Stimulation of intestinal fructose absorption by phorbol 12-myristate 13-acetate (PMA) results from rapid insertion of GLUT2 into the brush-border membrane and correlates with protein kinase C (PKC) \( \beta II \) activation. We have therefore investigated the role of phosphatidylinositol 3-kinase- and mammalian target of rapamycin in the regulation of fructose absorption by PKC \( \beta II \) phosphorylation. In isolated jejunal loops, stimulation of fructose absorption by PMA was inhibited by preperfusion with wortmannin or rapamycin, which blocked GLUT2 activation and insertion into the brush-border membrane. Antibodies to the last 18 and last 10 residues of the C-terminal region of PKC \( \beta II \) recognized several species differentially in Western blots. Extensive cleavage of native enzyme (80/78 kDa) to a catalytic domain product of 49 kDa occurred. PMA and sugars provoked turnover and degradation of PKC \( \beta II \) by dephosphorylation to a 42-kDa species, which was converted to polyubiquitylated species detected at 180 and 250+ kDa. PMA increased the level of the PKC \( \beta II \) 49-kDa species, which correlates with the GLUT2 level; wortmannin and rapamycin blocked these effects of PMA. Rapamycin and wortmannin inhibited PKC \( \beta II \) turnover. PI3-kinase, PDK-1, and protein kinase B were present in the brush-border membrane, where their levels were increased by PMA and blocked by the inhibitors. We conclude that GLUT2-mediated fructose absorption is regulated through PI3-kinase and mammalian target of rapamycin-dependent pathways, which control phosphorylation of PKC \( \beta II \) and its substrate-induced turnover and ubiquitin-dependent degradation. These findings suggest possible mechanisms for short term control of intestinal sugar absorption by insulin and amino acids.

Intestinal glucose absorption comprises two components (for a review, see Ref. 1). It is well established that one of these is an active component mediated by the Na\(^+\)/glucose cotransporter, SGLT1. We have proposed that the other is a facilitated component mediated by glucose-dependent activation and recruitment of GLUT2 to the brush-border membrane. GLUT2 recruitment correlates with SGLT1-dependent activation of PKC\(^\beta II\) (2) so that SGLT1 is now seen to exert an important regulatory role in addition to its established functions as a scavenger and transporter (1). Regulation of the facilitated component may also involve mitogen-activated protein (MAP) kinase- and PI3-kinase-dependent signaling pathways (3).

The \( K_m \) and \( J_{\text{max}} \) of the facilitated component are 2- and 3-fold, respectively, of those for SGLT1 in vivo. After a meal, the average concentration across the luminal contents of rat jejunum reaches 48 mM (4), close to the \( K_m \) of the facilitated component, and much higher concentrations may well be present locally at the brush-border membrane, as a result of the hydrolysis of complex dietary sugars (5). The facilitated component therefore appears to provide the major route by which glucose is absorbed immediately after a meal. Moreover, the activation of GLUT2 and its rapid trafficking to and from the brush-border membrane in response to changing glucose concentrations provides a cooperative mechanism by which absorptive capacity is matched precisely to dietary intake.

GLUT2 transports not only glucose but also fructose (6). Fructose absorption across the brush-border membrane is therefore mediated by both GLUT5, which is specific for fructose, and GLUT2 (7). Phloretin, which inhibits GLUT2 but not GLUT5, may be used separate out the contributions of each transporter to fructose transport. In this way, we were able to show that a 4-fold stimulation of fructose absorption by PMA in isolated loops in vitro was matched by a 4-fold increase in the level of GLUT2 at the brush-border membrane and correlated with the activation of PKC \( \beta II \) (7). The level of GLUT5 and its contribution to absorption did not change significantly.

Support for the model of rapid, GLUT2-mediated up-regulation of sugar absorption has been provided by the observation that glucagon-like peptide 2 promotes rapid insertion of GLUT2 into the brush-border membrane (8). Moreover, a definitive confirmation of the model has recently been provided by the observation that when the intestine of wild type mice is challenged with sugars by gastric intubation, a large increase in fructose transport occurs within minutes; the increase in transport does not occur in GLUT2-null mice and is attributable entirely to GLUT2 (9).

The intrinsic activity of PKC \( \beta II \) is controlled by phosphorylation at three sites, one in the activation loop and two in the C-terminal region, located at Thr-500, Thr-641, and the hydrophobic site Ser-660, respectively (10–12). Phosphorylation at all three sites serves to lock the enzyme into a catalytically competent conformation, which is then regulated by membrane-bound phosphatidylycerine and also by second messengers Ca\(^{2+}\) and diacylglycerol. The first step in the production of kinase B; PI3-kinase, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin-dependent; PMA, phorbol 12-myristate 13-acetate.

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‡The abbreviations used are: PKC, protein kinase C; PKB, protein kinase B; PI3-kinase, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin-dependent; PMA, phorbol 12-myristate 13-acetate.
mature PKC βII is phosphorylation of Thr-500 by PDK-1, which is blocked by inhibitors of PI3-kinase, since the latter regulates PDK-1 (13–15). Phosphorylation of Thr-500 then permits autophosphorylation of Ca2+/calmodulin kinase II (17), which increases affinity for Ca2+ (17). Phosphorylation of the hydrophobic site in PKC δ and PKC ε is sensitive to rapamycin (18, 19), suggesting a role for mTOR in controlling phosphorylation of this site, possibly by regulating the activity of a phosphatase (12).

In our previous work, we implicated PKC βII in the PMA-induced stimulation of the GLUT2-mediated component of fructose absorption by using chelerythrine, which inhibits PKC βII activity by binding directly to the catalytic site (7). In the present work, we have now used inhibitors of PI3-kinase and mTOR, wortmannin and rapamycin, respectively, to inhibit the activities of the intracellular signaling pathways that stimulate the phosphorylation and intrinsic activity of PKC βII. Using this quite different approach, we have reproduced and extended our previous results to confirm the involvement of PKC βII in the regulation of GLUT2 trafficking to the brush-border membrane. The data suggest new mechanisms for the regulation of fructose and glucose absorption by insulin and amino acids.

**EXPERIMENTAL PROCEDURES**

**Animals—**Male Wistar rats (240–260 g) were fed ad libitum on standard Bantin and Kingman rat and mouse diet with free access to water.

**Perfusion of Jejunal Loops—**Rats were anesthetized by an intraperitoneal injection of a mixture of 1.0 ml of Hypnorm (Janssen) and 0.4 ml of Hypnovel (Roche Applied Science) per kg of body weight. The systems for the luminal perfusion of jejunal loops in vitro and in vivo have been described in detail previously (2, 7, 20). Because PKC activators and inhibitors take some time to become effective in whole tissue and because PKC activators and inhibitors take some time to become effective in whole tissue and 0.38 ml min⁻¹ was used for the luminal perfusion of jejunal loops and 0.38 ml min⁻¹ was used for the luminal perfusion of jejunal loops.

**In vitro** perfusion was terminated at a time point corresponding to half the time period over which the average rate of transport was determined as described above. Immediately after perfusion, each jejunal segment was flushed with ice-cold buffered mannitol (20 ml imidazole buffer, pH 7.5, containing 250 mM mannitol and 0.1 mM phenylmethylsulfonyl fluoride) to arrest trafficking of GLUT2 and to trap any labile intermediates of PKC βII at the membrane. Jejunum was then placed on an ice-cold glass plate and slit longitudinally so that the muscle of the jejunal flattened out on to the cold plate. Mucosal scrapings were taken with an ice-cold glass slide and homogenized immediately at 4 °C in buffered mannitol using a Kinematica Polytron homogenizer (4 × 30-s bursts using the large probe at setting 7). The rest of the preparation and its detailed characterization for purity are as described by Corpe et al. (20). The preparation was reevacuated and rinsed three times to ensure that all cytotoxic contaminants were removed. Enrichment of sucrase activity in these highly purified preparations ranged from 16- to 20-fold, there was no significant enrichment of Na⁺/K⁺-ATPase activity. Dephosphorylation of brush-border membrane vesicles with protein phosphatase 1A (PP1A) was performed as described by Keranen et al. (10).

**Affinity Purification of PKC βII—**All procedures took place at 4 °C. C10 antibody was coupled to protein A-Sepharose 4B fast flow resin (Sigma) with diisuccinimidyl suberate and packed into a Bio-Rad EconoColumn (30 × 0.5 cm). The column was washed and stored in ice-cold buffered saline (phosphate-buffered saline, pH 7.4) until use. Brush-border membrane vesicles were solubilized to 0.5 mg of protein/ml in lysis buffer (150 mM NaCl, 25 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 25 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, and 25 mM HEPES, pH 7.2, plus 1% (w/v) Triton X-100, 1% (w/v) deoxycholate, and 0.2% (w/v) SDS) and centrifuged at 13,000 rpm to separate soluble and insoluble fractions. The soluble fraction was diluted 1:1 with phosphate-buffered saline (pH 7.4) and loaded on the protein A-Sepharose column. The flow-through (protein eluate) from this column was loaded onto the antibody column at <0.2 ml min⁻¹, after which the column was washed (150 mM NaCl, 25 mM Tris-HCl, pH 7.2) to remove unbound proteins. Bound proteins were then eluted with 1 M NaCl, 25 mM Tris-HCl (pH 7.2), plus 0.2% (w/v) SDS. The collected fractions were precipitated with 6% (v/v) perchloric acid and then spun down, and the pellet was resuspended in SDS sample buffer. Separated samples were transferred to polyvinylidene difluoride membrane by Western blotting and immunoblotted using a polyclonal antibody to ubiquitin raised in rabbit kindly supplied by Professor R. J. Mayer (Nottingham).

**Western Blotting—**SDS-PAGE and Western blotting was performed as described previously using ECL (enhanced chemiluminescence) detection (7, 20). C10 antibody to the last 10 C-terminal amino acids of PKC βII (Fig. 1) was raised in rabbit by Professor N. Groome (Oxford Brookes University). A C18 antibody to the last 18 amino acids of PKC βII (Fig. 1, not phosphorylated at Ser-660) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies to the last 14 amino acids at the C terminus of GLUT2 and GLUT5 were raised in rabbits. All antibodies were polyclonal. Neutralization of each antibody by preincubation with excess of the corresponding antigenic peptide abolished labeling, confirming the specificity of the antibodies. Quantitation of Western blots was performed using a Flowgen AlphaImager 1200 analysis system (Alpha Innotech Corp.). Protein levels (as indicated by band intensity) were determined in Western blots made from jejunal preparations from jejunum were expressed relative to those in control preparations. A liver GLUT2 standard was routinely used in blotting experiments. The linear range of intensity response in ECL photographs was established using a 20-fold range of the amount of an actin standard (2–40 μg). After background correction, the response was linear (correlation coefficient 0.996) for integrated density values ranging from 13,024 to 485,029. As much as possible, exposures were such that the intensity values fell within the middle third of the linear response range. The same loading of 15 μg of protein was used for all samples. Comparison of relative levels of target proteins was made on a total protein basis to minimize potential complications that might be caused by the trafficking of other proteins in response to the same stimuli that affect the trafficking of target proteins.

**Statistical Analysis—**Values are presented as means ± S.E. and were tested for significance using Student’s t test.
RESULTS

For each and every perfusion, fructose (5 mM) was present in the perfusate throughout the entire perfusion. To establish the effect of an inhibitor on the stimulation of the absorption of fructose by PMA, isolated loops of rat jejunum in vitro were perfused with PMA (200 nM) in the absence or presence of inhibitor. The concentrations of rapamycin and wortmannin were 25 and 200 nM, respectively; at these concentrations, the inhibition of PMA-stimulated brush border fructose absorption by rapamycin (rap) and wortmannin (wort) was 80% and 85%, respectively, for control perfusions and 30% for all other perfusions. Wortmannin remained at control levels. GLUT5 levels were not significantly decreased by PMA and/or inhibitor, either rapamycin or wortmannin as described in the legend for Fig. 1. Perfusion was terminated by flushing with ice-cold perfusate to arrest trafficking and to trap all intermediates at the membrane. For full details, see “Experimental Procedures.” Vesicle protein (15 μg) was then separated on 10% SDS-PAGE gels, transblotted onto polyvinylidene difluoride membrane, and Western blotted for GLUT5, GLUT2, and PKC βII using C18 and 10 antibodies.

Fig. 1. The inhibition of PMA-stimulated brush border fructose absorption by rapamycin (rap) and wortmannin (wort). The jejunum of an anesthetized rat was perfused for 30 min in vivo and then in vitro for 30 min with 5 mM D-fructose (black bar) and then for 30 min in vitro with 5 mM D-fructose and 0.2 mM phloretin (white bar). The phloretin-sensitive and -insensitive components represent the GLUT2 and the GLUT5 contributions to absorption, respectively. For each and every perfusion, fructose was present in the perfusate throughout the entire perfusion. In perfusions with PKC activator, PMA (200 nM) was present throughout the entire perfusion. When present, rapamycin (25 nM) or wortmannin (200 nM), were added to the perfusate 15 min before the PMA; for full experimental details, see Ref. 7. Values are given as mean ± S.E. (n = 12 for control perfusions and 3–5 for all other perfusions). ***, p < 0.001, by unpaired t test for comparison with rate in the absence of phloretin.

Fig. 2. The effect of PMA, rapamycin (rap), and wortmannin (wort) on the levels of GLUT5 and GLUT2 and on the turnover of PKC βII. Brush-border membrane vesicles were prepared from rat jejunum perfused with 5 mM D-fructose in the presence and absence of PMA and/or inhibitor, either rapamycin or wortmannin as described in the legend for Fig. 1. Perfusion was terminated by flushing with ice-cold perfusate to arrest trafficking and to trap all intermediates at the membrane. For full details, see “Experimental Procedures.” Vesicle protein (15 μg) was then separated on 10% SDS-PAGE gels, transblotted onto polyvinylidene difluoride membrane, and Western blotted for GLUT5, GLUT2, and PKC βII using C18 and 10 antibodies.

Before excision of jejunum (7, 21). The timing of the final in vitro perfusion was such as to get as direct a correspondence as possible between rates of transport and extent of trafficking. Perfusions for vesicle preparations were therefore terminated at a time point corresponding to half the time period over which the average rate of absorption was determined as described above. Vesicles were then Western blotted using antisera to the C-terminal 14 amino acids of GLUT5 or GLUT2. GLUT5 appeared as a triplet from 53 to 58 kDa and GLUT2 as a tightly spaced doublet from 61 to 63 kDa (Fig. 2). The distinct banding patterns confirmed that there was no cross-reactivity between antibody to one transporter and the other transporter; in addition, antibody to a sequence within the extracellular loop of GLUT2 gave similar results to the C-terminal antibody (8). All bands were eliminated by preincubation of antiserum with excess C18 peptide (data not shown). Fig. 2 shows that PMA increased the level of GLUT2 3.9 ± 0.6-fold as compared with untreated control jejunum. Stimulation reflected a 4-fold increase in the GLUT2-mediated component and was completely blocked by preperfusion with either rapamycin or wortmannin, each of which alone had no effect on overall fructose absorption and its components. Thus, rapamycin and wortmannin had effects on PMA-stimulated fructose transport similar to those found for chelerythrine (7). Ro-31-8220 exerts similar effects to chelerythrine (data not shown).

In that previous work with chelerythrine, we provided evidence that the increase in GLUT2-mediated absorption induced by PMA is matched by a corresponding increase of GLUT2 in the brush-border membrane. These changes correlated with activation of PKC βII, and there was little change in GLUT5-mediated transport and GLUT5 level (7). Vesicles were therefore prepared from jejunal perfused first in vivo and in vitro to determine the levels of transporters and PKC βII in response to rapamycin and wortmannin. Protein trafficking was arrested by flushing with ice-cold perfusate immediately after excision of jejunum (7, 21). The timing of the final in vitro perfusion was such as to get as direct a correspondence as possible between rates of transport and extent of trafficking. Perfusions for vesicle preparations were therefore terminated at a time point corresponding to half the time period over which the average rate of absorption was determined as described above. Vesicles were then Western blotted using antisera to the C-terminal 14 amino acids of GLUT5 or GLUT2. GLUT5 appeared as a triplet from 53 to 58 kDa and GLUT2 as a tightly spaced doublet from 61 to 63 kDa (Fig. 2). The distinct banding patterns confirmed that there was no cross-reactivity between antibody to one transporter and the other transporter; in addition, antibody to a sequence within the extracellular loop of GLUT2 gave similar results to the C-terminal antibody (8). All bands were eliminated by preincubation of antiserum with excess C18 peptide (data not shown). Fig. 2 shows that PMA increased the level of GLUT2 3.9 ± 0.6-fold as compared with control. Rapamycin blocked PMA-induced trafficking of GLUT2 to the brush-border membrane so that GLUT2 remained at control levels. GLUT5 levels were not significantly different from control levels in any other condition. Wortmannin behaved similarly to rapamycin.

Western blots were performed with C18 antibody raised against the C-terminal sequence residues 656–673 of PKC βII. C18 antibody recognized a doublet at 80 and 78 kDa and a single band at 49 kDa. All bands were eliminated by preincubation of antiserum with excess C18 peptide (data not shown). PMA strongly increases the intensity of the 49-kDa band. It also increases the intensities of the doublet, although to a
lesser extent. Translocation of conventional PKC isoenzymes to a target membrane is widely taken as an indicator of activation, which is often accompanied by calpain-dependent cleavage in the hinge region to a constitutively active catalytic domain of ~50 kDa. The activity of this form is independent of any activators, such as diacylglycerol, phosphatidylserine, and Ca\(^{2+}\) because of the separation of the catalytic unit from the regulatory domain and coincidentally from the pseudosubstrate in the N-terminal sequence (22, 23). Since the 80-/78-kDa bands correspond to those for two control standards (rat brain homogenate and a postnuclear liver membrane preparation, data not shown) and the standards either do not show or show only a light 49-kDa band, the 80-/78-kDa species must represent native (uncleaved) forms of the enzyme, and the 49-kDa band must represent the C-terminal catalytic domain. PKC \(\beta\)II in intestine appears to be particularly susceptible to cleavage, a phenomenon we have seen under many other conditions. The catalytic domain does not possess binding sites for diacylglycerol and phosphatidylserine in the membrane, yet is found in vesicles. It should therefore be noted that at the end of a preparation, vesicles are revesiculated several times through a 21-gauge needle so that the presence of the catalytic domain in vesicles is not caused by simple, physical trapping of cytosolic proteins as vesicles resel during preparation. That we detect the catalytic domain strongly in vesicles most likely reflects the fact that jejunum is flushed with ice-cold perfusate immediately before excision to prevent the rapid trafficking of GLUT2 away from the membrane. Coincidentally, this procedure traps the catalytic domain cleavage product in association with its (unknown) substrate. Rapamycin completely blocks the PMA-induced increase in the intensity of the 49-kDa species and, on its own, decreases band intensity as compared with control. Wortmannin decreased the PMA-induced increase in the intensity of the 49-kDa species, although not as effectively as rapamycin. When perfused alone, wortmannin had no effect on band intensity as compared with control. PKC \(\beta\)II activation, as indicated by the intensity of the 49-kDa band, correlates with the increase in brush border GLUT2 level, as reported previously (2, 3, 7).

Western blots of brush-border membrane vesicles from jejunum perfused first \textit{in vivo} and \textit{in vitro} were also performed using C10 polyclonal antibody raised against the C-terminal sequence residues 664–673 of PKC \(\beta\)II. The banding pattern with C10 antibody was very different from that with C18 antibody. The C10 antibody detects the 80-/78-kDa doublet only very weakly. However, it recognizes the catalytic domain at 49-kDa strongly; in addition, it also recognizes strong bands at 42 and 180 kDa and a lighter band at 250 kDa. Thus, in the absence of luminal perfusion with sugars, either alone (control) or in the presence of PMA (200 nM), brush-border membrane vesicles were then prepared and Western blotting was performed after dephosphorylation with PP1A as described by Keranen et al. (10).

![Fig. 3. Dephosphorylation converts the 49-kDa species to the 42-kDa species of PKC \(\beta\)II.](image)

All the bands detected by C10 and C18 antibodies represent different species of PKC \(\beta\)II. Thus, no bands are detected when antibody is first preabsorbed with the corresponding antigenic peptide. BLAST searches of the NCBI and SWISS-PROT databases with either the C18 or the C10 sequence reveal only PKC \(\beta\)II. All the bands are related to each other as shown by the fact that rapamycin alone significantly diminishes or eliminates them. Since rapamycin and wortmannin both inhibit the phosphorylation of PKC, the reciprocal relationship of the intensities of the 49- and 42-kDa bands suggests the 42-kDa species is a dephosphorylated form of 49-kDa species. Treatment of vesicles with PP1, which dephosphorylates all three C-terminal phosphorylation sites, converts the 49-kDa species to the 42-kDa species and implies that the 42-kDa species is likely to be fully dephosphorylated (Fig. 3). That activation at the membrane results ultimately in dephosphorylation is in keeping with the fact that activation induces an open conformation, which increases the sensitivity of the C terminus to dephosphorylation by more than 2 orders of magnitude (24). In addition, the isolated catalytic domain naturally has an open conformation (14, 25).

PKC species with apparent molecular weights much higher than that of the native, uncleaved enzyme have been reported to be polyubiquitylated intermediates on a pathway targeted for proteasomal degradation (26, 27). The Western blot in Fig. 4 (lane 1) shows that brush-border membrane vesicles from PKC-perfused intestine contain several ubiquitylated proteins, presenting for the most part as a broad smear with occasional discrete bands. An affinity column, in which C10 antibody was covalently linked to the support, was therefore used to purify the 180-kDa species to show that this species of PKC \(\beta\)II was strongly cross-reactive with anti-ubiquitin (lane 2). In some blots, very faint bands could also be seen at 250+ kDa.

The data thus far are consistent with the existence of two processes: the initial one is calpain-dependent cleavage of the native enzyme at the membrane on activation, and the second one is a turnover and degradation pathway, which involves dephosphorylation of the 49-kDa species to the 42-kDa species. Sugars also activate the degradation pathway and interconversion of the different species. This was clearly demonstrated when jejunum was perfused solely \textit{in vivo} with Krebs-Henseleit buffer alone (no sugar, Fig. 5); blots with C10 antibody showed a strong 49-kDa band and blots with C18 antibody showed the 80-/78-kDa doublet in addition to strong 49-kDa band. However, when jejunum was perfused with different sugar substrates, namely glucose (100 mM), mannitol (100 mM), fructose (100 mM), and maltose (200 mM), C10 antibody also detected a well defined 42-kDa band and an intense 180-kDa band, as well as a lighter 250+ kDa band. Thus, in the absence of luminal sugar \textit{in vivo} (no \textit{in vitro} perfusion), there is substantial cleavage of the native enzyme but effectively no turnover to species on the ubiquitin-dependent degradation pathway. However, luminal perfusion with sugars \textit{in vivo} (no \textit{in vitro} perfusion)
results in the activation of the degradation pathway in minutes. Since the 42-kDa species is derived by dephosphorylation of the 49-kDa species (Fig. 3), it seems that proteasomal degradation follows dephosphorylation in agreement with Hansra et al. (24). The order of production of detected species from the 80–78-kDa native enzyme therefore appears to be 49, 42, 180, and 250+ kDa. Our data, of course, cannot preclude the possibility that some polyubiquitylated degradation intermediates can be directly derived from the native enzyme, although it seems that proteolytic cleavage is favored in intestine.

The turnover provoked by mannitol was surprising since it is not thought to be transported. However, these effects were not caused by an increase in osmolarity at the high sugar concentrations used as compared with buffer alone since perfusion with 5 mM fructose in vivo was sufficient to induce equally definite turnover; moreover, analysis of mannitol revealed no detectable reducing sugars (data not shown). These observations therefore point toward a possible sensing mechanism (see below). As reported previously, however, the activation of PKC βII and GLUT2 insertion into the brush-border membrane was favored by glucose and fructose as compared with mannitol.

Western blots reveal that both PI3-kinase and PDK-1, which phosphorlates Thr-500 in the activation loop of PKC βII, are present at the brush-border membrane (Fig. 6). Also present is PKB, which is also a substrate for PDK-1. The levels of all three signaling enzymes in the presence of PMA are increased between 48 and 64% as compared with the corresponding control; levels in all other conditions are similar to control. Thus, the necessary signaling enzymes for wortmannin and rapamycin action are present. Interestingly, the upstream pathway is activated by PMA, and activation is blocked by both inhibitors. If we assume that PMA is indeed specific for the conventional isoforms of PKC, then the implication is that one of those isoforms is also involved in the upstream regulation of the pathway. It is not without precedent for one isoform of PKC to regulate the activity of another; for example, PKC ε seems to be involved in the regulation of novel PKC isoforms (12).

**DISCUSSION**

**Beware the Antibody**—The difference in banding patterns obtained with the C10 and C18 antibodies is remarkable given that the C10 sequence is contained within the C18 sequence. The differences in detection patterns in Western blots could not be accounted for by failure to generate antibodies with C18 peptide to epitopes within the C10-C1 region, for bands detected by C18 antibody were either partially or fully eliminated by preincubation of C18 antibody with C10 peptide (data not shown). Moreover, as detailed above, all species could be eliminated or strongly diminished simultaneously by perfusion with rapamycin; alternatively, they could be converted into each other by perfusion with PMA or sugars. All the evidence is therefore consistent with the view that each band represents a different species of PKC βII detected differentially by the two antibodies.

The use of antibodies against relatively short peptide sequences of signaling proteins is widespread. Our data emphasize that even with antibodies to overlapping sequences, use of antibody to just one sequence may not reveal significant as...
Physiological Significance of Phosphorylation and Turnover of PKC \( \beta \) II for Intestinal Sugar Absorption—We have previously reported that sugar absorption in rat small intestine is up-regulated by activation of PKC \( \beta \) II favoring insertion of GLUT2 into the brush-border membrane (2, 7). Up-regulation is blocked by chelerythrine or Ro 31-8220, which acts by binding to the catalytic site of PKC \( \beta \) II. We have now shown that the inhibitors rapamycin and wortmannin, which inhibit at different points in the signaling pathway controlling PKC maturation, have the same effects on absorption and GLUT2 trafficking. The data therefore provide an independent confirmation in scientific terms of our previous work on which the GLUT2 model of rapid regulation of intestinal sugar absorption was based (1).

Our data suggest that the signaling pathway controlling the involvement of PKC \( \beta \) II in the regulation of intestinal sugar absorption comprises two main parts: phosphorylation of PKC \( \beta \) II is followed by cleavage, turnover, and degradation (Fig. 7). Phosphorylation of Thr-500 in the activation loop by PDK-1 downstream of PI3-kinase is followed by autophosphorylation of Thr-641 and Ser-660 in the C-terminal sequence (13–15, 25); PI3-kinase-independent mechanisms may also exist in some circumstances (28). The formation of competent PKC \( \beta \) II is associated in intestine with binding to the brush-border membrane and substantial cleavage of native enzyme to a 49-kDa catalytic domain. Activation of PKC \( \beta \) II by PMA at the brush-border membrane favors insertion of GLUT2 into the brush border and so increases sugar absorption; further cleavage also occurs, increasing the level of the 49-kDa species at the brush-border membrane (Figs. 1 and 2) (7). Since the intestinal absorptive cell is polarized, it is supplied with low concentrations of glucose from the blood. Yet luminal perfusion with Krebs-Henseleit buffer alone does not promote further cleavage and turnover, whereas perfusion with 5 mM fructose (Figs. 2 and 5) or low concentrations of glucose (2) does. It therefore seems that there must be an additional sensing mechanism at the brush-border membrane by which sugars supplied in the lumen activate PKC \( \beta \) II (Fig. 7); such a mechanism presumably involves increases in cytosolic Ca\(^{2+}\) and diacylglycerol (1, 2). As noted, mannitol promotes turnover and degradation but is not transported. Whether there is a specific sugar sensor is open to debate, but it is of interest to note that SGLT3, which binds but does not transport glucose, can depolarize membranes in which it resides. Luminal sugars promote rapid turnover of PKC \( \beta \) II by dephosphorylation of the 49-kDa species to the 42-kDa species followed by polyubiquitylation to the 180- and 250-kDa species prior to degradation at the proteasome.

On the basis of these observations, we can envisage a possible overall mechanism for the regulation of sugar absorption. In perfusions with fed animals, but when no sugar is present in the lumen at the start of an experiment, plasma sugar and insulin levels are normal. The amount of competent PKC \( \beta \) II available for activation is determined by the prevailing balance between its rate of formation by phosphorylation and the rate of cleavage, turnover, and degradation. Luminal perfusion of sugars causes translocation of competent PKC \( \beta \) II to the brush-border membrane through a brush border-sensing mechanism, resulting in activation, cleavage, and turnover and degradation. The balance of the different pathways is different with different sugars so that glucose and fructose promote the 49-kDa form of PKC \( \beta \) II and favor insertion of GLUT2 into the membrane as compared with mannitol (Fig. 5). Moreover, a high level of glucose favors rapid insertion of GLUT2 as compared with a low level of glucose (Fig. 3 of Ref. 2). Increased insertion of GLUT2 into the membrane results in increased absorption and therefore transintestinal delivery of sugars to the circulation; as plasma sugar levels increase, release of insulin is stimulated, activating PI3-kinase and thereby PDK-1 to increase the rate of formation of competent PKC \( \beta \) II. Amino acids have a similar, although less potent, effect on insulin release. Moreover, an amino acid-sensing pathway also promotes the formation of competent enzyme by activating mTOR, which is thought to prevent dephosphorylation of the hydrophobic site Ser-660, which is crucial for PKC \( \beta \) II activity, by inhibiting a phosphatase (12, 18, 19, 29). Thus, perfusion of jejunum with either wortmannin or rapamycin in the presence of fructose blocks the formation of competent PKC \( \beta \) II and the whole of the signaling pathway (Figs. 2 and 6). As absorption proceeds, the fall in luminal sugar concentrations both inactivates PKC \( \beta \) II and ultimately diminishes plasma sugar and insulin levels, by which time the initial level of competent PKC \( \beta \) II is restored by re-establishment of the balance between the rates of formation and cleavage, turnover, and degradation. This results in loss of GLUT2 from the brush-border membrane and down-regulation of sugar absorption. Wortmannin and rapamycin by inhibiting the formation of competent PKC \( \beta \) II alter the balance strongly in favor of turnover and degradation.

![Fig. 7. Potential signaling pathways for the regulation of GLUT2-mediated sugar absorption by insulin and amino acids through the control of PKC \( \beta \) II activity. For explanation of the pathways, see “Discussion.”](https://www.jbc.org/content/28649/1/28649.full.pdf)
It therefore appears that the control of PKC βII phosphorylation has the potential to provide a mechanism for the observation that intestinal sugar absorption is regulated by amino acids (5) and insulin (20, 30–32).

The PKC βII turnover and degradation pathway appears to be strongly emphasized in intestine. Moreover, the degradation of PKC βII is much faster than that of PKC α (24), which is also present in intestine (33), so that PKC βII seems more suited to the short term control of sugar absorption than PKC α. The significance of our findings for the regulation of intestinal sugar transport is therefore clear. Immediately after a meal, the absorptive capacity of the brush-border membrane is rapidly up-regulated to match dietary intake as a large local concentration of glucose is generated at the surface of the brush-border membrane by the hydrolysis of luminal disaccharides and α-limit dextrins so that PKC βII is rapidly activated and GLUT2 is inserted into the membrane (1). As sugars are absorbed and luminal concentrations decrease, so PKC βII is inactivated, and the GLUT2-mediated component is down-regulated by the inactivation and loss of GLUT2 from the membrane. Blood sugar and insulin levels peak within 1 h, returning to normal after about 2 h; the initial events controlling intestinal delivery of sugars occur on a time scale of minutes. Such rapid up- and down-regulation requires the dynamic control afforded by the rapid turnover and degradation of PKC βII provoked by dietary sugars.

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