Corp.-product no. 539513 from rat brain) was solubilized in 20 mM Tris, pH7.5, 50 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, and 10

mM DTT. Aloquots of 50 units each were stored frozen at -80°C.
ATP (100 mM) was prepared by solubilizing ATP in an equimolar
concentration of $MgCl_2$. ATP was vanadate free and the final
product was stored in aloquots of 200 ul at -80°C. ATP inhibi-
tion of the ionophore-dependent granule lysis was presented as
the percent change from the lysis rate in the absence of ATP.
All experiments were conducted at 37°C using a Beckman DU-64
spectrophotometer equipped with a constant temperature chamber,
6-unit sample changer, kinetic software, and a computerized data
capture system.

Evaluation of membrane fusion. The rate of dissolution of
the condensed secretory granule matrix was used to evaluate the
fusion of secretory granules with target membranes. It is be-
lieved that the intragranular environment is altered during
fusion in order to maintain the macromolecular products in the
condensed state. Solubilization of the granule product can be
measured as the decrease in OD at 540 nm. Purified secretory
granules (35 ug/ml protein) were added to cuvettes containing 3.0
ml of a solution of either (150 mM KCl, 20 mM HEPES, pH 7.0, 0.2
mM EGTA, and 0.2 mM $MgSO_4$) or (75 mM KCl, 10 mM HEPES, pH 7.0,
150 mM sucrose, 20 mM MOPS, 0.2 mM EGTA, and 0.2 mM $MgSO_4$) at
37°C. Target membrane (15 ug protein) was added to the secretory
granule suspension and the change in OD_{540} was recorded. The
change in OD_{540} due to granule/apical membrane fusion was con-
sistently compared to the change in OD_{540} of secretory granules
alone as a baseline control. Further additions to the
granule/apical membrane complex included 3 mM ATP and 50 units of

protein kinase A and 50 units of protein kinase C to designated cuvettes. The cuvettes were allowed to incubate for 15 min at 37°C after which the ionophores TPT (20 ul) and TCS (15 ul) were added to all but the controls (zymogen granules in KCl light scatter and in homogenization buffer only).

To test the effect of phosphorylation on apical membrane, Trypsin inhibitor (1 mg/ml) and PMSF (1 ul/ml) were added. Apical membranes were treated with 3 mM ATP and protein kinase A (1 unit/ul). Both treated and control preparations of apical membrane were incubated at 37°C for 30 min and were stored frozen at -80°C.

Results

The secretory granules, isolated from the rat pancreas, were stable in a 150 mM KCl solution at pH 7.0 and 37°C. The overall half-time for lysis was 64.5 min ±5.7 in these control experiments. In a 150 mM sucrose solution at 37°C and pH 7.0, the secretory granules exhibited similar stability and displayed a half-time lysis rate of 78.3 min ±6.1. Those granules that exhibited osmotic stability in KCl and sucrose environments were subsequently used to evaluate the regulation of zymogen granule K^+ transport and membrane fusion. Target membranes in fusion experiments were pancreatic apical membranes at a concentration of 15 ug/ml protein. Preparations of granules with a half-time lysis rate of 40 minutes or less were considered to be unstable and were discarded.

The use of salt solutions and ionophores have been successful in defining membrane permeabilities in many organelles from the osmotic influx of fluid and the subsequent swelling and lysis of the organelle (Gasser and Holda, 1993). The ionophore induced permeabilization of the membrane generates a driving force for the major ion in solution that is specifically targeted by the ionophore. In this experiment, K^+ transport was identified by permeabilizing the membrane to Cl^- through the use of the ionophores tripropyltin and TCS. The ionophores tripropyltin and TCS provide the equivalent of a Cl^- conductance and create a driving force for endogenous granule K^+ transport (Gasser and Holda, 1993). In a solution of 75 mM KCl and TPT-TCS, the rate of lysis of the zymogen granules had a half-life of 12.3 min. However,

the rate of lysis for zymogen granules suspended in 150 mM sucrose and TPT-TCS was significantly slower with a half-life of 40.4 min. The difference in rate between the KCl and sucrose solutions illustrates the selectivity for the K^+ ion. The control received no ionophores and only represents the intrinsic rate of granule lysis in a KCl/sucrose environment (Fig. 1).

Figure 2 represents the effect of ATP on K^+ transport in pancreatic zymogen granules. In the absence of ATP, the secretory granules displayed a K^+-dependent lysis rate of 12.3 min. However, the addition of ATP (1 mM, 2 mM, and 3 mM) resulted in a decrease in K^+ transport. The mean rates of zymogen granule lysis for 1 mM, 2 mM, and 3 mM ATP were 16.3 min, 20.5 min, and 28.2 min respectively. These results suggest that ATP inhibits the rate of K^+ transport in a concentration dependent manner. It was also determined that this inhibition is dependent upon external KCl concentration. Increased KCl concentration, in the presence of ATP, resulted in a slower lysis rate while a decrease in KCl concentration reduced ATP inhibition and resulted in a faster rate of granular lysis.

Protein kinase A is a signaling enzyme known to be activated during secretion-coupling in the pancreas. Previous reports have shown protein kinase A to have a stimulatory effect on other K^+ channels such as those within smooth muscle (Huang et al., 1994; Miyoshi and Nakaya, 1993; Zhang et al., 1994). Therefore, the ability of protein kinase A to activate zymogen granule K^+ transport and secretion was determined by a 15 minute incubation of the zymogen granules with protein kinase A in a 75 mM KCl and 150

mM sucrose environment. These results, given in Fig. 3, show that the rate of lysis for secretory granules in 3 mM ATP was 17.5 min and the rate of lysis for secretory granules incubated with protein kinase A was 6.2 min. As expected, the lysis rate of secretory granules was inhibited by ATP alone. However, protein kinase A stimulated K^+ transport and the inhibitory effects of ATP were reduced. This suggests that K^+ transport may be regulated by protein kinase A phosphorylation of the K^+ channel.

Protein kinase A has been shown to have an activating effect on K_{ATP} channels in this experiment. In addition, the role of protein kinase C on K_{ATP} channel regulation of pancreatic acinar cells was also investigated. In a previous paper (DeWeille et al., 1989), protein kinase C was shown to have stimulatory effects on the K_{ATP} channel in rat insulinoma cells. This proved not the case in our experiments. K^+-transport of the secretory granules in the presence of protein kinase C was not elevated over control levels in comparison to the rate of K^+-transport induced by protein kinase A phosphorylation (Table 1). Therefore, protein kinase C seemed to have no direct effect on K^+ transport.

Based on the results of protein kinase A stimulated K^+ transport in Fig. 3, this effect was further investigated in fusion experiments of both treated and untreated apical membranes (Table 2). Apical membranes were incubated in a buffer solution containing trypsin inhibitor and PMSF. Treated membranes received 3 mM ATP and protein kinase A. Measurement of membrane

fusion with light scatter at 540 nm determines the granule lysis rate derived from the change in OD_{540}. As described earlier (Stahl and Gasser, 1996), as zymogen granules fuse with the target membrane, the changing luminal environment causes the condensed core to solubilize resulting in a decrease in OD. The rate of fusion for the untreated membranes was 11.8 ± 4.1 min while the fusion rate for treated membranes was 7.3 ± 1.8 min. Clearly, membranes treated with protein kinase A fused much faster than those without. It would seem that the K^+ transport by apical membranes is sensitive to ATP, but at the same time it is also stimulated by the addition of protein kinase A.

Discussion

It is possible that the zymogen granule regulates electrolyte transport as a means to increase fusion efficiency and exocytosis. This belief is based upon experiments on the fusion of intracellular secretory granule membranes (zymogen granules) with apical plasma membranes. Upon fusion, the granule enclosed product is released into the extracellular environment and additional membrane, including granular membrane proteins, is inserted into the plasma membrane. Within the membrane of the zymogen granule exists a Cl^- channel (DeLisle and Hopfer, 1986; Gasser et al., 1988a, 1990; Fuller et al., 1989a; Goddard et al., 1988; Arvan et al., 1984; Pazoles and Pollard, 1978) and an ATP-sensitive K^+ channel (Gasser et al., 1988b; Gasser and Holda, 1993a; Thevenod et al., 1992).

Electrolyte transport by the zymogen granule involves the Cl^- and K_{ATP} granule ion channels and their contribution to swelling as a way of promoting membrane fusion which culminates in exocytosis. Swelling of the zymogen granule causes the membrane to thin and exposes the hydrophobic core of the phospholipid bilayer. As the membrane continues to stretch, due to the osmotic effects of net salt and fluid influx into the granule lumen, the membrane surface area increases. This in turn increases the exposure of the hydrophobic core of the granule membrane. It is the hydrophobic attraction between the phospholipid bilayers of the granular and apical membranes that induces membrane fusion according to Helm et al. (1992). This overall process would, in effect, contribute to the exocytotic event.

Other reports do suggest that granule electrolyte transport plays a primary role in fusion efficiency and exocytosis. In a report by Fuller et al. (1989b), the rate of product release from pancreatic acinar cells was lowered when intracellular Cl^- was depleted. In addition, pancreatic acinar cells with the mutant cystic fibrosis transmembrane conductance regulator protein, which lacks protein kinase A stimulated Cl^- channels, have a reduced Cl^- transport capability and a significantly reduced rate of exocytosis compared to those cells containing the wild-type CFTR (Bradbury et al., 1992). These findings coincide with the prediction that reduced electrolyte transport across the vesicle membrane decreases the rate of exocytosis.

Electrolyte transport by the zymogen granule is activated by the coordinated efforts of the Cl^- and K^+ channels to prevent premature swelling and intracellular lysis of the granule. This can be achieved by regulating either the Cl^- or the K^+ channel. Both channels are regulated by distinct mechanisms. The Cl^- channel (Fuller et al., 1989a) is activated by cAMP-dependent protein kinase A. Protein kinase A phosphorylates the Cl^- channel and enhances the zymogen granule's capacity to fuse with the apical plasma membrane (Gasser et al., 1993). In contrast, the granule K^+ channel is inhibited by intracellular concentrations of ATP. ATP maintains the channel in a closed state until it is phosphorylated by a signaling enzyme activated during secretion-coupling in the pancreas. Previous reports have shown this to be a protein kinase A regulatory effect. The focus of this experiment was then centered on K_{ATP} channel regulation as a way of

promoting fusion and exocytosis.

Protein kinase A, as an activator of ATP-sensitive K^+ channels, has been shown in other types of smooth muscle. Zhang et al. (1994) provides evidence that calcitonin gene-related peptide (CGRP) opens K_{ATP} channels in gallbladder smooth muscle through activation of adenylyl cyclase, elevation of cAMP, and stimulation of protein kinase A. In a study by Miyoshi and Nakaya (1993), K_{ATP} channels in coronary tissue were activated by protein kinase A via cAMP-dependent phosphorylation of the channel. Huang et al. (1994) also reports regulation of the K^+ channel from protein kinase A phosphorylation in their studies of cardiac smooth muscle.

K_{ATP} channel activation was promoted by protein kinase C in other cells. Protein kinase C activation of the K_{ATP} channel has been noted in insulinoma cells in which intracellular concentrations of ATP become less inhibitory on the K_{ATP} channel when activated by the kinase (DeWeille et al., 1989). However, stimulation of the K_{ATP} channel by protein kinase C did not cause a significant effect on K^+-transport in our experiments. The rate of K^+-transport was much lower in zymogen granules incubated with protein kinase C than those that were incubated with protrein kinase A.

Our experiments do verify the stimulatory effects of protein kinase A on the K_{ATP} channel as described in other experiments. In the presence of ATP, K^+ transport in the zymogen granule is inhibited (Fig. 2). However, zymogen granules incubated with ATP and protein kinase A showed an increased rate of K^+-transport.

This would suggest that K_{ATP} channels are regulated by protein kinase A. Further evidence comes from the fusion experiments of protein kinase A treated and untreated apical membranes. Membranes treated with protein kinase A exhibited an enhanced fusion rate compared to untreated membranes, thus exhibiting a possible regulatory effect by direct phosphorylation of the K^+ channel.

Regulation of the K_{ATP} channel was initially determined by the rate of K^+-transport in zymogen granules in a 75 mM KCl and a 150 mM sucrose environment. It was determined that K^+-transport occurs more readily in a KCl environment and that ATP inhibition is dependent on KCl concentration. For example, the rate of K^+-transport decreased when ATP was present in a higher concentration of KCl. When ATP was not present, K^+-transport was higher in KCl versus sucrose environmnets. These results imply the need for electrolyte transport to contribute to granular swelling, membrane fusion, and exocytosis.

A model for regulating the K_{ATP} channel, as well as exocytosis, requires a non-secreting acinar cell containing secretory granules with closed ion channels. Cl^- transport would be inhibited by a dephosphorylated Cl^- channel and the K^+ channel would be inhibited by ATP. In pancreatic acinar cells, stimulus-secretion coupling involves an increase in the cytosolic concentration of cAMP and Ca^{2+} as a result of secretogogue activation of the acinar cell. Cyclic AMP and Ca^{2+} are second messengers that activate intracellular effector enzymes, such as protein kinases and phospholipases. These enzymes would transform the zymogen granules into an active state through Cl^- channel phosphorylation

by protein kinase A and alteration of the granule membrane by phospholipase A_2. Evidence suggests that the activated granules have a greater capacity for fusion with the apical plasma membrane in cell-free reconstituted systems (Karli et al., 1990; Gasser et al., 1993).

The results suggest that committed fusion could be attributed to K_{ATP} channel activation by protein kinase A after contact with the apical plasma membrane. It is also suggested that the apical plasma membrane would contribute the necessary G-protein or a coupling protein for association with the K^+ channel. Activation of these pathways would promote granule swelling and therefore increase fusion efficiency. Activated granules that have not made contact with the apical membrane do not swell and are transferred back to the resting state once the stimulatory signal (cAMP) has been terminated. This would occur by dephosphorylation of the Cl^- channel by endogenous cellular phosphatases. The final step would be the return of the secretory granule membranes to their original composition and electrolyte transport capacity.
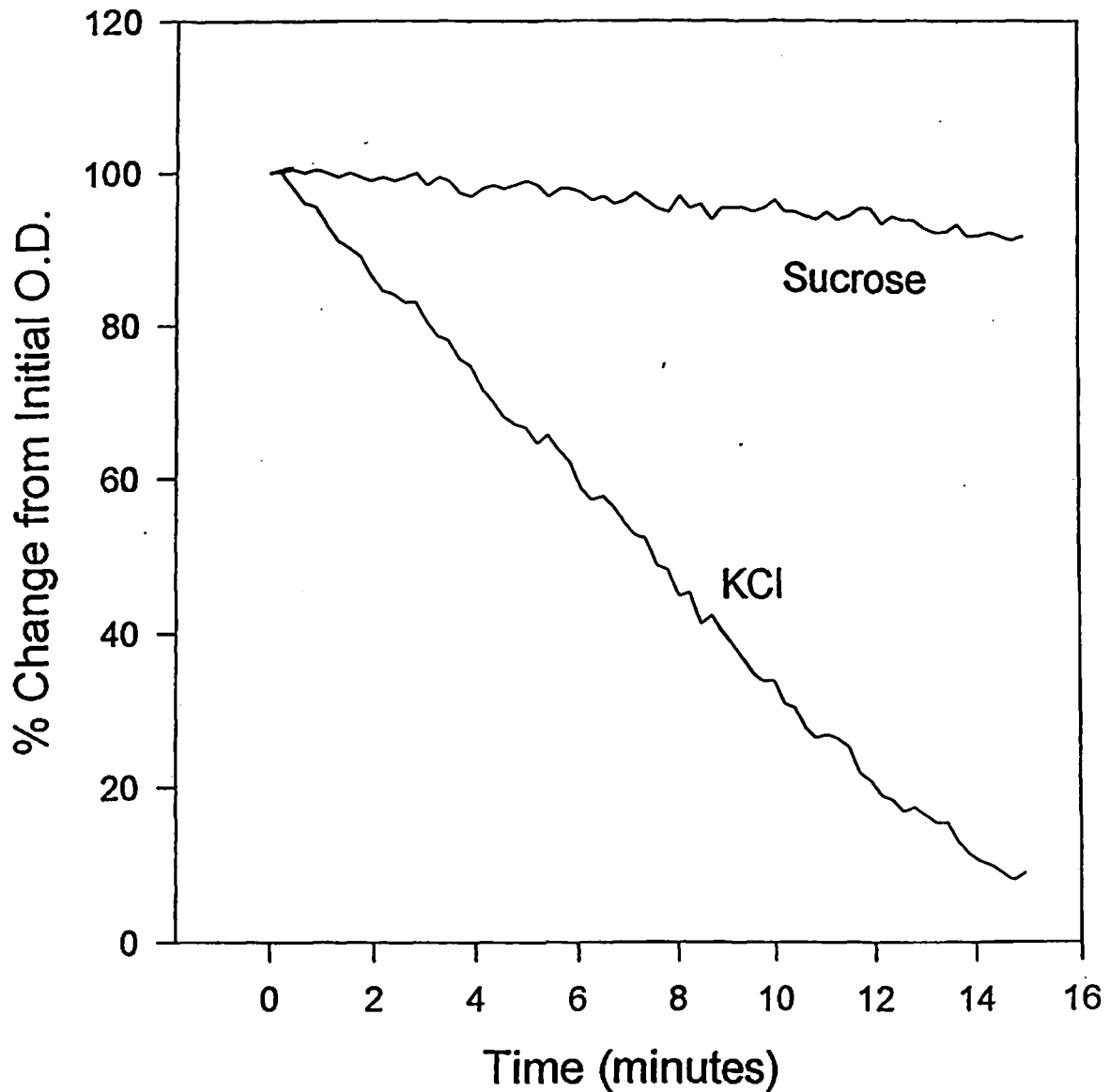
Figure 1. The effect of an ionophore induced Cl⁻ conductance on granule lysis in KCl and sucrose suspension solutions. The secretory granules were incubated in 37C solutions containing 20 mM HEPES at pH 7.0, 0.2 mM $MgSO_4$, 0.2 mM EGTA and either 150 mM KCl or 300 mM sucrose. A Cl⁻ conductance was induced by the addition of the ionophores tri-propyl tin and tetrachloro-salicylanilide. The figure is a representative experiment that illustrates the K^+ dependence of granule lysis as measured by the decrease in optical density at 540 nm.
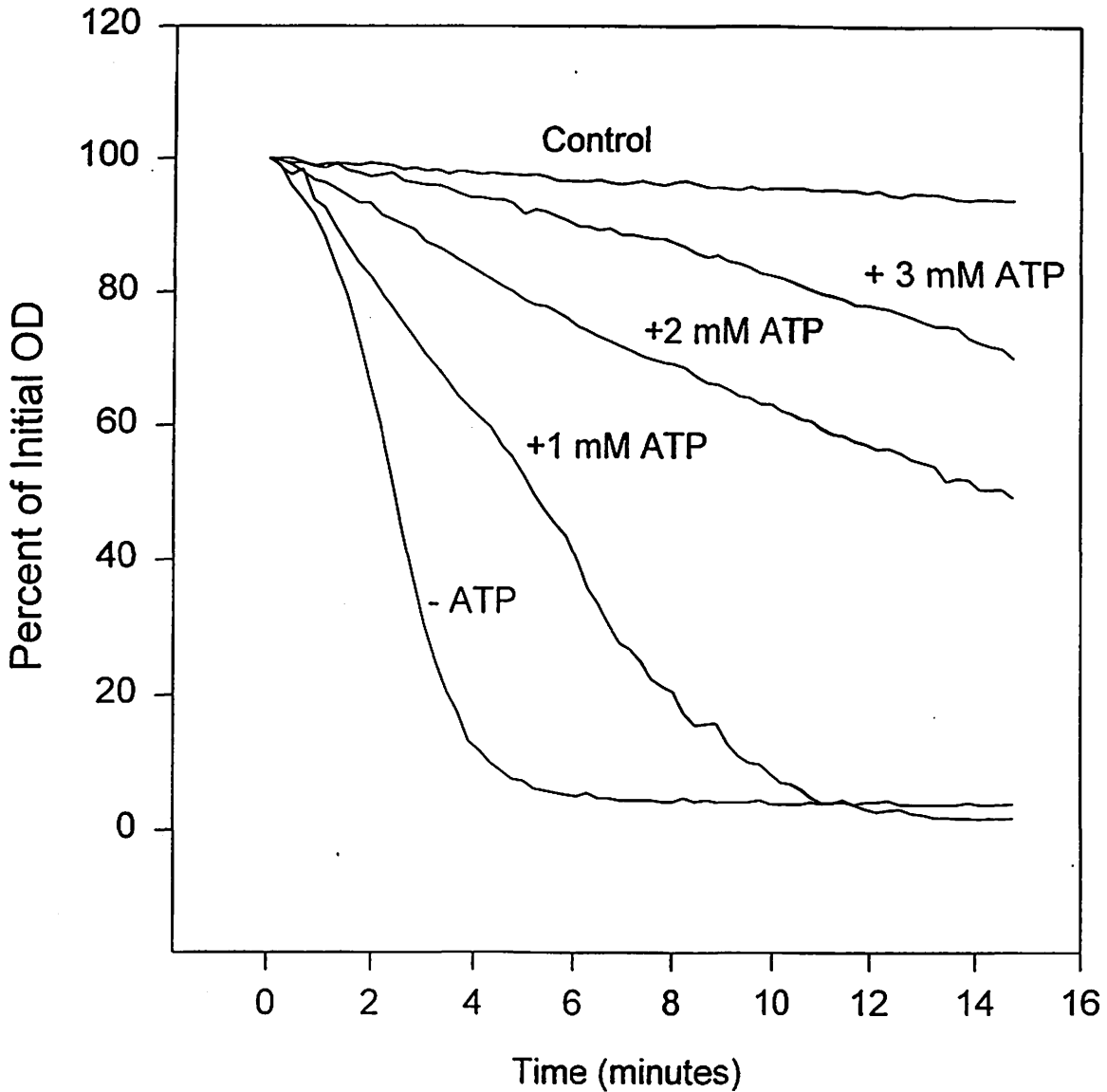
Figure 2. The inhibition of ionophore-induced granule lysis by ATP. The secretory granules were suspended in a 37C solution of 75 mM KCl, 150 mM sucrose, 20 mM HEPES at pH 7.0, 0.2 mM EGTA, 0.2 mM MgSO₄, and the listed concentration of ATP. K⁺ transport was measured as the rate of secretory granule lysis after inducing a driving force for solute accumulation using the ionophore combination TPT and TCS. The figure is a representative experiment showing the ATP dose-dependent reduction in secretory granule K⁺ transport and is indicative of the K_{ATP} K^+ transport channels.
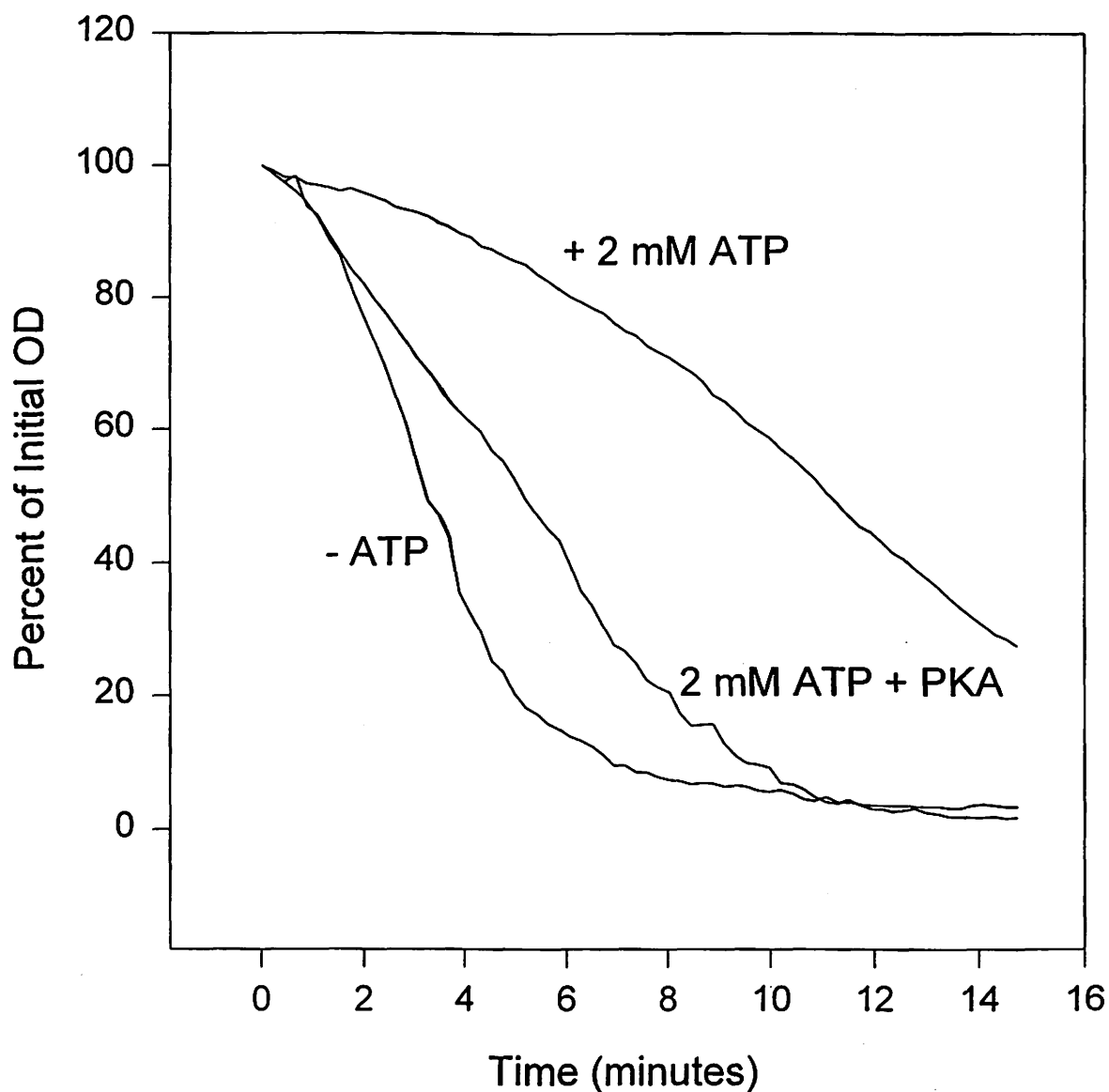
Figure 3. The effect of phosphorylation with protein kinase A on the activity of ATP-inhibited secretory granule K^+ transport. The secretory granules were suspended in a 37C solution of 75 mM KCl, 150 mM sucrose, 20 mM HEPES at pH 7.0, 0.2 mM EGTA, 0.2 mM $MgSO_4$, and 3 mM ATP. Granules were further treated by preincubation with 50 units of the catalytic subunit of protein kinase A for 15 minutes at 37C prior to the addition of ionophores (trace labeled +PKA). The figure is a representative experiment showing the intrinsic rate of granule K+ transport (control), the inhibited rate of transport (+ATP), and the activation in the presence of ATP by protein kinase A (ATP + PKA).

Table 1. The effect of protein kinase phosphorylation on the rate of ATP-inhibited secretory granule K+ transport

Treatment	K^+ transport rate (half-time; min.)
Control	2.3 ± 3.1 (n=14)
+ 3 mM ATP	28.2 ± 2.9 (n=14)
ATP + PKC	26.1 ± 3.1 (n=3)
ATP + PKA	15.4 + 2.5 (n=9)

Secretory granules were pretreated with protein kinases (50 units) for 15 minutes at 37C prior to the initiation of solute accumulation with the ionophores TPT and TCS. The values are the mean half-times for granule lysis in a KCl solution (mean ± standard error).

Table 2. The effect of protein kinase A phosphorylation on the rate of secretory granule fusion with apical plasma membranes.

Treatment	Fusion rate (half-time; min.)
Control (untreated)	11.8 ± 4.1 (n=12)
+ATP	19.3 ± 6.1 (n=6)
ATP + PKA	7.3 ± 1.8 (n=5)

Apical plasma membrane were preincubated with protein kinase A (50 units) for 30 minutes with 3 mM ATP. Granule and apical plasma membranes were incubated together in a KCl solution to measure the rate of exocytotic membrane fusion, measured as the decrease in OD_{540}. Control experiments were run in the absence of kinase and ATP.

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