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Phosphorylation of MP26, a Lens Junction Protein, Is Enhanced by Activators of Protein Kinase C

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Summary. MP26, a protein thought to form gap junctional channels in the lens, and other lens proteins were phosphorylated under conditions that activate protein kinase C. Phosphorylation was detected both in lens fiber cell fragments in an "in vivo" labeling procedure with ^{32}P-phosphate and in cell homogenates with ^{32}P-ATP. In these experiments, both calcium and 12-O-tetradecanoylphorbol 13-acetate (TPA) were necessary for maximal phosphorylation of MP26. Calcium stimulated the phosphorylation of MP26 approximately fourfold and TPA with calcium led to a sevenfold increase. If TPA was present, 1 μM calcium was sufficient for maximal labeling. Phosphoamino acid analysis demonstrated approximately 85% phosphoserine, 15% phosphothreonine, and no phosphotyrosine when MP26 was phosphorylated in lens homogenates in the presence of TPA and calcium and then electrophoretically purified. Phosphorylation occurred near the cytoplasmic, C-terminal of MP26. The possible involvement of other kinases was also examined. The Walsh inhibitor, which affects cAMP-dependent protein kinases, had no influence on the TPA-mediated increase in phosphorylation. In studies with isolated membranes and added kinases, MP26 was also found to not be a substrate for calcium/calmodulin-dependent protein kinase II. Thus, protein kinase C may have phosphorylated MP26 in a direct manner.

Key Words lens · gap junctions · MP26 · protein kinase C · phosphorylation

Introduction

Gap junctions are thought to serve significant cellular functions in such processes as development [14, 59], growth control [1, 3, 39] and tissue homeostasis [52] by allowing the exchange of small molecules between adjacent cells. Careful regulation of these hydrophilic channels would be expected because cell-to-cell communication varies spatially and temporally within different tissues. There is evidence for gap junction regulation in the synthesis of junctional RNA/protein [55], in protein degradation [13, 62], in channel formation [for a review, *see* 29] and in channel gating [for a general review, *see* 54].

Since protein phosphorylation could affect some of these processes, reagents that affect kinase activity have been used in a variety of studies to investigate the regulation of gap junctions. These studies have demonstrated long-term changes in the extent of gap junction communication [31, 49] and rapid changes that may result from channel gating via direct phosphorylation of channel proteins [9, 37, 50, 54]. Modulation of cAMP-dependent protein kinase (A kinase) activity has led to both increases and decreases in gap junction communication [37, 40, 49, 50, 60]. Recently, results with cAMP derivatives have demonstrated an increase in the phosphorylation of the 27-kDa liver gap junction protein [50, 58], which was correlated with an increase in gap junction conductance in hepatocyte pairs [50]. Other kinases have also been explored. The tyrosine kinase, pp60^{src} [1–3], and protein kinase C (C kinase) [12, 15, 61, 63] have been implicated in a reduction of gap junction communication. Thus, the activation of protein kinases appears to play a critical role in the regulation of junctional communication.

Because the lens is thought to contain the most extensive system of junctional structures known, it may be useful in the study of junction regulation by phosphorylation. Lens membranes contain a major intrinsic protein often referred to as MP26 or MIP26 due to its apparent molecular weight on polyacrylamide gels [4, 8]. Studies on junction isolation [4, 8, 20], membrane reconstitution [7, 10, 11, 18, 64], EM immunolocalization [6, 10, 28, 51], and antibody-mediated reduction of channel activity [19, 28] all indicate that MP26 is capable of forming a cell-to-cell membrane channel. The protein sequence of MP26 has been determined from cDNA clones and a model for the structure of MP26 within a lipid bilayer (based on hydrophobicity analysis and other techniques) is also consistent with MP26 monomers associating to form a hydrophilic channel [21]. However, the sequence of MP26 has no homology

with at least two other junctional proteins from heart and liver [5, 47], while a 70-kDa lens protein has significant homology with these proteins in its N-terminal region [32]. Thus, the lens fiber cell may have multiple proteins involved in cell-to-cell channels. This topic is discussed more extensively in a later section.

Considerable evidence indicates that MP26 in lens fiber cells is a phosphoprotein. MP26 in isolated membranes ("in vitro") has been phosphorylated by both A kinase [16, 27, 41] and C kinase [36]. MP26 was also phosphorylated in a "in vivo" type system when fragments of fetal, bovine lenses were incubated in the presence of ^{32}P-orthophosphate [26]. In the same study, MP26 phosphorylation was shown to be calcium dependent and responsive to both cAMP and forskolin when whole lens homogenates were incubated with [γ-^{32}P]ATP. These two results could be related because the activity of adenylate cyclase in the lens has been found to be calcium dependent [42]. Alternatively, other calcium-sensitive kinases could be involved, independently or in concert with the A kinase. We report here that both calcium and TPA can also regulate the phosphorylation of MP26. Data are presented that suggest protein kinase C directly phosphorylated MP26. We believe this is the first report of TPA-stimulated phosphorylation of a putative gap junction protein.

Materials and Methods

Materials

L-1-Tosylamide-2-phenylethylchloromethylketone-treated trypsin was from Cooper Biomedical (Malvern, PA). Lysylendopeptidase-C was from Calbiochem Brand Biochemicals (San Diego, CA). [γ-^{32}P]ATP (3 Ci/mmol) and ^{32}P-phosphate (10 mCi/ml, HCl-free) were from Amersham (Arlington Heights, IL). TPA was obtained from LC Services Corp. (Woburn, MA). Walsh inhibitor, phosphoserine, phosphothreonine, and phosphotyrosine were purchased from Sigma Chemical Co. (St. Louis, MO). Ionophore A23187 was from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other reagents were reagent grade.

Tissue Labeling

Lens tissue was labeled as previously described with minor modifications [26]. For tissue fragments, young calf lenses were decapsulated and the cortex was dissected from the lens core. The cortex was fragmented into pieces several millimeters in diameter, suspended and twice washed in phosphate-free minimum essential media with Earle salts (Flow Laboratories), and adjusted to 155 μM free calcium with ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). In a few experiments where whole lenses were used, no overall phosphorylation differences were detected from cortex alone, although the relative increases caused by TPA and calcium appeared slightly greater in the cortical samples. In specified cases (Fig. 1, lanes, *2, 3, 7, 10*), the media washes had enough EGTA added to lower the free calcium to nanomolar concentrations. Free calcium concentrations were calculated using a computer program which considered pH and the dissociation constants for calcium, magnesium, EGTA and in some cases ethylenediaminetetraacetic acid (EDTA) and ATP [56]. ^{32}P-phosphate (0.2 mCi) and various specified effectors (TPA, EGTA, A23187) were added to start the reaction. The TPA (10 and 100 nM) and A23187 (7.5 μM) concentrations used in this study were in the ranges commonly used by many investigators [12, 15, 45] and were prepared by dilution from stock solutions that were 500–1000× their final reaction concentration in 100% dimethylsulfoxide (DMSO). DMSO (0.5%) controls had no effect on phosphorylation levels. Incubations continued for 60 min at 37°C and were terminated by dilution in buffer (5 mM phosphate, 5 mM EGTA, pH 7.0). Membranes were purified and run on sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE) as indicated below.

Phosphorylation in lens homogenates was as previously described [26] except the incubation media had different effectors as specified in the figure legends. Lenses were decapsulated and homogenized in an equal ratio (wt/vol) of 20 mM imidazole and 10 mM $MgCl_2$. ATP (3 Ci/mmol) was 25 μM and calcium and TPA were varied as indicated. If EGTA was added, the concentration of free calcium was subnanomolar. In the experiments listed in the Table, TPA (20 nM), EDTA (1 mM) and/or calcium (10 μM) were added as indicated to lens homogenates with $MgCl_2$ and ATP. Lens membranes were purified and run on SDS-PAGE. Bands at 18, 26, and 60–70 kDa were excised and subjected to scintillation counting. Results from two incubations and four SDS-PAGE separations were averaged and standard deviations were calculated. In Fig. 3 experiments, calcium was buffered with EGTA and EDTA because the Walsh inhibitor contained EDTA. Walsh inhibitor was added to a concentration that would inhibit 36 phosphorylating units (Sigma Chemical Co). Protease studies were performed at a 1:20 ratio of protease/lens protein on membranes which were phosphorylated in the presence of TPA (20 nM) and calcium (10 μM). After 1 hr at room temperature, membranes were pelleted at 12,000 × *g*, purified [20], and run on SDS-PAGE as indicated below.

Lens Membranes

Urea-washed membranes were prepared as previously described [20]. This procedure involved two buffer washes and two urea washes (4 and 7 M) that removed most of the extrinsic proteins, and a final water wash to remove the urea. Lens membrane protein concentrations were determined by the bicinchoninic acid method [53] in the presence of 0.5% SDS.

SDS-PAGE of Labeled Membranes

SDS-PAGE was performed on 12.5% polyacrylamide gels following the method of Laemmli [35] with Coomassie blue staining. After drying the gels under vacuum, autoradiography was performed overnight using Kodak XAR 5 film without a screen. In Figs. 1–3, 5 and 6, the Coomassie-stained gel profile is in panel *A* and the corresponding autoradiograph is shown in *B*.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was essentially by the method of Hunter and Sefton [25]. MP26 was phosphorylated in homogenates (four lenses, as above) in the presence of calcium (10 μM)

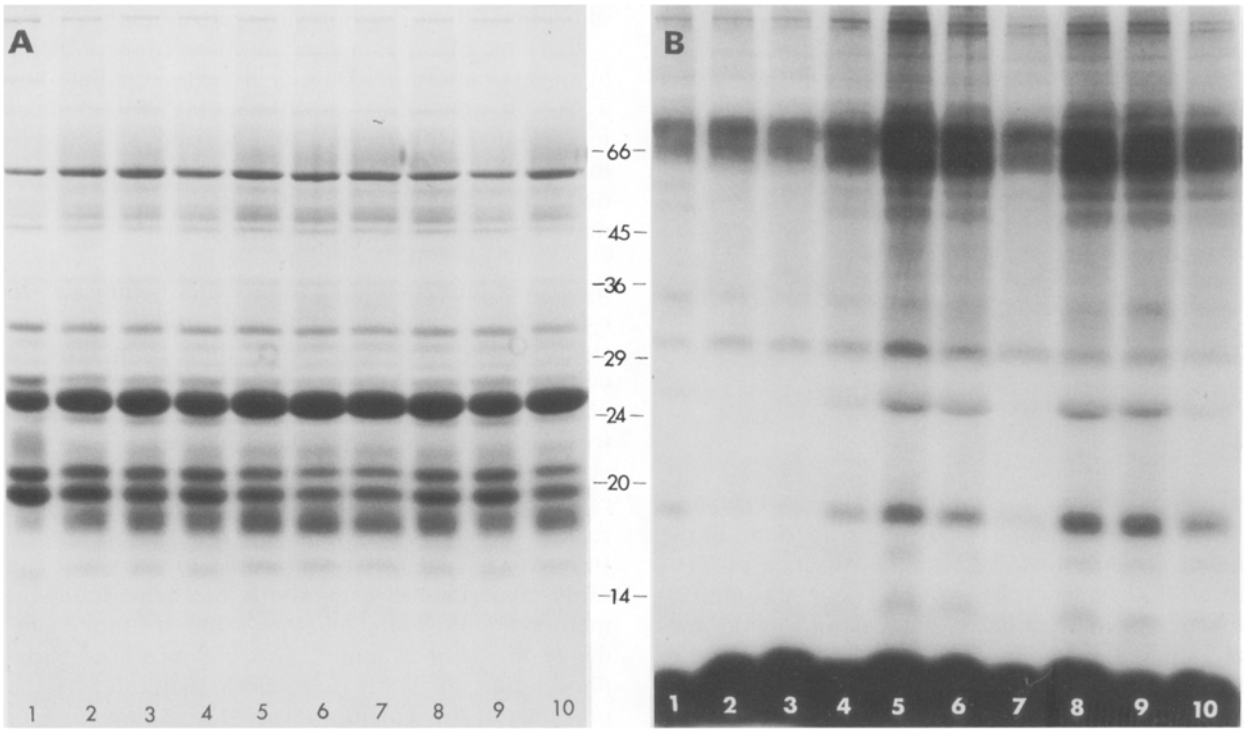

Fig. 1. SDS-PAGE following the phosphorylation of membrane proteins in lens tissue fragments. Lenses were fragmented into several pieces (1–2 mm in diameter) in minimal essential media that contained 155 μM free calcium and no phosphate. The fragments were washed and incubated for 60 min in 0.2 mCi ^{32}P-phosphate (all lanes). No TPA was added to the reaction mixtures in lanes *1–4*, while lanes *5–7* received 10 nM TPA and lanes *8–10* received 100 nM TPA. Extracellular calcium was maintained at 155 μM for lanes *1*, *5*, and *8*. EGTA (5 mM) was added in lane *2*. EGTA (5 mM) and A23187 (7.5 μM) were added in lanes *3*, *7*, and *10*. A23187 (7.5 μM) was added alone (lane *4*) and with TPA (lanes *6* and *9*). Cell fragments were homogenized, membranes were purified, and SDS-PAGE analysis was performed. The Coomassie-blue-stained gel (*A*) and corresponding autoradiograph (*B*) are shown

and TPA (20 nM) and lens membranes were purified as described above. MP26 was resolved on a 12.5% acrylamide, 3.0 mm-thick gel and identified by soaking in 4 M sodium acetate, pH 7.0 [24]. The band was excised and electroeluted. TCA was added to 25% concentration and 50 μg bovine serum albumin was added as a carrier. After 1 hr at 4°C the precipitate was collected at 12,000 × *g*. The precipitate was dissolved in 6 M HCl and hydrolyzed under nitrogen for 1–2 hr. Nonradioactive phosphoamino acid standards (3 μg/lane) were added to the samples. Phosphoamino acid analysis was performed at both pH 1.9 (water/acetic acid/formic acid, 897 : 78 : 25) and pH 3.5 (water/acetic acid/pyridine, 945 : 50 : 5) in one dimension at 750 V on Whatman No. 3 paper. Phosphoamino acid spots were detected by spraying with ninhydrin, excised and scintillation counted or the whole chromatogram was subjected to autoradiography for 25–72 hr with an intensifying screen.

Phosphorylation of Purified Membranes by Calcium/Calmodulin-Dependent Protein Kinase II (CaM Kinase)

Membranes were purified as described above. CaM kinase and synapsin were the generous gifts of Dr. Angus Nairn (Rockefeller University). A complete reaction mixture contained 50 mM N-2-hydroxyethylpiperazine-N′-2-hydroxypropanesulfonic acid (HEPES) at pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1.0 μM calmodulin, 1.5 mM $CaCl_2$, 20 μM [γ-^{32}P]ATP, CaM kinase and 10 μg of either lens membranes or synapsin. Incubations were for 30 min at room temperature and were terminated and run on SDS-PAGE as indicated in the figure legend.

Results

Calcium and TPA Effects on Phosphorylation

Figure 1 shows the effect of TPA and calcium on phosphorylation in tissue fragments. This preparation was the most native used in these studies. Considerable tissue viability remained as illustrated by the incorporation of ^{32}P-phosphate into the ATP pool and subsequent phosphorylation of proteins. The Coomassie blue staining (Fig. 1*A*) reveals relatively even loading and the similar composition of the various samples. Slight variations can be expected because each lane represents a separate homogenization and purification. MP26 is the major band in the gel and the only phosphoprotein around 26 kDa [26].

The corresponding autoradiograph in Fig. 1*B* illustrates the influence of both TPA and calcium on

Table. The effects of calcium and TPA on the phosphorylation ratio of lens proteins[a]

	Relative level of phosphorylation[b]		
	Ca^{2+}	TPA	TPA & Ca^{2+}
18 kDa	4.2 ± 1.8	2.6 ± 0.7	6.32 ± 1.9
MP26	4.1 ± 1.4	1.8 ± 0.1	7.1 ± 1.3
60–70 kDa	1.8 ± 0.4	1.4 ± 0.1	2.7 ± 0.5

[a] Phosphorylation was in lens homogenates. Calcium (10 μM) and TPA (20 nM) were added as indicated.
[b] The level of radioactivity was normalized to phosphorylation levels at subnanomolar calcium. The standard deviation for four separations is given.

the phosphorylation of MP26 and an 18-kDa protein. A limited amount of labeling was found in the control preparations, i.e., in the absence of TPA and A23187 (lane *1*). The low level of phosphorylation observed may be due to calcium in the medium, since the labeling was reduced by addition of EGTA alone (lane *2*) or by EGTA plus ionophore (lane *3*). Moreover, the simple addition of the ionophore to the standard medium (containing 155 μM calcium) served to increase the level of phosphorylation (lane *4*). However, 10 nM TPA was a more potent stimulator of this process (lane *5*). Even here calcium appeared to be involved, as the TPA increase was thoroughly blocked when EGTA and ionophore were added along with the TPA (lane *7*). Finally, some inhibition of phosphorylation occurred when A23187 was added with the TPA (*compare* lanes *5* and *6*), possibly reflecting the effects of elevated cytoplasmic calcium on protein kinase C. The inhibition of this kinase by higher calcium levels has been observed previously [43].

Studies were also performed with higher levels of TPA (100 nM). Again, TPA displayed a dramatic effect on phosphorylation (lane *8*). In this case, the results were much less sensitive to changes in calcium levels. First, the addition of ionophore along with TPA had no inhibitory influence on the labeling of MP26 (*compare* lanes *8* and *9*). Second, only a partial inhibition was observed when TPA was combined with EGTA and ionophore (lane *10*). C kinase is known to be less dependent on calcium at higher TPA concentrations [45].

While the above discussion relates to the phosphorylation of both MP26 and the 18-kDa protein, it is clear that other phosphoproteins exist within the lens membrane and that TPA and calcium also influenced the phosphorylation of these proteins. Most notably, there is a broad band in the 50-70 kDa range which probably represents several different proteins. Although TPA enhanced this labeling, the phosphorylation did not vary substantially over a wide range of calcium concentrations. The labeling at approximately 30 kDa was likewise stimulated by TPA, but was not as sensitive as MP26 to changes in calcium. However, a minor band at approximately 50 kDa does follow calcium and TPA dependence in a manner similar to MP26, and this band may represent a dimer of MP26.

Thus far, the results discussed have involved the use of lens fragments to provide the most natural system for analyzing the regulation of phosphorylation. The remaining results come from work on cell homogenates. We have utilized this second approach for several reasons: (i) it enables us to control much more precisely the concentrations of calcium, (ii) it allows us to add protein inhibitors of protein kinases, and (iii) it minimizes the problems related to penetration and accessibility of reagents. In instances where we were able to carry out the same experiment, using both the lens fragment method and the homogenate method, we found similar results. For example, TPA enhanced the phosphorylation of MP26 with both methods, in the presence of low calcium levels (Fig. 1, lane *5*; Fig. 2, lane *6*).

With the homogenate system, Fig. 2 shows the effect of increasing calcium concentrations on phosphorylation as a function of TPA levels. A series of four different calcium concentrations (from nanomolar to 100 μM) was evaluated with TPA. In the first series (lanes *1–4*), no TPA was added. In the next two, a low level of TPA (10 nM, lanes *5–8*) and a high level of TPA (100 nM, lanes *9–12*) were applied. When the free calcium concentrations were in the nanomolar range, only limited phosphorylation was observed in MP26 and the 18-kDa protein. Some increase was noted as the calcium level was elevated to 100 μM in the absence of TPA (lane *4*). The addition of TPA led to a striking enhancement in phosphorylation. While the lower TPA level still required at least 1 μM calcium for a strong effect (lane *6*), the higher TPA treatment led to some labeling even in the presence of EGTA (lane *9*).

In order to quantify the effects of TPA and calcium on phosphorylation, the relative ratio of label in the major phosphoproteins was determined under different conditions (Table). TPA (20 nM) in the presence of calcium (10 μM) led to an approximate sevenfold increase, over EGTA control levels, in the phosphorylation of an 18-kDa protein and MP26. Calcium without TPA yielded a fourfold change. However, phosphorylation in the 60–70 kDa range was only increased 2.7-fold in the presence of calcium and TPA.

In Fig. 3, the Walsh inhibitor was added to evaluate possible A kinase effects on phosphoryl-

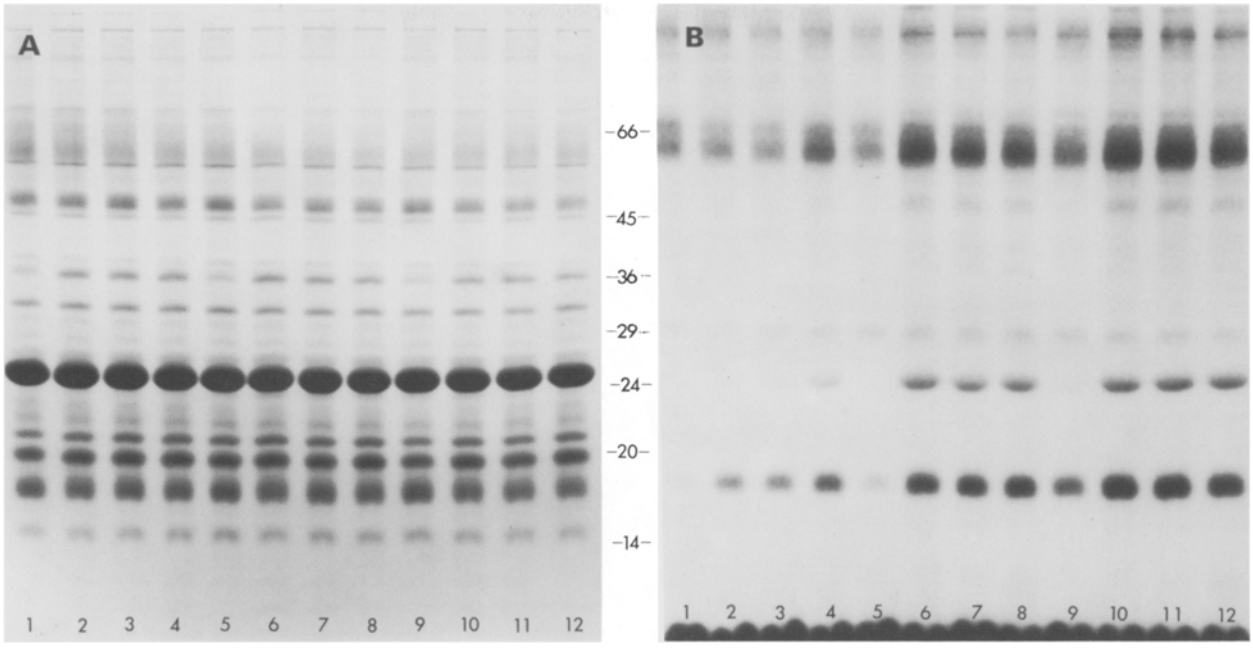

Fig. 2. SDS-PAGE following the phosphorylation of membrane proteins in lens homogenates. Lenses were homogenized in 20 mM imidazole and 10 mM $MgCl_2$. Each sample received 25 μM [γ-^{32}P]ATP. Varying calcium and TPA were added to the reaction mixtures. No TPA was added in lanes *1–4*, while TPA was at 10 nM in lanes *5–8*, and 100 nM in lanes *9–12*. Calcium was increased through each of the TPA concentrations from nanomolar calcium (1 mM EGTA, lanes *1, 5, 9*), to 1 μM added $CaCl_2$ (lanes *2, 6, 10*), 10 μM added $CaCl_2$ (lanes *3, 7, 11*), and 100 μM added $CaCl_2$ (lanes *4, 8, 12*). Membranes were purified and SDS-PAGE analysis was performed. The Coomassie-blue-stained gel (*A*) and corresponding autoradiograph (*B*) are shown

ation. Phosphorylation was in lens homogenates as described above to permit the addition of the Walsh inhibitor protein. The reaction mixture represented in lane *1* contained no TPA and those in lanes *2–5* had 10 nM TPA. Calcium was present at approximately 10 μM (lanes *1, 3* and *4*) or 0.01 μM (lanes *2* and *5*). As in Fig. 2, TPA had a dramatic effect, even in the presence of Walsh inhibitor (lanes *4* and *5*). When TPA was present the reactions with lower free calcium (lanes *2* and *5*) yielded much less phosphorylation. These results extended those from Fig. 2 and further established a calcium and TPA interdependence. The extent of TPA-enhanced phosphorylation was not altered at 10 μM calcium when the Walsh inhibitor was added (*compare* lanes *3* and *4*). At low calcium concentrations (0.01 μM) the Walsh inhibitor appeared to have a very slight effect on the phosphorylation level (*compare* lanes *2* and *5*). TPA-enhanced phosphorylation activity was particularly sensitive in this calcium range. Indeed, the minor difference observed may actually be a calcium effect because the Walsh inhibitor preparation contained EDTA. An effort was made to compensate for this difference by adding the same amount of EDTA to the mixture without the Walsh inhibitor, but without using large amounts of EDTA and calcium to provide an effective buffer at this free calcium concentration, slight differences were likely.

Phosphoamino acid analysis was performed on MP26 that had been phosphorylated in the presence of TPA and calcium (Fig. 4). Phosphorylation was in lens homogenates and at calcium and TPA concentrations designed to maximize phosphorylation. Phosphoamino acid electrophoresis of gel-purified and hydrolyzed MP26 was performed at both pH 3.5 and 1.9, to clearly separate phosphoserine, phosphothreonine and phosphotyrosine. Phosphotyrosine and phosphothreonine are not separated at pH 1.9. Internal nonradioactive standards were detected using ninhydrin and their relative positions are indicated. Most of the label was found in phosphoserine with a smaller amount in phosphothreonine. No label was detected in phosphotyrosine even after prolonged exposure. Excision of the ninhydrin spots from two experiments and scintillation counting showed approximately 85% of the counts in phosphoserine and 15% in phosphothreonine.

Protease digestion of MP26 was performed to determine the portion of MP26 that contains the phosphorylated residues. Digestion of MP26 yields a stable 22-kDa fragment with the hydrolysis of peptide occurring almost exclusively from the C-termi-

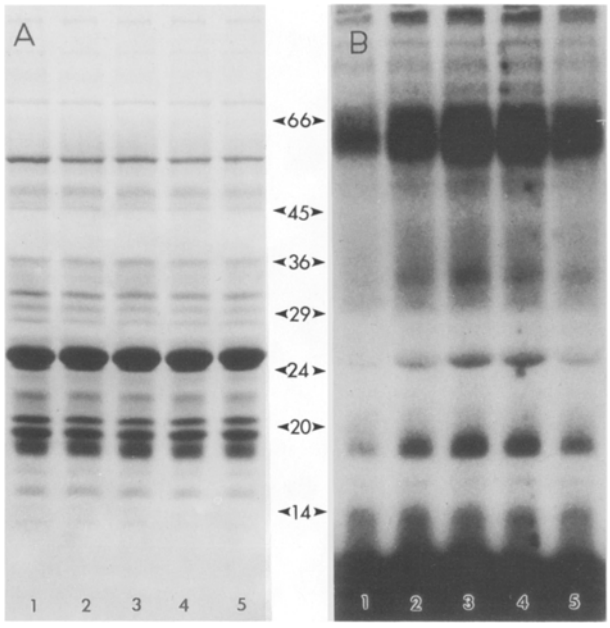

Fig. 3. SDS-PAGE following the phosphorylation of membrane proteins in the presence of Walsh inhibitor in lens homogenates. All reaction mixtures contained 5 mM $MgCl_2$ and 25 μM [γ-^{32}P]ATP. TPA (10 nM) was added to the reaction mixture in lanes *2–5*. In lanes *1*, *3* and *4* calcium was added to a free concentration of approximately 10 μM using limited EDTA and EGTA buffering. In lanes *2* and *5* the free concentration of calcium was estimated to be 0.01 μM. The reaction mixtures in lanes *4* and *5* contained 36 units of Sigma Walsh inhibitor. Note that this inhibitor of A kinase has no demonstrable effect on TPA-stimulated phosphorylation (*compare* lanes *3* and *4*). Membranes were purified and SDS-PAGE analysis was performed. The Coomassie-blue-stained gel (*A*) and corresponding autoradiograph (*B*) are shown

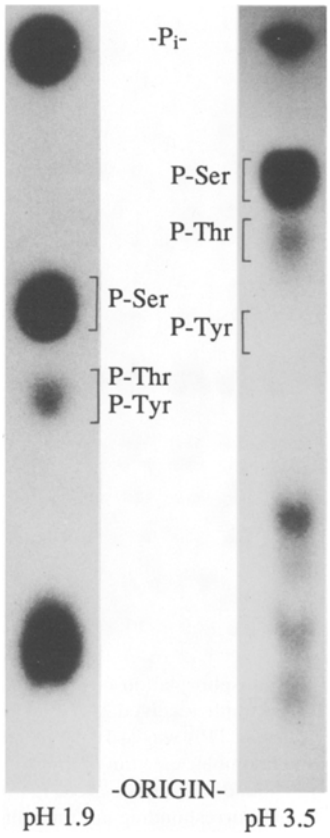

Fig. 4. Phosphoamino acid analysis of MP26 phosphorylated in the presence of TPA. Phosphorylation was in lens homogenates under conditions that activate C kinase. MP26 was electrophoretically purified, electroeluted, hydrolyzed and electrophoretically chromatographed in the presence of nonradioactive standards (3 μg/lane) at both pH 3.5 and 1.9 to clearly separate the three phosphoamino acids. The positions of the ninhydrin positive spots corresponding to the phosphoamino acids are indicated by brackets. Most of the label was found in phosphoserine, with approximately 15% being phosphothreonine

nal end [30]. This approach has been used previously to localize the site of phosphorylation of both A and C kinases to the C-terminal portion removed by proteolysis. Following TPA-stimulated phosphorylation of MP26, protease digestion by both lysylendopeptidase-C and trypsin also showed no significant phosphorylation remaining in the 22-kDa fragment (Fig. 5). Only three lysylendopeptidase-C cleavage sites exist in MP26 and they all reside after residue 227 of the C-terminal tail [21]. Thus, phosphorylation stimulated by TPA and calcium occurred on the C-terminal tail.

Phosphorylation of Lens Membranes by Calcium/Calmodulin-Dependent Protein Kinase II (CaM Kinase)

Since calcium levels were shown to be important for the phosphorylation of MP26 and other lens proteins, we sought to discover whether CaM kinase, which is also calcium dependent, could phosphorylate MP26. Figure 6 shows the phosphorylation of isolated lens membranes by CaM kinase, using conditions where both A kinase and C kinase are known to phosphorylate MP26 [16, 27, 36, 41]. Lane *1* represents a complete mixture of lens membranes, calcium, magnesium, calmodulin, ^{32}P-ATP and CaM kinase. No phosphorylation of MP26 or an 18-kDa protein occurred, but significant phosphorylation of higher molecular weight proteins was observed. Lane *2* membranes were treated the same as those in lane *1* except they were trypsinized after phosphorylation. Controls indicated that the kinase was calcium and calmodulin dependent (lanes *3* and *4*). In lane *5* synapsin, a known substrate for CaM

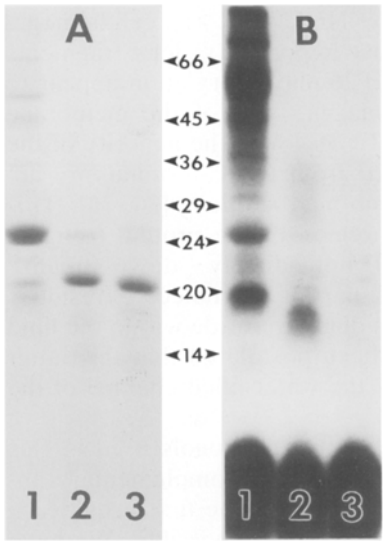

Fig. 5. SDS-PAGE following the phosphorylation of membrane proteins in lens homogenates and protease digestion. All reaction mixtures contained 5 mM $MgCl_2$ and 25 μM [γ-^{32}P]ATP, 10 μM $CaCl_2$ and 20 nM TPA. Membranes were purified and incubated for 1 hr with buffer (lane *1*), lysylendopeptidase-C (lane *2*) or trypsin (lane *3*) at a ratio of 1 : 20 with membrane protein. Membranes were recentrifuged and run on SDS-PAGE. The Coomassie-blue-stained gel (*A*) and corresponding autoradiograph (*B*) are shown. Note that the major proteolytic fragments of MP26 do not display significant phosphorylation

kinases, was phosphorylated to a very high degree. Thus, although the kinase displayed substantial activity, very few lens membrane proteins were phosphorylated.

Discussion

The vertebrate lens is an avascular organ of considerable dimension with surprisingly long cellular lifetimes and limited protein turnover. As investigators have evaluated these unusual properties of the lens, the junctions between lens fiber cells have attracted considerable attention. As a result, lens junctions have emerged as a model system for studying the structure, composition and permeability of cell-to-cell channels. The lens itself has been recognized as a system which epitomized the ability of cells to exchange substantial amounts of small metabolites via these channels. Thus, a strong interest has developed in identifying the protein or proteins which form the junctions between the lens fiber cells.

Four criteria have been outlined by Hertzberg et al. to aid in the identification of proteins as components of gap junctions [22]. These include: (i) characterization of proteins associated with isolated

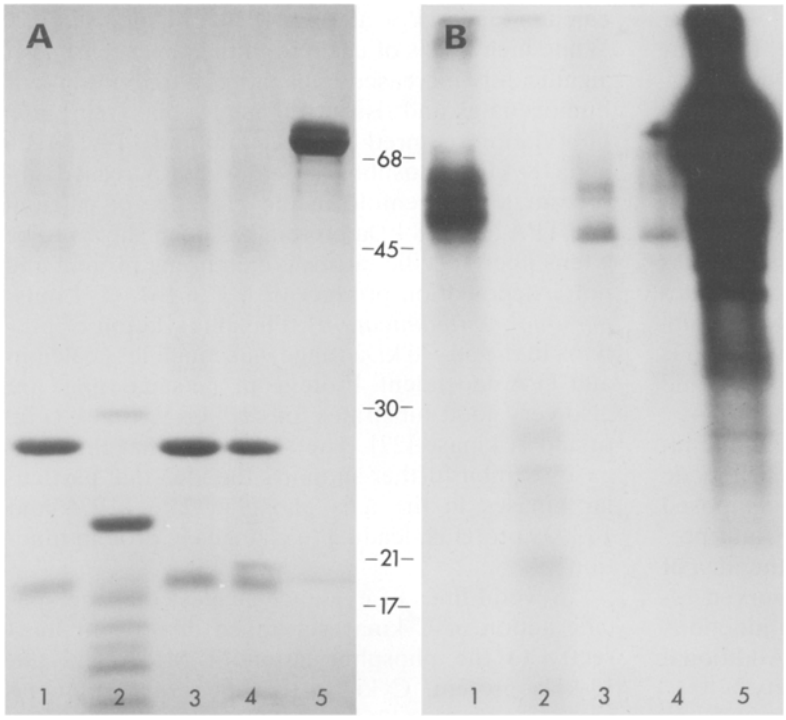

Fig. 6. SDS-PAGE following the phosphorylation of isolated lens membranes by CaM kinase. A complete assay (lane *1*) contained 50 mM HEPES, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1.0 μM calmodulin, 1.5 mM $CaCl_2$, 20 μM [γ-^{32}P]ATP and CaM kinase. Incubation was for 30 min at room temperature. The membranes were centrifuged and either washed (lanes *1*, *3*, *4*) or incubated with trypsin at a ratio of 1 : 20, protease/membrane protein (lane *2*) and recentrifuged (as in Fig. 5). Lane *3* represents a complete assay mixture with calcium omitted; in lane *4*, calmodulin was left out. In lane *5*, the reaction was terminated by the addition of SDS to 1%. SDS-PAGE analysis was performed and the Coomassie-blue-stained gel (*A*) and corresponding autoradiograph (*B*) are shown

gap junctions; (ii) preparation of antibodies for immunocytological localization of the proteins in question; (iii) determination of antibody effects on electrical coupling and dye coupling between cells; and (iv) analysis of the biophysical properties of channels in the isolated and reconstituted material. Although the work is ongoing, data from several different laboratories concerning each of these criteria support a role for MP26 as a protein of cell-to-cell channels. (i) MP26 is by far the major protein of junction-rich urea and detergent-treated membranes [e.g., 4, 8, 20]. (ii) Different investigators have found MP26 within lens junctions of both "thick" and "thin" types [6, 10, 28, 51], while others have found no immunogold labeling of thick junctions with MP26 antibodies [48]. These conflicting results may be explained by changes in the immunoreactivity of junctional MP26, which may occur with the different labeling procedures being employed [28]. (iii) Antibodies to a C-terminal peptide of MP26 blocked channel activity in a vesicle assay [19]. MP26 antibodies have also been reported to reduce dye transfer between cells in chicken lens cultures [28]. (iv) With MP26 purified from urea-treated membranes by organic solubilization and membrane reconstitution, MP26 concentration-dependent increases in intervesicle conductance were reported when two vesicles were manipulated into contact [7]. In addition, HPLC purified monomeric MP26 incorporated into planar bilayers yielded voltage-dependent channels [11, 64].

A 70-kDa protein from lens has also been suggested as a gap junction protein candidate. It fits the above criteria in the following ways: (i) One laboratory has found significant amounts of the protein associated with junction-rich membranes from the outermost region of the lens cortex, but MP26 was still the major protein in these preparations [33]. (ii) The same laboratory has immunolocalized this protein exclusively within "thick" junction structures [33]. (iii and iv) No antibody or biophysical studies have been published yet. The best evidence supporting a gap junction role for this 70-kDa protein is its N-terminal sequence homology with liver and heart gap junction proteins [32].

The most encompassing explanation would allow for either multiple junctional types, each made up of one protein, or one junctional type composed of multiple proteins. Freeze-fracture EM and preliminary antibody injection data support the idea of multiple types of junctions [28]. The observed restriction of the 70-kDa protein to cortical junctions [33] is also consistent with this idea. Additional work is required to evaluate this possibility.

The phosphorylation of MP26 has been demonstrated by studies with isolated membranes and different protein kinases [16, 27, 36, 41], cell homogenates [26], and, most importantly, lens fragments [26]. The sites of MP26 phosphorylation appear to reside on the cytoplasmic side of the membrane [this study, 16, 26, 27, 36], with the majority of the sites being separated from the C-terminus by approximately 20 amino acid residues [26, 36]. This corresponds to the region of the protein (roughly residues 220 to 240) immediately "down-stream" from an amphipathic part of the molecule (residues 197–219), which is thought to reside within the lipid bilayer [21]. It has been postulated that this latter region actually lines the water-filled channel of the junction [21].

The two tissue labeling protocols used in this study were devised to provide complementary approaches to phosphorylation. The first provided a system which was as native as possible. We have previously shown in studies with tissue fragments that the preparation had been capable of ATP synthesis and that phosphate was incorporated into the donor ATP pool [26]. Protein synthesis was also shown to be linear over a period of several hours [26]. Finally, A23187 had a calcium-dependent effect on phosphorylation levels in tissue fragments, suggesting that membrane compartmentalization had been maintained. In the second protocol, lens homogenates allowed for careful control of the levels of calcium and treatment of the system with protein kinase inhibitors.

The results presented here clearly establish that calcium and TPA affect MP26 phosphorylation. While high levels of calcium in the absence of TPA significantly increased phosphorylation both in lens homogenates and tissue fragments, maximal phosphorylation required both calcium and TPA. MP26 and 18 kDa phosphorylation were increased approximately sevenfold in the presence of calcium and TPA. The 18-kDa protein has been shown to be a lens fiber-specific intrinsic membrane protein and not a degradation product of MP26 [44, C. Louis, *personal communication*]. Phosphorylation of proteins in the 50–70 kDa range was much less calcium and TPA dependent. Proteins in the same range are also very good substrates for both CaM kinase (Fig. 6) and A kinase [27]. The selectivity that these kinases exhibit further supports the idea that particular kinases in the lens phosphorylate MP26 and 18-kDa proteins, leading to changes in protein function.

Several lines of evidence suggest that the specific action of C kinase is linked directly or indirectly to the phosphorylation of MP26 and the 18-kDa protein. C kinase has been shown to be present in the lens [36] and phosphorylation of these proteins can be increased many fold by TPA in con-

cert with calcium. TPA and other phorbol esters have been shown to be highly specific activators of C kinase; they are not known to activate any other kinase [45]. This high specificity of activators cannot be matched by C kinase inhibitors. Several C kinase "inhibitors" actually act through the calcium or phospholipid dependence of C kinase [17, 23] and hence they provide for generalized effects and are not suited for this study.

Given the specific action of C kinase in the lens, one then asks whether this kinase directly phosphorylates MP26. The direct phosphorylation of a protein by a particular kinase is difficult to prove in a living cell, due to the number of different kinase systems and interactions between some of these systems. However, the existing data indicate that the C kinase likely phosphorylated MP26 in a direct manner when stimulated by TPA. First, it is known that C kinase prepared from either brain or lens is able to phosphorylate MP26 in isolated membranes [36]. Second, in the work with C kinase and isolated membranes, the proportions of phosphoserine and phosphothreonine obtained from MP26 [36] are similar to those reported here with the TPA-stimulated phosphorylation. Third, the lack of any phosphotyrosine within MP26 serves to eliminate tyrosine kinases from consideration. Fourth, as considered below, two other calcium-sensitive kinase systems in the lens are unlikely to be involved following TPA treatment.

Since A kinase in the lens responds indirectly to calcium, following the activation of adenylate cyclase [42], A kinase could conceivably be involved in the phosphorylation of MP26 upon exposure to calcium and TPA. We have addressed this alternative by using the Walsh inhibitor. The negligible effect of the Walsh inhibitor indicates that A kinase is not involved in the TPA-mediated increase in MP26 phosphorylation (Fig. 3). In addition, while A kinase phosphorylates only serines within MP26 [16, 36], it has been shown here that both serine and threonine are labeled following TPA treatment (Fig. 4). Phosphorylation occurred on the cytoplasmic, C-terminal tail (Fig. 5). Another calcium-sensitive kinase is the CaM kinase. In this case, MP26 is an unlikely substrate because purified CaM kinase was unable to phosphorylate this protein in isolated membranes (Fig. 6). Therefore, while other kinases could be involved following TPA stimulation, the direct phosphorylation of MP26 by C kinase appears to be the most likely explanation.

The stoichiometry of MP26 phosphorylation in these experiments appears to be quite low. While MP26 is the major protein of lens membranes, other proteins (e.g., the 18-kDa protein) were more highly phosphorylated. With this result, one could possibly argue that the phosphorylation at 26 kDa was due to a co-migrating protein, and not MP26. However, the label is consistently associated with MP26, during separation by means of six different electrophoretic and chromatographic methods (K. Johnson, *personal communication*). Thus, low stoichiometry appears to be a genuine feature of MP26 phosphorylation. In fact, we have also used a totally different approach than that reported here to detect the phosphorylation of MP26. Employing chemical methods on a protease derived peptide that contains three of the six C-terminal tail serines, we have found that at least 5% of MP26 from untreated membranes was phosphorylated (*unpublished observations*). Even if some native phosphorylation is lost during isolation, we are still clearly left with a low stoichiometry. Moreover, by applying protein kinases and labeled ATP to isolated membranes, only a small amount of additional phosphorylation has been observed in previous studies [16, 27, 36, 41].

Therefore, the question becomes whether phosphorylation at the 5% level could lead to important regulatory changes in the MP26 molecule. A useful perspective comes from work on the phosphorylation of the liver 27-kDa protein [50], which also involves a low stoichiometry (less than 2.5%). In that study, an increase of 25–75% in gap junction conductance between hepatocyte pairs was observed under conditions where glucagon and 8-Bromo-cAMP increased gap junction protein phosphorylation. Different factors could help explain the low stoichiometry. One possibility is that only one of the proteins that makes up the dodecameric channel (two opposed hexamers in each bilayer [34]) would need to be phosphorylated to regulate channel activity. In this case, a 5% change in phosphorylation could lead to a maximal change of 60% in junctional conductance.

These last considerations assume that the phosphorylation of MP26 directly regulates channel permeability in the lens as it appears to do in the liver [50]. However, this posttranslational modification may control other aspects of MP26 function. For example, phosphorylation/dephosphorylation could be involved in the assembly of junctions and have no influence over the permeability of junctional channels. Since 80% of MP26 has been estimated to be junctional [30], the removal of phosphate could be associated with the incorporation of MP26 into junctional aggregates. Other possibilities also exist.

Multiple kinase systems appear to be involved in the phosphorylation of lens MP26, as is the case in hepatocytes. Different kinases have been shown to phosphorylate junctional protein in isolated membranes from both lens [16, 27, 36, 41] and liver

[50, 57]. In addition, both A and C kinases appear to phosphorylate MP26 in native systems of an *in vivo* type [26 and this report]. Effectors of various kinase systems including C kinase [12, 15, 61, 63], A kinase [9, 31, 37, 49, 60] and pp60^{src} [1–3] have been shown to alter junctional activity in other cell types. Different groups have proposed that one kinase may increase and another decrease junctional activity [15, 40]. The effects of different kinases on other protein functions have also been pursued in varied cell types. In human erythrocytes, phosphorylation by both A and C kinase reduced the ability of band 4.1 to promote spectrin binding to F actin [38]. However, they caused opposite effects on actin polymerization [46]. These two kinases in the lens may phosphorylate at the same site and act in a similar manner or at different sites to yield opposed or synergistic effects. Phosphoamino acid sequencing studies are in progress on MP26 to address this issue.

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References

1. Atkinson, M.M., Menko, A.S., Johnson, R.G., Sheppard, J.R., Sheridan, J.D. 1981. Rapid and reversible reduction of junctional permeability in cells infected with a temperature-sensitive mutant of avian sarcoma virus. *J. Cell Biol.* **91:**573–578
2. Azarnia, R., Loewenstein, W.R. 1984. Intercellular communication and the control of growth. X. Alteration of junctional permeability by the *src* gene. A study with temperature-sensitive mutant Rous sarcoma virus. *J. Membrane Biol.* **82:**191–205
3. Azarnia, R., Reddy, S., Kmiecik, T.E., Shalloway, D., Loewenstein, W.R. 1988. The cellular *src* gene regulates junctional cell-to-cell communication. *Science* **239:**398–400
4. Benedetti, E.C., Dunia, I., Bentzel, C.J., Vermorken, A.J.M., Kibbilaar, M., Bloemendal, H. 1976. A portrait of plasma membrane specializations in eye lens epithelium and fibers. *Biochim. Biophys. Acta* **457:**353–384
5. Beyer, E.C., Paul, D.L., Goodenough, D.A. 1987. Connexin43: A protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* **105:**2621–2629
6. Bok, D., Dockstader, J., Horwitz, J. 1982. Immunocytochemical localization of the lens main intrinsic polypeptide (MIP26) in communicating junctions. *J. Cell Biol.* **92:**213–220
7. Brewer, G.J., Takemoto, L.J., Draper, K.L. 1987. Gap junction reconstitution in two large spherical membranes: Junctional membrane conductance increase with the second power of the input protein concentration and individual current jumps are larger and more frequent after adhesion. *J. Cell Biol.* **105:**227*a*
8. Broekhuyse, R.M., Kuhlmann, E.D. 1974. Lens membranes: I. Composition of urea-treated plasma membranes. *Exp. Eye Res.* **19:**297–302
9. DeMello, W. 1984. Effect of intracellular injection of cAMP on the electrical coupling of cardiac cells. *Biophys. Biochem. Res. Commun.* **119:**1001–1007
10. Dunia, I., Manenti, S., Rousselet, A., Benedetti, E.L. 1987. Electron microscopic observations of reconstituted proteoliposomes with the purified major intrinsic membrane protein of eye lens. *J. Cell Biol.* **105:**1679–1689
11. Ehring, G.R., Zamphigi, G.A., Horwitz, J., Bok, D., Hall, J.E. 1988. Channels incorporated into planar lipid bilayer membranes from HPLC purified MIP26 or from membrane fragments enriched for lens fiber junctions are nearly identical. *Biophys. J.* **53:**51*a*
12. Enomoto, T., Yamasaki, H. 1985. Phorbol ester-mediated inhibition of intercellular communication in BALB/c 3T3 cells: Relationship to enhancement of cell transformation. *Cancer Res.* **45:**2681–2688
13. Fallon, R., Goodenough, D.A. 1981. Five-hour half-life of mouse liver gap junction protein. *J. Cell Biol.* **90:**521–526
14. Fraser, S.E., Green, C.R., Bode, H.R., Gilula, N.B. 1987. Selective disruption of gap junctional communication interferes with a patterning process in hydra. *Science* **237:**49–55
15. Gainer, H., Murray, A. 1985. Diacylglycerol inhibits gap junctional communication in cultured epidermal cells: Evidence for a role of protein kinase C. *Biophys. Biochem. Res. Commun.* **126:**1109–1113
16. Garland, D., Russell, P. 1985. Phosphorylation of lens fiber cell membrane proteins. *Proc. Natl. Acad. Sci. USA* **82:**653–657
17. Garland, L.G., Bonser, R.W., Thompson, N.T. 1987. Protein kinase C inhibitors are not selective. *Trends Pharmacol. Sci.* **8:**334
18. Girsch, S.J., Peracchia, C. 1985. Lens cell-to-cell channel protein: I. Self-assembly into liposomes and permeability regulation by calmodulin. *J. Membrane Biol.* **83:**217–225
19. Gooden, M.M., Rintoul, D.A., Takehana, M., Takemoto, L. 1985. Major intrinsic polypeptide (MIP26k) from lens membrane: Reconstitution into vesicles and inhibition of channel forming activity by peptide antiserum. *Biophys. Biochem. Res. Commun.* **128:**993–999
20. Goodenough, D.A. 1979. Lens gap junctions: A structural hypothesis for non-regulated low-resistance intercellular pathways. *Invest. Ophthalmol. Visual Sci.* **18:**1104–1122
21. Gorin, M.B., Yancey, S.B., Cline, J., Revel, J.P., Horwitz, J. 1984. The major intrinsic protein (MIP) of the bovine fiber membrane: Characterization and structure based on cDNA cloning. *Cell* **39:**49–59
22. Hertzberg, E.L., Spray, D.C., Cook, R. 1988. Biochemical, immunological and topological studies of gap junctions. *In:* Gap Junctions: Modern Cell Biology Series. Vol. 7, pp. 9–28. E.L. Hertzberg and R.G. Johnson, editors. Alan R. Liss, New York
23. Hidaka, H., Hagiwara, M. 1987. Pharmacology of the isoquinoline sulfonamide protein kinase C inhibitors. *Trends Pharmacol. Sci.* **8:**162–164
24. Higgins, R.C., Dahmus, M.E. 1979. Rapid visualization of protein bands in preparative SDS-polyacrylamide gels. *Anal. Biochem.* **93:**257–260
25. Hunter, T., Sefton, B. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* **77:**1311–1315
26. Johnson, K.R., Lampe, P.D., Hur, K.C., Louis, C.F., Johnson, R.G. 1986. A lens intercellular junction protein, MP26, is a phosphoprotein. *J. Cell Biol.* **102:**1334–1343
27. Johnson, K.R., Panter, S.S., Johnson, R.G. 1985. Phosphorylation of lens membranes with a cyclic AMP-dependent protein kinase purified from the bovine lens. *Biochim. Biophys. Acta* **844:**367–376
28. Johnson, R.G., Klukas, K.A., Lu, T.-H., Spray, D.C. 1988. Antibodies to MP28 are localized to lens junctions, alter intercellular permeability and demonstrate increased expres-

sion during development. *In*: Gap Junctions: Modern Cell Biology Series. Vol. 7, pp. 81–98. E.L. Hertzberg and R.G. Johnson, editors. Alan R. Liss, New York

29. Johnson, R.G., Meyer, R.A., Lampe, P.D. 1988. Gap junction formation: A self-assembly model involving membrane domains of lipid and protein. *In*: Cell Interactions and Gap Junctions. Vol. 1, N. Sperelakis and W.C. Cole, editors. CRC, Boca Raton (*in press*)
30. Keeling, P., Johnson, K., Sas, D., Klukas, K., Donahue, P., Johnson, R.G. 1983. Arrangement of MP26 in lens junctional membranes: Analysis with proteases and antibodies. *J. Membrane Biol.* **74**:217–228
31. Kessler, J.A., Spray, D.C., Saez, J.C., Bennett, M.V.L. 1984. Determination of synaptic phenotype: Insulin and cAMP independently initiate development of electrotonic coupling between cultured sympathetic neurons. *Proc. Natl. Acad. Sci. USA* **81**:6235–6239
32. Kistler, J., Christie, D., Bullivant, S. 1988. Homologies between gap junction proteins in lens, heart and liver. *Nature (London)* **331**:721–723
33. Kistler, J., Kirkland, B., Bullivant, S. 1985. Identification of a 70,000-D protein in lens membrane junctional domains. *J. Cell Biol.* **101**:28–35
34. Kuszak, J.R., Rae, J.L., Pauli, B.U., Weinstein, R.S. 1982. Rotary replication of lens gap junctions. *J. Ultrastruct. Res.* **81**:249–256
35. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685
36. Lampe, P.D., Bazzi, M.D., Nelsestuen, G.L., Johnson, R.G. 1986. Phosphorylation of bovine lens membranes by protein kinase C. *Eur. J. Biochem.* **156**:351–357
37. Lasater, E.M., Dowling, J.E. 1985. Dopamine decreases the conductance of the electrical junctions between cultured retinal horizontal cells. *Proc. Natl. Acad. Sci. USA* **82**:3025–3029
38. Ling, E., Danilov, Y.N., Cohen, C.M. 1988. Modulation of red cell band 4.1 function by cAMP-dependent kinase and protein kinase C phosphorylation. *J. Biol. Chem.* **263**:2208–2216
39. Loewenstein, W.R. 1979. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta* **560**:1–65
40. Loewenstein, W.R. 1985. Regulation of cell-to-cell communication by phosphorylation. *Biochem. Soc. Symp. London* **50**:43–58
41. Louis, C.F., Johnson, R.G., Johnson, K.R., Turnquist, J. 1985. Characterization of the bovine lens plasma membrane substrates for cAMP-dependent protein kinase. *Eur. J. Biochem.* **150**:279–286
42. Louis, C.F., Mickelson, J.R., Turnquist, J., Hur, K.C., Johnson, R. 1987. Regulation of lens cyclic nucleotide metabolism by calcium plus calmodulin. *Invest. Ophthalmol. Vis. Sci.* **28**:806–814
43. Momoi, T. 1986. Activation of protein kinase C by ganglioside GM3 in the presence of TPA. *Biochem. Biophys. Res. Commun.* **138**:865–871
44. Mulders, J.W., Voorter, C.E.M., Lamers, C., Haard-Hoekman, W.A. de, Montecucco, C., Ven, W.J.M. van de, Bloemendal, H., Jong, W.W. de 1988. MP17, a fiber specific intrinsic membrane protein from mammalian eye lens. *Curr. Eye Res.* **7**:207–219
45. Nishizuka, Y. 1986. Perspectives on the role of protein kinase C in stimulus-response coupling. *J. Natl. Canc. Inst.* **76**:363–370
46. Ohta, E., Akiyama, T., Nishida, E., Sakai, H. 1987. Protein kinase C and cAMP-dependent protein kinase induce opposite effects on actin polymerizability. *FEBS Lett.* **222**:305–310
47. Paul, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.* **103**:123–134
48. Paul, D.L., Goodenough, D.A. 1983. Preparation, characterization, and localization of antisera against bovine MP26, an integral protein from lens fiber plasma membrane. *J. Cell Biol.* **96**:625–632
49. Radu, A., Dahl, G., Loewenstein, W.R. 1982. Hormonal regulation of cell junction permeability: Upregulation by catecholamine and prostaglandin E_1. *J. Membrane Biol.* **70**:239–251
50. Saez, J.C., Spray, D.C., Nairn, A.C., Hertzberg, E., Greengard, P., Bennett, M.V.L. 1986. cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principle gap junction polypeptide. *Proc. Natl. Acad. Sci. USA* **83**:2473–2477
51. Sas, D.F., Sas, J., Johnson, K., Menko, A.S., Johnson, R.G. 1985. Junctions between lens fiber cells are labeled with a monoclonal antibody shown to be specific for MP26. *J. Cell Biol.* **100**:216–225
52. Sheridan, J.D. 1976. Cell coupling and cell communication during embryogenesis. *In*: The Cell Surface in Animal Embryogenesis. G. Poste and G. Nicholson, editors. pp. 409–447. Elsevier, New York
53. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurements of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85
54. Spray, D.C., Bennett, M.V.L. 1985. Physiology and pharmacology of gap junctions. *Annu. Rev. Physiol.* **47**:281–303
55. Spray, D.C., Fujita, M., Saez, J.C., Choi, H., Watanabe, T., Hertzberg, E., Rosenberg, L.C., Reid, L.M. 1987. Proteoglycans and glycosaminoglycan induce gap junction synthesis and function in primary liver cultures. *J. Cell Biol.* **105**:541–552
56. Storer, A.C., Cornish-Bowden, A. 1976. Concentration of $MgATP^{2-}$ and other ions in solution. *Biochem. J.* **159**:1–5
57. Takeda, A., Hashimoto, E., Yamamura, H., Shimazu, T. 1987. Phosphorylation of liver gap junction protein by protein kinase C. *FEBS Lett.* **210**:2681–2688
58. Traub, O., Look, J., Paul, D., Willecke, K. 1987. Cyclic AMP stimulates biosynthesis and phosphorylation of the 26 kDa gap junction protein in cultured mouse hepatocytes. *Eur. J. Cell Biol.* **43**:48–54
59. Warner, A., Guthrie, S.C., Gilula, N.B. 1984. Antibodies to gap junctional proteins selectively disrupt junctional communication in the early amphibian embryo. *Nature (London)* **311**:127–131
60. Wiener, E.C., Loewenstein, W.R. 1983. Correction of cell–cell communication defect by introduction of a protein kinase into mutant cells. *Nature (London)* **305**:433–435
61. Yada, T., Rose, B., Loewenstein, W.R. 1985. Diacylglycerol downregulates junctional membrane permeability. TMB-8 blocks this effect. *J. Membrane Biol.* **88**:217–232
62. Yancey, S.B., Nicholson, B.J., Revel, J.P. 1981. Dynamic state of liver gap junctions. *J. Supramol. Struct. Cell. Biochem.* **16**:221–232
63. Yotti, L.P., Chang, C.C., Trosko, J.E. 1979. Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science* **206**:1089–1091
64. Zampighi, G.A., Hall, J.E., Kreman, M. 1985. Purified lens junctional protein forms channels in planar lipid films. *Proc. Natl. Acad. Sci. USA* **82**:8468–8472

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