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cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principal gap junction polypeptide

(hepatocytes/intercellular communication/glucagon/electrical coupling/MP27 protein)

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ABSTRACT **Membrane-permeant cAMP derivatives (dibutyryl- and 8-bromo-cAMP) increase gap-junctional conductance within minutes when applied to voltage-clamped pairs of rat hepatocytes. Glucagon also increases junctional conductances, but the response has a more rapid onset and is more rapidly reversible. The glucagon effect can be prevented by intracellular injection of the protein inhibitor of the cAMP-dependent protein kinase (Walsh inhibitor), indicating that the catalytic subunit of cAMP-dependent protein kinase is directly involved. The 27-kDa major gap junction polypeptide is phosphorylated when liver cells dissociated into small groups are incubated with ^{32}P. Addition of 8-bromo-cAMP to cells increases the incorporation of ^{32}P into the 27-kDa junctional protein. Serine is the amino acid residue that is phosphorylated. When isolated liver gap junctions are incubated in the presence of catalytic subunit of the cAMP-dependent protein kinase, the 27-kDa gap junction polypeptide is phosphorylated with low stoichiometry on serine. The rapid increases in gap junctional conductance caused by agents that elevate cAMP and phosphorylation of the gap junction protein by cAMP-dependent protein kinase suggest that cAMP-dependent phosphorylation of the gap junction channel modulates the conductance of liver gap junctions.**

Gap-junction channels mediate exchange of ions and small molecules between coupled cells. As with many other channels in biological membranes, conductance is altered by certain treatments (1). Recently, cAMP has been shown to act on gap junctions in a number of systems. Increase in coupling and/or incidence of gap junctions has been observed over periods of hours or days in cultured mammalian cells treated with substances that increase cytoplasmic cAMP (2–7). These slow effects are generally dependent on protein synthesis (2, 3, 6, 7), suggesting that the control is on formation of gap junctions. In a few cases, the effects of cAMP are more rapid, occurring within minutes to tens of minutes; examples include increases in coupling in insect salivary gland (4) and vertebrate heart muscle (5). Coupling between fish retinal horizontal cells is reversibly decreased by increases in cAMP with a time course fast enough to suggest a direct gating process (8, 9). The fast permeability changes at gap junctions caused by agents that increase cAMP levels could be the consequence of direct phosphorylation of the channel-forming protein by cAMP-dependent protein kinase. Alternatively, cAMP-dependent protein kinase may phosphorylate a modulating protein that affects the channel properties.

Gap junctions can be isolated by subcellular fractionation following alkali or detergent treatment (10, 11). While there are some discrepancies, it is generally agreed that the constituent protein is ≈27 kDa (cf. ref. 12). Each channel is thought to be a dodecamer of this protein, a hexamer forming a hemichannel in each of the joined membranes. Antibodies raised against the isolated junctions bind to the 27-kDa protein in immunoblots (11). The antisera also label the cytoplasmic aspect of the junctions and block their conductance in a variety of tissues (13).

We report here that membrane-permeant derivatives of cAMP increase junctional conductance between liver cells in culture. Glucagon, which increases cAMP levels in these cells (14), also increases junctional conductance. Participation of cAMP-dependent protein kinase is suggested by the inhibition of the glucagon effect by Walsh inhibitor. Furthermore, phosphorylation of the 27-kDa junctional protein of intact cells is increased by dibutyryl-cAMP (Bt_2cAMP), and cAMP-dependent protein kinase catalyzes phosphorylation of the 27-kDa protein of isolated junctions.

Preliminary reports of these findings have appeared (15, 16).

METHODS

Preparation of Partially Dissociated Hepatocytes. Cells were isolated from livers of adult Sprague–Dawley rats (Charles River Breeding Laboratories) by using a collagenase perfusion technique (17). Pairs and small aggregates of hepatocytes resulting from incomplete dissociation were abundant. For the electrophysiological studies, cells were plated (10^5 cells per culture dish) on plastic culture dishes (35-mm diameter) with Waymouth's medium containing 10% fetal calf serum (GIBCO) for 0.5–3 hr at 37°C. The medium was replaced with Dulbecco's phosphate-buffered saline (GIBCO) prior to recording. For the ^{32}P labeling experiments, cells were maintained in an ice bath until use.

Reagents. DL-*o*-phosphoserine, DL-*o*-phosphothreonine, bovine serum albumin, dithiothreitol, EGTA, ATP, Hepes, Bt_2cAMP, 8-bromo-cAMP (8Br-cAMP), Triton X-100, and glucagon were obtained from Sigma. K glutamate was from City Chemical (New York), $^{32}P_i$ and [γ-^{32}P]ATP were from New England Nuclear, and thin-layer cellulose sheets (type 13255) were from Eastman Kodak. Catalytic subunit of cAMP-dependent protein kinase and Walsh inhibitor were purified as described (18, 19).

Electrophysiology. Cell pairs were recorded from with either conventional or patch-clamp (20) microelectrodes (15–20 MΩ, filled with 2 M K citrate or 2–5 MΩ, filled with 0.15 M KCl or K glutamate/10 mM EGTA/5 mM Hepes, adjusted to pH 7.2 with KOH). The glass used contained a fiber for a quick filling. Patch electrodes were connected to single-electrode voltage clamps. Cells were clamped to a common holding potential; when one cell was stepped to a

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Abbreviations: Bt_2-cAMP, N^6,O^2-dibutyryl-cAMP; 8Br-cAMP, 8-bromo-cAMP; g_j, gap junctional conductance.

command potential, gap junctional conductance (g_j) was measured as the current in the other cell divided by the transjunctional voltage (20, 21). Drugs were applied to the bath with a peristaltic pump at a flow rate of ≈0.5 ml/min (dish volume, 1 ml).

^{32}P Labeling and Immunoprecipitation of the 27-kDa Phosphoprotein. Liver cells (10^6 cells per 250 μl) were incubated in a phosphate-free Krebs buffer [containing 150 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$ (pH 7.4)] for 45 min at room temperature (20°C). Cells were centrifuged (200 × *g* for 5 min in a centrifuge at 4°C), resuspended, and incubated at 37°C for 90 min in Krebs buffer containing 10 mM pyruvate and ^{32}P (50 μCi per 250 μl of cell suspension; 1 Ci = 37 GBq). Cells were then incubated in the absence or presence of 8Br-cAMP (1 mM final concentration) for different periods of time, following which $NaDodSO_4$ (1% final concentration) was added and the solution was mixed gently using a Vortex until a homogeneous solution was obtained. Each sample was incubated for 5 min with an equal volume of buffer A (5% Nonidet P-40/20 mM EDTA/250 mM NaCl/20 mM sodium pyrophosphate/100 mM NaF/100 mM phosphate buffer, pH 7.4). To minimize nonspecific absorption to protein A, 15 μl of protein A-bearing *Staphylococcus* [Pansorbin, 10% (wt/vol) in buffer A containing 2.5% bovine serum albumin] was added to the samples and incubation was carried out for an additional 10 min at room temperature. The samples were centrifuged for 4 min in a Beckman Microfuge to obtain the supernatant. Each sample was divided into two aliquots, which were incubated for 60 min at room temperature with 15 μl of either immune antiserum specific for the 27-kDa protein (11) or 15 μl of preimmune serum. Protein A-bearing *Staphylococcus* suspension (50 μl) was added to each sample and incubated for an additional 30 min. The samples were centrifuged for 4 min in a Microfuge, the supernatants were aspirated, and the pellets, containing the antibody–antigen complex, were washed in buffer A and centrifuged again. The resulting pellets were resuspended in $NaDodSO_4$-containing solution (22) and aliquots were subjected to 12.5% $NaDodSO_4$/PAGE. The gels were dried and autoradiographed. Gel pieces corresponding to immunoprecipitated 27-kDa phosphoprotein were cut from the dried gels and counted by liquid scintillation spectrometry. The same gel pieces were then used for phosphoamino acid analysis as described (23). Briefly, the gel pieces were trypsinized and the eluted material was subjected to 2 hr of hydrolysis in 6 M HCl. Phosphoamino acid standards were added, the mixture was subjected to electrophoresis on cellulose sheets, and autoradiography was used to localize the labeled phosphoamino acid.

Phosphorylation of the Purified Gap Junctions. Gap junction membrane was purified by means of the alkali-extraction procedure described (11). The phosphorylation reaction mixture contained [γ-^{32}P]ATP (10^6 cpm/nmol), 10 mM Hepes (pH 7.6), 10 mM Mg acetate, 1 mM EGTA, bovine serum albumin (1 mg/ml), 1 mM dithiothreitol, gap junction membrane (15 μg of protein), and catalytic subunit of the cAMP-dependent protein kinase (4.6 μg/ml). The reaction was run at room temperature (20°C) in a 50 μl final vol for various times (5, 10, 15, 30, and 60 min). The reaction was stopped by the addition of 5 μl of $NaDodSO_4$-containing solution (22). Samples were resolved and subsequently analyzed as described above for the intact cells. In addition, tryptic fingerprints of the 27-kDa major gap-junctional protein were obtained by standard techniques (23).

RESULTS

Membrane-Permeant cAMP Derivatives Increase g_j. Bath application of Bt_2-cAMP or 8Br-cAMP (1 mM) to pairs of hepatocytes led to a gradual increase in g_j in pairs of cells measured under voltage-clamp conditions (illustrated for Bt_2-cAMP in Fig. 1*A*). The increases in g_j caused by these cAMP derivatives reached plateau values within 5–10 min and were partially reversed by prolonged rinsing in normal medium (not shown). Maximal increases in g_j after 5–10 min of exposure to 1 mM Bt_2-cAMP were smaller than those obtained with 8Br-cAMP (Fig. 1*B*), perhaps reflecting differences in membrane permeability to these compounds or possibly due to acidification accompanying Bt_2-cAMP treatment.

Cytoplasmic pH is known to affect g_j in hepatocytes and other tissues (1). cAMP derivatives might alter cytoplasmic pH either through metabolic changes or, in the case of Bt_2-cAMP, by enzymatic hydrolysis of the ester. To determine whether cAMP derivatives changed cytoplasmic pH, the cytoplasmic pH of one cell was monitored with a pH-sensitive microelectrode while Bt_2-cAMP (Fig. 1*A*) or 8Br-cAMP was added. Cytoplasmic pH decreased 0.1 to 0.3 pH units upon each addition of Bt_2-cAMP and then recovered rapidly when the cells were rinsed; the pH changes were too small to affect g_j and the increase in g_j continued throughout this period. With 8Br-cAMP, no substantial change in cytoplasmic pH was found (not shown).

Glucagon Increases g_j and This Effect Is Prevented by Walsh Inhibitor. Bath application of glucagon (10 μg/ml) to pairs of

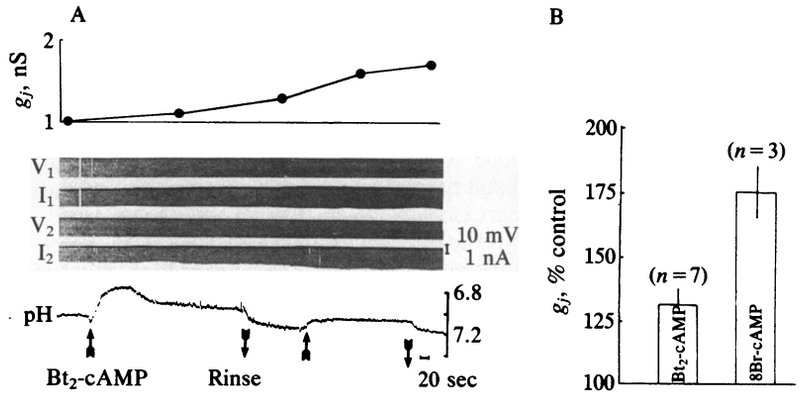

FIG. 1. Electrical coupling between pairs of hepatocytes measured under voltage clamp is increased by bath application of Bt_2-cAMP or 8Br-cAMP. (*A*) A representative experiment. The g_j values plotted on the graph were calculated from the voltage (V) and current (I) measurements (V_1, I_1, V_2, I_2). Intracellular pH (bottom trace) was monitored with a pH-sensitive microelectrode placed in cell 1. The arrows under the pH record indicate times at which 1 mM Bt_2-cAMP was applied or washed out. (*B*) Percent maximal increase in g_j induced by Bt_2-cAMP and 8Br-cAMP (1 mM, obtained within 5–10 min). Vertical lines indicate SD; *n*, number of experiments.

hepatocytes increased g_j to a maximal value within 2–3 min (Fig. 2*A*), and the effect was generally completely reversed by rinsing the cells in normal saline. After Walsh inhibitor was injected intracellularly into both cells or when patch pipettes containing Walsh inhibitor were used for recordings, glucagon application was ineffective (Fig. 2*A Bottom*). In one series using nine different cell pairs, bath application of glucagon in the absence of Walsh inhibitor led to a substantial increase in g_j, a maximum response occurring within 3 min (Fig. 2*B*). When pipettes containing Walsh inhibitor were subsequently used on seven cell pairs, g_j did not change within the first 3 min (Fig. 2*B*). In several of these experiments (3/7), g_j was slowly reduced so that by 5 min it was ≈93% of control value (95%, 92%, 92%).

The 27-kDa Gap Junction Polypeptide Is a Phosphoprotein Whose Phosphorylation in Intact Cells Is Elevated by Increasing the Intracellular cAMP Concentration. If g_j is increased as a consequence of direct phosphorylation of the 27-kDa gap junction protein, membrane-permeant cAMP derivatives should increase the phosphorylation of the 27-kDa protein within the time course of the g_j change (Figs. 1*A* and 2*A*). To determine whether phosphorylation of gap junction protein occurs in liver, partially dissociated liver cells were labeled with ^{32}P as described in *Methods*. The cells were preincubated in ^{32}P for 1 hr, then incubated in the absence or presence of 1 mM 8Br-cAMP for various times, following which the 27-kDa protein was immunoprecipitated. The autoradiogram of the 12.5% NaDodSO$_4$/polyacrylamide gel used to separate the immunoprecipitated proteins showed that the 27-kDa protein was phosphorylated in the absence of 8Br-cAMP (Fig. 3*A*). ^{32}P incorporation was increased ≈1.6-fold by 8Br-cAMP within 60 min (Fig. 3*A*). Without the addition of 8Br-cAMP, phosphorylation of the 27-kDa protein did not change within 30 min following the prelabeling period, but when 8Br-cAMP was supplied a gradual increase in the phosphorylation was seen (Fig. 3*B*). Phosphoamino acid analysis revealed that serine was the only residue phosphorylated (not shown).

The 27-kDa Protein of Isolated Gap Junctions Is Phosphorylated with Low Stoichiometry by the Catalytic Subunit of cAMP-Dependent Protein Kinase. Alkali-extracted gap junctions from adult rat liver were incubated for various times with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP. Autoradiographs of NaDodSO$_4$/polyacrylamide gels showed that the 27-kDa protein was phosphorylated, as was a 47-kDa band, which apparently is a dimer of the 27-kDa polypeptide (11) (Fig. 4*A*). The incorporation of ^{32}P into the 27-kDa protein reached a plateau after ≈15 min (Fig. 4*B*). The maximal stoichiometry of phosphorylation was 0.025 mol/mol. Maximally phosphorylated 27-kDa phosphoprotein was used for phosphoamino acid analysis and tryptic fingerprinting. The phosphoamino acid analysis revealed that serine was the only amino acid residue phosphorylated (Fig. 5*A*). One major phosphopeptide was obtained after digestion with trypsin (Fig. 5*B*).

In an attempt to increase ^{32}P incorporation, the isolated gap

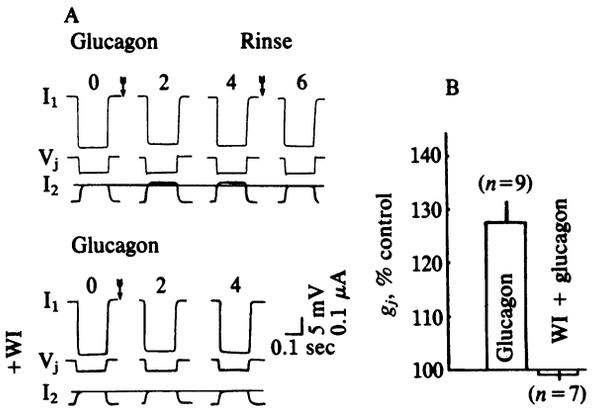

FIG. 2. Glucagon increases g_j, an effect blocked by Walsh inhibitor. (*A*) Representative regions of chart records from single experiments involving glucagon application with or without prior injection of Walsh inhibitor (WI). I_1, current in cell 1; V_j, voltage in cell 1, the transjunctional voltage. I_2 with reference line, current in cell 2 equal to junctional current since potential of cell 2 was held constant. Time is indicated in min. When glucagon (10 μg/ml) was added (first arrow, upper record) junctional current was increased, and this effect was reversed when glucagon was removed (second arrow, upper record). After Walsh inhibitor was injected into both cells of a pair through the recording pipettes, the glucagon effect was blocked (lower record). (*B*) Mean of normalized maximal increase in g_j with glucagon alone and glucagon after Walsh inhibitor. Vertical lines indicate SD; n is the number of experiments.

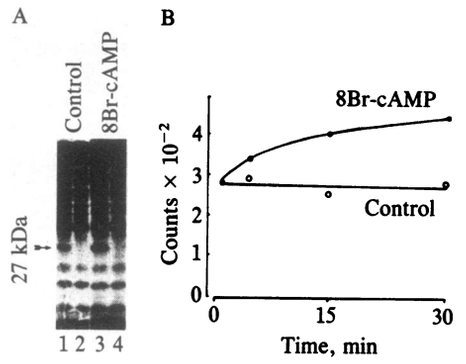

FIG. 3. Phosphorylation of 27-kDa gap junction protein in partially dissociated cells. (*A*) Autoradiogram of polyacrylamide gels after immunoprecipitation of the 27-kDa gap junction protein following incubation of cells with $^{32}P_i$ for 60 min in the presence (lanes 3 and 4) and absence (lanes 1 and 2) of 8Br-cAMP. Immunoprecipitation was carried out with antibody to the 27-kDa protein (lanes 1 and 3) and with preimmune serum (lanes 2 and 4). The high background observed in all lanes could be reduced by carrying out two cycles of immunoprecipitation, which did not change phosphorylation of the 27-kDa protein (not shown). (*B*) Time course of ^{32}P incorporation into the 27-kDa protein (counts in each sample per 5 min) in the absence (control) or presence (8Br-cAMP) of 1 mM 8Br-cAMP.

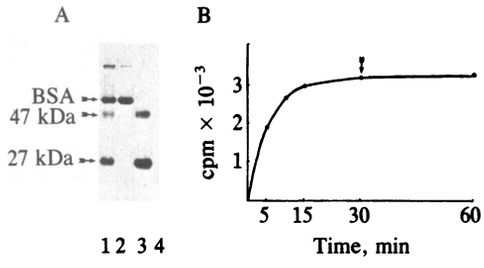

FIG. 4. Phosphorylation of the 27-kDa protein in isolated gap junctions. (*A*) Lanes 1 and 2 show protein staining of the NaDodSO$_4$/polyacrylamide gel. Lane 1 contains the catalytic subunit of cAMP-dependent protein kinase, bovine serum albumin (BSA) (to stabilize enzyme activity), and purified liver gap junctions. Prominent bands are at 27 kDa, the major gap junction protein; 47-kDa, a dimer of the 27-kDa protein; and ≈68-kDa, bovine serum albumin. Lane 2 contains the same reagents but no gap junctions. The 27- and 47-kDa bands are absent. Lanes 3 and 4 are the respective autoradiographs of lanes 1 and 2. The gap-junction bands are labeled. (*B*) The time course of 27-kDa protein phosphorylation by the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation was measured in terms of radioactivity of the junctional protein isolated by gel electrophoresis as on the left. At the arrow, additional enzyme was added in order to demonstrate that the reaction had gone to completion.

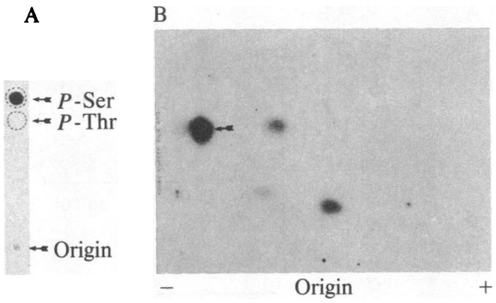

FIG. 5. Analysis of the 27-kDa protein phosphorylated in preparations of purified gap junctions and isolated by $NaDodSO_4$/PAGE. (*A*) Autoradiograph showing the phosphoamino acid analysis. A single spot appears at the location of phosphoserine (origin at the bottom). Dashed lines indicate position of ninhydrin stain of standard phosphoamino acids; phosphoserine (*P*-Ser) and phosphothreonine (*P*-Thr). Tryptic fingerprint of the 27-kDa protein. The products of the tryptic digestion were separated on thin-layer cellulose plates by electrophoresis followed by ascending chromatography. A single phosphopeptide predominates (arrow).

junctions were subjected to the following treatments before the phosphorylation reaction: (i) dephosphorylation using phosphoprotein phosphatase, (ii) treatment with detergent (0.2% Triton X-100), and/or (iii) sonication (20 sec) in a microbath sonicator. None of these treatments caused a marked change in ^{32}P incorporation (results not shown). Phosphorylation of synapsin I by the catalytic subunit of cAMP-dependent protein kinase (23) was not affected by addition of the purified gap-junction preparation, suggesting that this preparation does not contain an inhibitor of cAMP-dependent protein kinase (data not shown).

DISCUSSION

The structure and physiology of gap junctions in these liver cell preparations have been characterized (24). The present experiments indicate that g_j between isolated pairs of liver cells can be enhanced by increasing the intracellular concentration of cAMP, either with permeant derivatives of cAMP or through the effect of glucagon. The onset of the effects of Bt_2cAMP, 8Br-cAMP, and glucagon is rapid. The cAMP derivatives increase cAMP intracellularly by diffusion across the cell membrane, while glucagon binds to a membrane receptor with high affinity (25) and activates adenylate cyclase (26). The outcome of both treatments is to increase intracellular cAMP, which activates cAMP-dependent protein kinase (26) and increases protein phosphorylation (27, 28). Inhibition of the glucagon effect by Walsh inhibitor suggests that the effect of glucagon is mediated through the catalytic subunit of cAMP-dependent protein kinase. The effects of Walsh inhibitor also indicate that the increase in g_j involves activation of cAMP-dependent protein kinase rather than a direct cAMP action (e.g., binding of cAMP to the channel), as has been recently suggested for cGMP in photoreceptors (29).

The mechanism by which phosphorylation of the channels leads to increase in g_j is unknown, but presumably the number of open channels or the time they spend open is increased. Single-channel recording from reconstituted gapjunction membranes (30) may resolve this issue. The conductance in intact cells may result from a background state of phosphorylation, or channels not phosphorylated may have a reduced but finite conductance or probability of being open. Injection of Walsh inhibitor in the absence of glucagon treatment only occasionally caused a small decrease in g_j, suggesting the latter possibility.

The 27-kDa protein was found to be phosphorylated both *in vitro* and *in vivo*. The serine residue phosphorylated appears to be located in the cytoplasmic domain of the 27-kDa protein, since in intact cells only that part of the channel would have been accessible to the kinase. The stoichiometry of phosphorylation obtained using purified gap junctions was ≈0.025 mol/mol of the 27-kDa protein. This ratio is lower than would be expected if only a single site of the channel dodecamer were phosphorylated. Possibly during isolation of the gap junctions, the accessibility of the phosphorylatable residues is altered by partial denaturation or there is partial proteolysis of the protein since no protease inhibitors were used. Although the main protein component of gap junction between liver and lens is different (31), phosphorylation of the lens gap junction protein *in vitro* by cAMP-dependent protein kinase also gives a low stoichiometry of phosphorylation (32).

cAMP affects many other channels in biological membranes (e.g., see refs. 18 and 33–37), but in only a few cases has direct phosphorylation of the channel molecule been demonstrated (33, 34). Effects of cAMP on electrical coupling between cells of a variety of tissues have been reported (1–9); in most cases, the onset of the effect is hours or many minutes after application, and in one case hours were required for an effect of cAMP-dependent protein kinase (38). An exception is the dopaminergic uncoupling in retinal horizontal cells of fish and turtle, which appears to be mediated by increase in cAMP. In these cells, g_j decreases within 1 min of dopamine application and recovers over a period of 10–20 min (8, 9).

Our results show that the 27-kDa protein is phosphorylated in adult liver cells, consistent with the previous report of phosphorylation of the gap junction protein in cultured embryonic hepatocytes (39). In addition, we show that the phosphorylation is stimulated by cAMP and that this phosphorylation correlates with short-term increases in g_j.

The effects of cAMP on g_j reported here imply that cAMP and agents that elevate it, including various hormones and neurotransmitters, may quickly modify the strength of coupling between communicating cells. For excitable cells, the expected functional consequence is change in synchrony of coupled elements or in efficacy of electrotonic spread. For inexcitable cells, one would expect changes in buffering of intracellular ions and small molecules, where opening or closing gap-junction channels in effect would modulate cytoplasmic volume (capacity). The exocrine function of liver is one potential physiological target for this modulation. Many hepatic enzyme systems are phosphorylated via a cAMP-dependent kinase (27, 28, 40–42), causing, for example, increased glycogenolysis and gluconeogenesis (43, 44). The increase in g_j caused by cAMP would tend to equalize the distribution of small molecules (including cAMP: see refs. 45 and 46) among all the coupled cells. Thus, if coupled cells did not share receptors for hormones such as glucagon, increase in cAMP in one would increase gap-junction phosphorylation and increase spread of the response to other cells lacking the receptors. The net effect of this amplification could be increased glucose release in response to elevated circulating glucagon levels.

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