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Hormonal Regulation of Cell Junction Permeability: Upregulation by Catecholamine and Prostaglandin E_1

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Summary. By cellular activation with hormones, we test the proposition (Loewenstein, W.R., *Physiol. Rev.* **61**:829, 1981) that the permeability of cell junction is upregulated through elevation of the level of cyclic AMP. Cultured rat glioma C-6 cells, with β-adrenergic receptors, and human lung WI-38 cells, with prostaglandin receptors, were exposed to catecholamine (isoproterenol) and prostaglandin E_1, respectively, while their junctions were probed with microinjected fluorescent-labelled mono-, di-, and triglutamate. Junctional permeability, as indexed by the proportion of cell interfaces transferring the probes, rose after the hormone treatments. The increase in permeability took several hours to develop and was associated with an increase in the number of gap-junctional membrane particles (freeze-fracture electron microscopy). Such interaction between hormonal and junctional intercellular communication may provide a mechanism for physiological regulation of junctional communication and (perhaps as part of that) for physiological coordination of responses of cells in organs and tissues to hormones.

Key Words: cell junction permeability · cell-to-cell communication · membrane permeability · cell-to-cell membrane channels · gap junction · hormones · catecholamine · prostaglandin E_1 · cyclic AMP

Introduction

The present work was prompted by the findings that junctional permeability in various cultured mammalian cell types depends on the intracellular concentration of cyclic AMP ($[cAMP]_i$): the permeability rises when $[cAMP]_i$ is elevated by administration of exogenous cAMP, by phosphodiesterase inhibitor, serum deprivation, or downsteps of cell density; it falls when $[cAMP]_i$ is lowered by serum application or upsteps of cell density. The rise of permeability takes several hours to develop and goes hand in hand with a (protein-synthesis dependent) increase in the number of gap-junctional membrane particles (Flagg-Newton & Loewenstein, 1981; Flagg-Newton, Dahl & Loewenstein, 1981). A dramatic demonstration of this cAMP effect was provided by Cl-1D cells, a cell type that, in ordinary confluent culture, makes no permeable junctions: the cells developed permeable junctions when they were supplied with exogenous cAMP or subjected to choleragen or other conditions elevating $[cAMP]_i$ (Azarnia, Dahl & Loewenstein, 1981).

These findings led to the hypothesis that junctional permeability, P_j, is somehow regulated by the level of cAMP according to the schemes:

$$[cAMP]_i\uparrow \rightarrow P_j\uparrow$$
$$[cAMP]_i\downarrow \rightarrow P_j\downarrow$$

where $\uparrow$ and $\downarrow$ denote increases and decreases of base levels (Flagg-Newton & Loewenstein, 1981; Loewenstein, 1981). On this basis, one would expect an upregulation of junctional permeability when one stimulates cells with hormones elevating $[cAMP]_i$. To test this point, we chose a cell type with β-adrenergic receptors – the rat glioma (astrocytoma) cell line C-6 – and a cell type with prostaglandin E_1 receptors – the human lung fibroblast line WI-38. Both are known to respond to the hormones with large $[cAMP]_i$ elevations (Gilman & Nirenberg, 1971; Kelly & Butcher, 1974; Kurtz, Polgar, Taylor & Rutenburg, 1974).

We find that the junctional permeability of these cells is indeed enhanced by hormone action, and that this enhancement is associated with proliferation of gap-junctional membrane particles. A preliminary report of the results dealing with permeability has appeared (Radu & Loewenstein, 1982).

Materials and Methods

Cell Cultures

Rat glioma C-6 cells (Benda, Lightbody, Sato, Levine & Sweet, 1968; Benda, Someda, Messer & Sweet, 1971) were grown in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Human lung WI-38 cells (Hayflick

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& Moorhead, 1961; Saksela & Moorhead, 1963; Hayflick, 1965) were grown in Eagle's basal medium with 10% bovine serum. The medium was renewed every 2–3 days, but not during the experiments nor during the 1-day period before the experiments, to avoid effects of serum on junctional permeability (Flagg-Newton & Loewenstein, 1981). The cultures were in plastic dishes (Falcon, 35 mm) in an incubator, 5–10% CO_2 in air, 37 °C.

Hormones

For treatment, a 10-µl stock solution of the hormone was added to the culture medium (2 ml) in the dishes. The same volume of culture medium alone was added in the controls. The cells were in the hormone medium throughout the experiments, including the period of junctional permeability probings. Hormone stock solutions: L-isoproterenol *A* bitartrate (N-isopropylnorepinephrine, Chemalogue) 10 or 20 mM in water; prostaglandin E_1 methyl ester, in 10% ethanol and 90% water or phosphate buffer (pH 7.2), plus 0.2 mg/ml Na_2CO_3. The ethanol, at the final concentration used, had by itself no effect on junctional communication.

Junctional Permeability Probing

Junctional permeability was probed with the fluorescent (lissamine rhodamine B-labelled) glutamic acid series listed in Table 1. Preparation and purification of these tracers is described in Simpson, Rose and Loewenstein (1977) and Socolar and Loewenstein (1979). During the probings, limited to periods of up to 30 min for any given culture dish, the cells were at about 25 °C, exposed to room air. (At all other times, except for a few minutes when the hormone solutions were added to the dishes, the cultures were in the incubator.)

The tracers were injected into the cells with the aid of an intracellular microelectrode, carried by clipped sinusoidal currents, 5×10^{-8} amp, 0.5–2 kHz, in trains of 0.3 sec duration with 2–3 sec intervals. Total injection time, 10–20 sec. (Currents of this sort were also helpful for smooth penetration of the microelectrode into the cell; the current was turned on as soon as the electrode made contact with the cell membrane.) The intracellular spread of the tracer was observed by incident-light fluorescence microscopy during the injection. The exciting light (560 nm) was then blocked, to be re-admitted only after 3 min for a count of the number of fluorescent cell neighbors. This protocol minimized photobleaching and harm to the cells. The fluorescence intensity in the injected cell (source cell) was $\gg$ detection threshold, well within the range of source concentration where, due to saturating interaction of the charged probes with the cell-to-cell channels, junctional flux apparently is sensibly independent of chemical driving force and dependent on junctional permeability alone (Flagg-Newton & Loewenstein, 1980; Loewenstein, 1981).

Table 1. Junctional probes

	Charge[a]	mol wt
LRB-Glu	2	688
LRB-Glu-Glu	3	817
LRB-Glu-Glu-Glu	4	946

[a] Net negative charge.
LRB = Lissamine rhodamine B (red fluorescent); Glu = glutamic acid. For preparation, characteristics, and purity of the probes, *see* Simpson et al. (1977) and Socolar and Loewenstein (1979); for molecular dimensions, *see* Schwartzmann et al. (1981).

The membrane potential of the injected cell was continuously monitored (through the injecting microelectrode) throughout the 3-min period. Potentials ranged −20 to −50 mV. Cells with smaller potentials were rejected.

All experiments were "blind": During the permeability measurements the experimentor did not know which dishes had been hormone-treated. The hormone and control solutions were prepared and coded by an assistant and – in all experiments, except series C, Table 2 – also applied to the cultures by the assistant.

Fluorescence Loss

Intracellular fluorescence of the tracers was measured as a photocurrent, *F* (proportional to fluorescence intensity), by means of a photodiode system onto which the cells were projected through the microscope (Flagg-Newton et al., 1981). The measurements of fluorescence loss were taken on single cells, without neighbors in contact; the excitation pulses were of 5-sec duration at 1/min or 0.5/min. In the consecutive runs before and after treatment, the cells were injected to approximately matched time-zero *F* levels (Fig. 5).

Intracellular cAMP

Intracellular cAMP was determined by radio-immunoassay (Steiner, Parker & Kipnis, 1972). $8–14 \times 10^6$ cells were used for each assay. Cells were harvested with the aid of a rubber policeman, without the use of trypsin.

Electron Microscopy

For freeze-fracture the cells were grown on dishes with thin bottoms (Falcon, "film liner"; bottom, ~0.15 mm thickness). The procedures for fixation, freeze-fracture, electron microscopy, gap junction quantitation, and estimation of *equivalent cell area* are described elsewhere (Flagg-Newton et al., 1981), but the unit area of the grid mesh used for scanning of the present replica samples was 3,900 μm^2.

Statistical Treatment

Statistical confidence levels of the junctional permeability data were determined by the nonparametric, Mann-Whitney U method (one-tailed) (Sohkal & Rohlf, 1969); those of the electron microscopic data, by standard *t* test.

Culture Parallelism

In all series of experiments, the permeability tests and their respective controls were carried out on subcultures from the same stock that had been subjected to the same feeding schedules, had undergone the same number of passages, and had the same cell densities. This close parallelism was important, because junctional permeability varies with the serum content of the medium, with cell density and, at least in some cell types, with the number of cell passages (Flagg-Newton et al., 1981; Flagg-Newton & Loewenstein, 1981).

Results

Junctional Permeability

The series of fluorescent glutamic acid molecules of Table 1 has several advantageous features for probing changes of junctional permeability: (a) The molecules are too large to permeate the ion

channels of nonjunctional membrane (Flagg-Newton, Simpson & Loewenstein, 1979). (b) They permeate the cell-to-cell channel of junctional membrane, yet they are interactive enough with it so that their throughput saturates with moderate source concentrations, like those we work with (Flagg-Newton & Loewenstein, 1980; Loewenstein, 1981). One can thus compare junctional transfer in different conditions without exactly having to match the concentrations of the cells injected with the probes (source cells). (c) All three molecules have the same width, 16 Å (the permeation-limiting, abaxial dimension), and they carry negative charges that increase with their length. Concomitantly with their increasing charge and length, their permeance for the cell-to-cell channel – which discriminates against negative charges (Flagg-Newton et al., 1979) – falls off steeply from Glu to Glu-Glu-Glu, but without their ever exceeding the 16–20 Å steric limit for channel permeation (Schwartzmann et al., 1981).

Such a uniformly graded series offers a convenient choice of probes for setting up threshold conditions for junctional transfer – and it is near threshold where increases in transfer are most sensitively detected. Thus, in multicell systems, these probes allow one to conveniently and sensitively detect increases in junctional permeability as increases in the number of cell interfaces that transfer the probes.

In preliminary runs we first established, for each cell type, which of the (permeant) molecules was closest to transfer threshold in control conditions. This turned out to be LRB-Glu for the C-6 glioma cells and LRB-Glu-Glu-Glu for WI-38 lung cells. These molecules (in the case of C-6, also the next member in the series) were then adopted as probes for the experiments. The number of fluorescent cells in contact with the source cell – the number of transferring cell interfaces of the source cell (hereafter the *number of transferring cell interfaces*) – served as index of permeability. To minimize variation of junctional permeability due to variation of cell density (Flagg-Newton et al., 1981), the probings were carried out at equal cell densities for a given cell type, on (single-layer) cell topologies where the number of cell interfaces (cells in contact as seen in phase contrast) of any given injected cell varied less than 4% (SD). This also made it unnecessary to normalize the data for the total number of cell interfaces.

Catecholamine-sensitive Cells. C-6 Glioma Cells. Treatment of C-6 cells with isoproterenol caused increase in junctional permeability. Figure 1 gives an example, with LRB-Glu-Glu as the probe:

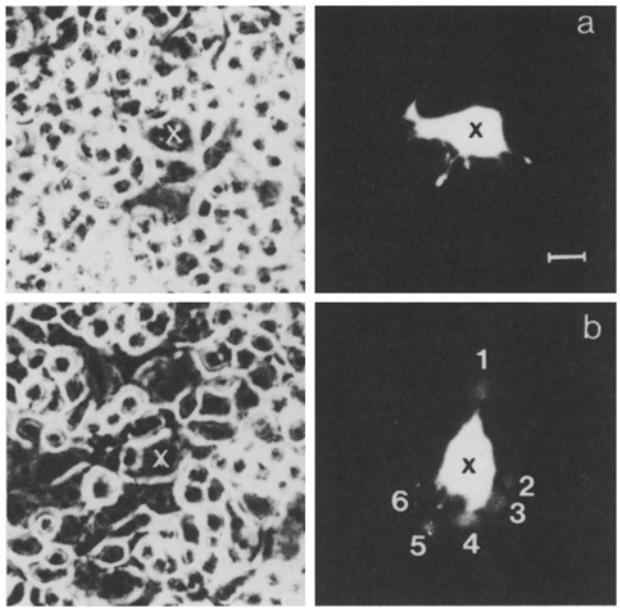

Fig. 1. Enhancement of junctional cell-to-cell transfer by catecholamine treatment of C-6 glioma cells. Fluorescent LRB-Glu-Glu is injected into a cell (*x*) in control condition (*a*) and 7.1 hr after application of 50 μM isoproterenol (*b*). The injected cell has seven contiguous neighbors in each case, six of which exhibit the fluorescent tracer in the treated condition, but none in control. (In the photographs the tracer shows up in the thickest regions of the neighbors; however, in direct microscope views, a dark-adapted observer generally can see the tracer everywhere in these cells.) Confluent culture. *Left,* phase contrast micrograph; *Right,* darkfield. Calibration, 15 μm

whereas none of the interfaces of the cell injected with the probe transferred it in the control condition, several did so in the treated condition (50 μM). We tested in this way an estimated total of 55 and 80 cell interfaces in the control and hormone-treated condition, respectively, at a cell density of 1.1×10^5 cells/cm^2 (superconfluent). In half of the injection trials the number of transferring interfaces rose within 7.75–8.25 hr of the treatment above the zero level of the controls, on the average to two interfaces. Figure 2*B* gives the frequency distribution of the number of transferring interfaces of a representative run, and Table 2, the data detail.

An increase in junctional permeability by the hormone was also manifest with Glu as the probe, although this molecule was above control transfer threshold in a proportion of the trials. We carried out three independent series of experiments, testing an estimated total of 245 and 250 cell interfaces in the control and treated condition (50–100 μM), respectively, each series at the same cell density (superconfluent range). The mean number of transferring junctions increased three- to 17-fold within 7.25 to 8.25 hr of the hormone treatment; the statistical confidence levels ranged <0.001 to

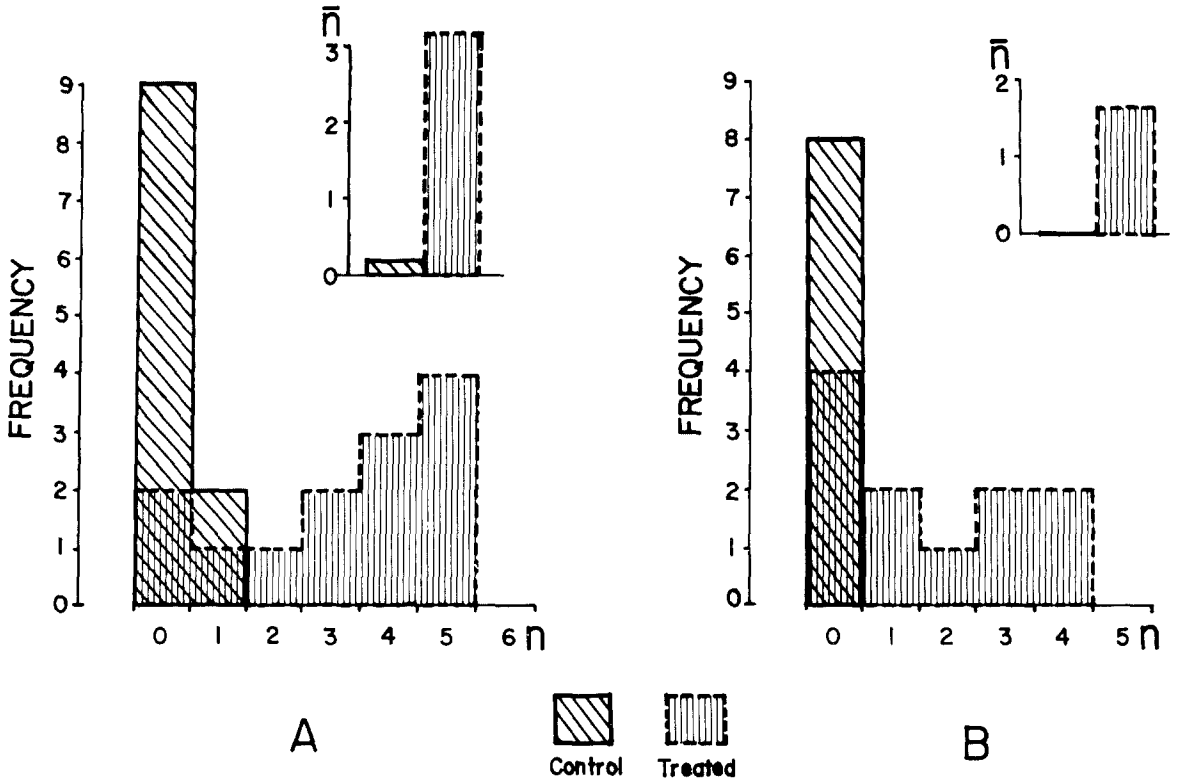

Fig. 2. Frequency distribution of transferring C-6 cell interfaces before and after hormone treatment. Two representative runs with LRB-Glu (*A*) and LRB-Glu-Glu (*B*) as probes, in controls and, in parallel subcultures, after a 6.75–8.25 hr exposure to isoproterenol (100 μM in *A;* 50 μM in *B*). *Abscissae:* the number (*n*) of interfaces transferring the probes in the various injection trials (each injection trial tests 6–7 interfaces); *ordinates:* their frequency. Data detail in Table 2; *A* and *B* correspond, respectively, to series A and D of that table. Cell density, 1.1×10^{-5} cell/cm^2. *Top insets:* mean number ($\bar{n}$) of transferring interfaces per injected cell (zero in the controls of *B*)

Table 2. Effect of isoproterenol on junctional transfer. C-6 Glioma Cells

Probe	Series	Condition	Treatment time (hr)	Number of transferring cell interfaces									
				Mean[a]	*P*	Frequency[b]							
						0	1	2	3	4	5	6	7	8
Glu	A	Control		0.18 (11, 2)	<0.001	9	2	0	0	0	0	0	0	0
		Treated, 100 μM	6.75–7.25	3.15 (13, 2)		2	1	1	2	3	4	0	0	0
	B	Control		0.73 (11, 2)	<0.05	7	2	0	2	0	0	0	0	0
		Treated, 100 μM	9.0–9.5	2.5 (10, 2)		3	0	1	2	3	1	0	0	0
	C	Control		1.1 (13, 4)	<0.01	6	4	2	0	0	0	1	0	0
		Treated, 50 μM	7.25–7.75	3.5 (13, 4)		3	1	1	1	1	2	3	0	1
Glu-Glu	D	Control		0 (8, 3)	<0.025	8	0	0	0	0	0	0	0	0
		Treated, 50 μM	7.75–8.25	2.0 (12, 4)		4	2	1	2	2	0	1	0	0

All determinations in the control and treated condition of each series are on parallel subcultures at the same cell densities and at the same times. Series A and B are parallel subcultures. Series A and D correspond to Fig. 2*A* and *B*, respectively.

[a] Mean number of transferring cell interfaces, i.e., the number of fluorescent neighbors of the injected cell; in parentheses, in the following order: the number of injection trials and the number of culture dishes. Each injection trial tests 6–8 cell junctions. *P*, the statistical confidence level of the difference between data in the control- and treated condition.

[b] Frequency distribution of the number of transferring junctions from 1 to the maximum 8 fluorescent junctions for each injection trial.

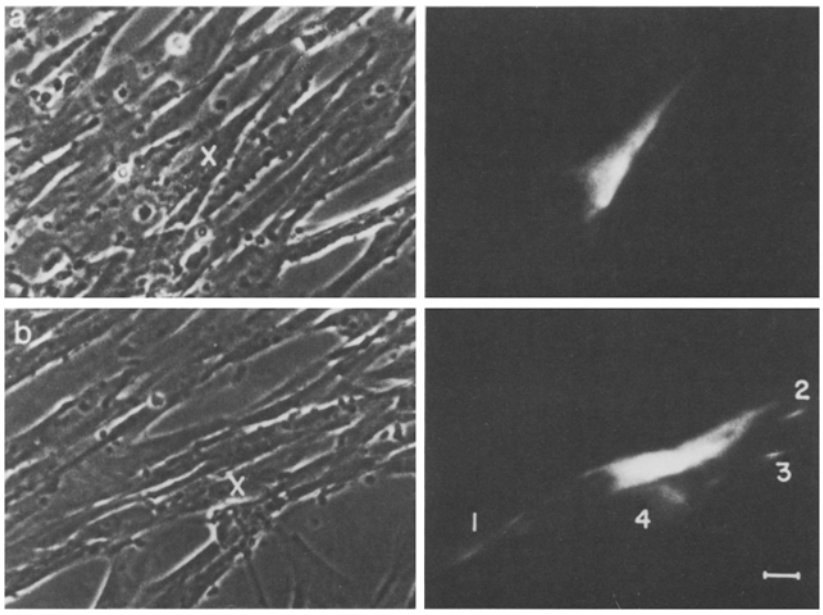

Fig. 3. Enhancement of junctional transfer by prostaglandin E_1 in WI-38 lung cells. Junctional probe, LRB-Glu-Glu-Glu. Injected cell, *x*. (*a*): control; no transfer. (*b*): 7.25 hr after application of 17 μM prostaglandin E_1; transfer to four neighbors

<0.05 in the three series. Figure 2*A* gives the frequency distribution of the transferring junctions in one series, and Table 2, the data of all three.

The effect of the hormone took several hours to develop. In a series of experiments testing junctional transfer 4.5, 5.75, 7 and 8 hr after hormone application, we found a significant increase in the number of transferring junctions at the last two time points. This result guided our choice of the times for permeability testing in the experiments of Table 2. The 5.75–7 hr time, however, is by no means a true time of onset of the effect; it is merely the time when a statistically clear effect was obtainable with a manageable number of permeability determinations. The actual lag time may have been shorter, perhaps about 5 hr.

Prostaglandin E_1-Sensitive Cells. WI-38 Lung Cells. Treatment of the WI-38 cells with prostaglandin E_1 led to a similar increase in junctional permeability. These cells have a higher junctional permeability to start with than the C-6 cells. Nevertheless, when probed with the long and charged LRB-Glu-Glu-Glu molecule, there was a clear increase in the number of transferring junctions after hormone treatment (Figs. 3 and 4). The mean number of transferring junctions increased about tenfold ($P<0.005$) within 7.25–7.75 hr of application of 17 μM prostaglandin E_1 (cell density, 7.5×10^4 cells/cm^2) (Table 3; Fig. 4).

Controls against Permeabilities not Mediated by Cell-to-Cell Channels. Two possible effects of the hormones needed to be considered in evaluating the enhancements of cell-to-cell transfer: (i) a reduction in nonjunctional membrane permeability and (ii) an increased frequency of (coarse) cytoplasmic bridges between incompletely divided or fused cells. The first possibility was examined by measuring the rate of the probes' fluorescence loss from the cells. In both cell types used, the rate of loss for LRB-Glu-Glu and LRB-Glu-Glu-Glu was $<1\%$/min, negligible compared with the $\leqq$25-sec junctional transit times (and even negligible for the 3-min waiting period used for scoring of transferring cell interfaces; *see* Methods)[1]. This alone made possibility *i* seem unlikely. It was ruled out altogether by determinations of the loss rates after the hormone treatments (LRB-Glu-Glu in C-6, 7 hr; LRB-Glu-Glu-Glu in WI-38, 7.25 hr); the rates turned out to be unchanged (Fig. 5; *see* legend for protocol).

Possibility *ii* could also be ruled out: fluorescent-labelled fibrinopeptide and serum albumin – which do not permeate cell-to-cell channels (Simpson et al., 1977) – were neither transferred in the control- nor in the hormone-treated condition in the two cell types.

[1] The probes also were not significantly taken up by the cells from the outside: there was no detectable fluorescence in the cells after bathing them for 12 min in medium with 10^{-4} M probe concentration, before or after hormone treatment. The present cells behave in this regard like a number of other mammalian cell types in culture (Flagg-Newton et al., 1979, 1981).

Table 3. Effect of prostaglandin E_1 on junctional transfer. WI-38 lung cells

Probe	Condition	Treatment time (hr)	Number of transferring cell interfaces						
			Mean	*P*	Frequency				
					0	1	2	3	4
Glu-Glu	Control		0.17 (12,3)	<0.005	10	2	0	0	0
	Treated, 17 μM	7.25–7.75	1.91 (13,3)		4	2	1	3	3

Cell density, 7.5×10^4 cells/cm^2. Notation as in Table 2.

In conclusion, the enhancements of cell-to-cell transfer by the hormones reflect increases in junctional permeability.

Intracellular Cyclic AMP

Endogenous cAMP. When the cells are continuously exposed to hormone-containing medium, as they were in all our experiments (*see* Methods), the $[cAMP]_i$ peaks within 15–30 min (*cf.* Kelly & Butcher, 1974). We measured the endogenous $[cAMP]_i$ at that time: it rose 74- to 110-fold in C-6, and seven- to eightfold in WI-38 cells (Table 4). These increases are comparable to those found earlier with these cells (Gilman & Nirenberg, 1971; Browning, Schwartz & Breckenridge, 1974; Kelly & Butcher, 1974; Kelly, Hall & Butcher, 1974; Kurtz et al., 1974; *see also* Schwartz & Passonneau, 1974).

Exogenous cAMP Application. The effect of the hormones on junction lagged several hours behind their peak effect on endogenous cAMP. At about 7 hr, when the rise in junctional permeability was statistically clear, the $[cAMP]_i$ may still have been somewhat elevated, but it was certainly well below peak (Fig. 6) (*see also,* Gilman & Nirenberg, 1971; Kelly & Butcher, 1974; Kurtz et al., 1974). Such a lag is, of course, not inconsistent with our working hypothesis, but it was now interesting to test whether a *short* pulse of cAMP (without hormone stimulation) would elicit the junctional effect on its own.

We examined this point by means of 10-min applications of 1 mM dibutyryl cAMP plus 1 mM caffeine to the outside of C-6 cells, bypassing their hormone receptors. This gave a six- to ninefold elevation of intracellular cyclic nucleotide concentration, a pulse that peaked within 30 min and declined to near base level within 35–50 min. (The intracellular cyclic nucleotide concentration here included monobutyryl cAMP, a more slowly degraded derivative of dibutyryl cAMP, in addition to cAMP.) The effect of such pulses on junctional permeability was comparable to that of the hormone: the mean number of LRB-Glu-transferring cell interfaces rose four- to sixfold in 7 hr (Fig. 6)[2].

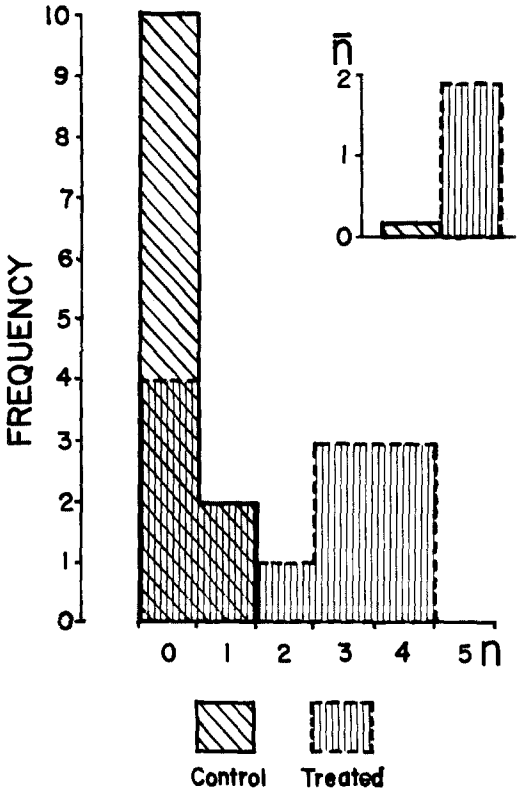

Fig. 4. Frequency distribution of transferring WI-38 cell interfaces. Junctional probe, LRB-Glu-Glu-Glu. 7.25–7.75 hr treatment with 17 μM prostaglandin E_1. Data detail in Table 3. Cell density 7.5×10^4 cells/cm^2. *Top:* mean number of transferring interfaces ($\bar{n}$) per injected cell. Notation as in Fig. 2

[2] The effect of these short pulses on junctional permeability is also not very different from the one produced by longer pulses of exogenous cyclic nucleotide in various other cell types (Flagg-Newton et al., 1981). Such short pulses, moreover, suffice to promote formation of gap junctions in Cl-1D cells that do not express them in basal conditions (G. Dahl, *unpublished*).

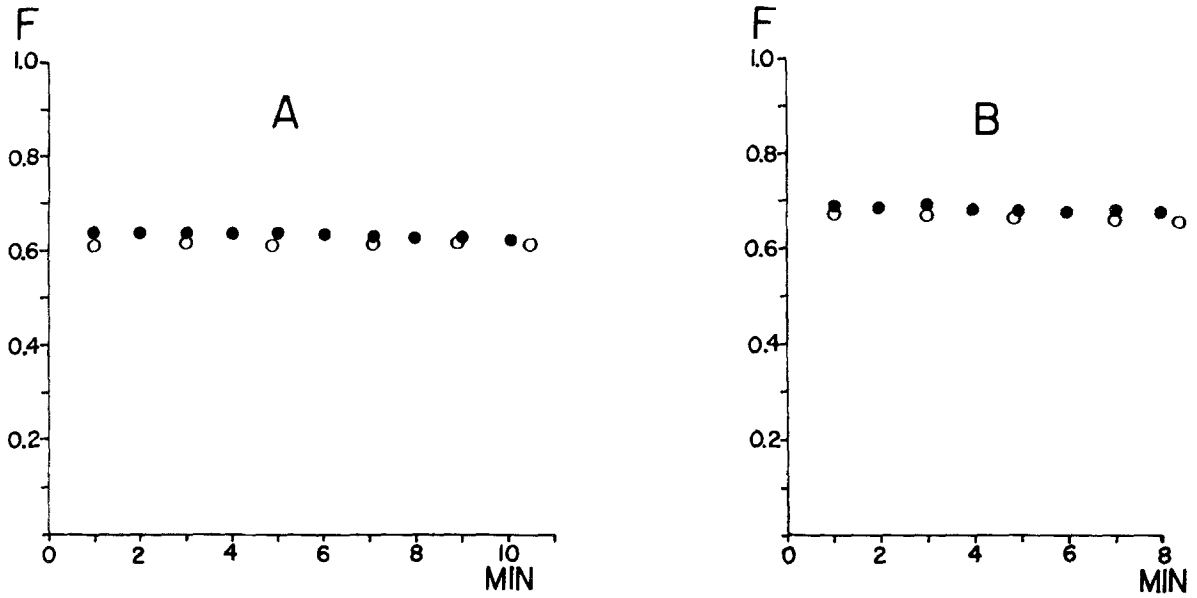

Fig. 5. Fluorescence losses. (*A*), Fluorescence intensity (*F*, photocurrent, relative units) of LRB-Glu-Glu in a C-6 cell; and (*B*), of LRB-Glu-Glu-Glu in a WI-38 cell as a function of time, before (black) and after (open circles) treatment with 50 μM isoproterenol and 17 μM prostaglandin E_1, respectively. Time zero is the end of the period of tracer injection for each run; the *F* plots begin 1 min thereafter, after the injection micropipettes had been withdrawn from the cells. The time relationships between *F* runs and hormone treatments were as follows: 'before-treatment' *F* runs end 15 min before the start of hormone treatments; 'after-treatment' runs start at 7 and 7.25 hr of isoproterenol and prostaglandin E_1 treatment, respectively. Fluorescence excitation with pulses of 5-sec duration

Table 4. Intracellular cAMP concentration

Cell type	cAMP (pmol/mg protein)	
	Control	Hormone-treated
C-6	14	1,036
	16	1,760
WI-38	42	294
	38	304

Assays 15–20 min after hormone application. Hormone treatments, 50 μM isoproterenol for C-6; 17 μM prostaglandin E_1 for WI-38. Densities (10^4 cell/cm^2): C-6 ~ 10–12; WI-38, 2–7.

Thus, the determination of the junctional effect, that is, the cellular reaction that gives rise to upregulation of junctional permeability, appears to occur within the half hour or so of intracellular cAMP elevation.

Gap-Junction Particles

Freeze-fractured junctions of C-6 cells exhibited a single kind of membrane differentiation in the electron microscope, the clusters of membrane particles of 'gap junction'. WI-38 cells had 'tight junctions' (*maculae occludentes*) in addition (Fig. 7).

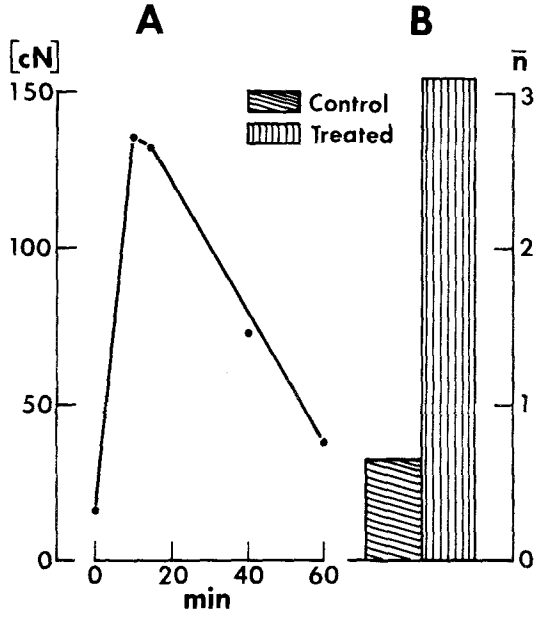

Fig. 6. A cyclic nucleotide pulse causes enhancement of junctional transfer in C-6 cells, in the absence of hormone stimulation. The cells were exposed for 10 min to medium containing 1 mM dibutyryl cAMP plus 1 mM caffeine; the intracellular cyclic nucleotide concentration ([*cN*]) is determined concurrently; junctional transfer is determined immediately before the exposure – the time zero and control for both determinations – and at 7 hr. (*A*): A plot of [*cN*], the combined concentrations of cAMP and monobutyryl cAMP (pmol/mg protein) (parallel subcultures) determined at the various times. (*B*): The mean number of transferring junctions ($\bar{n}$) at 7 hr

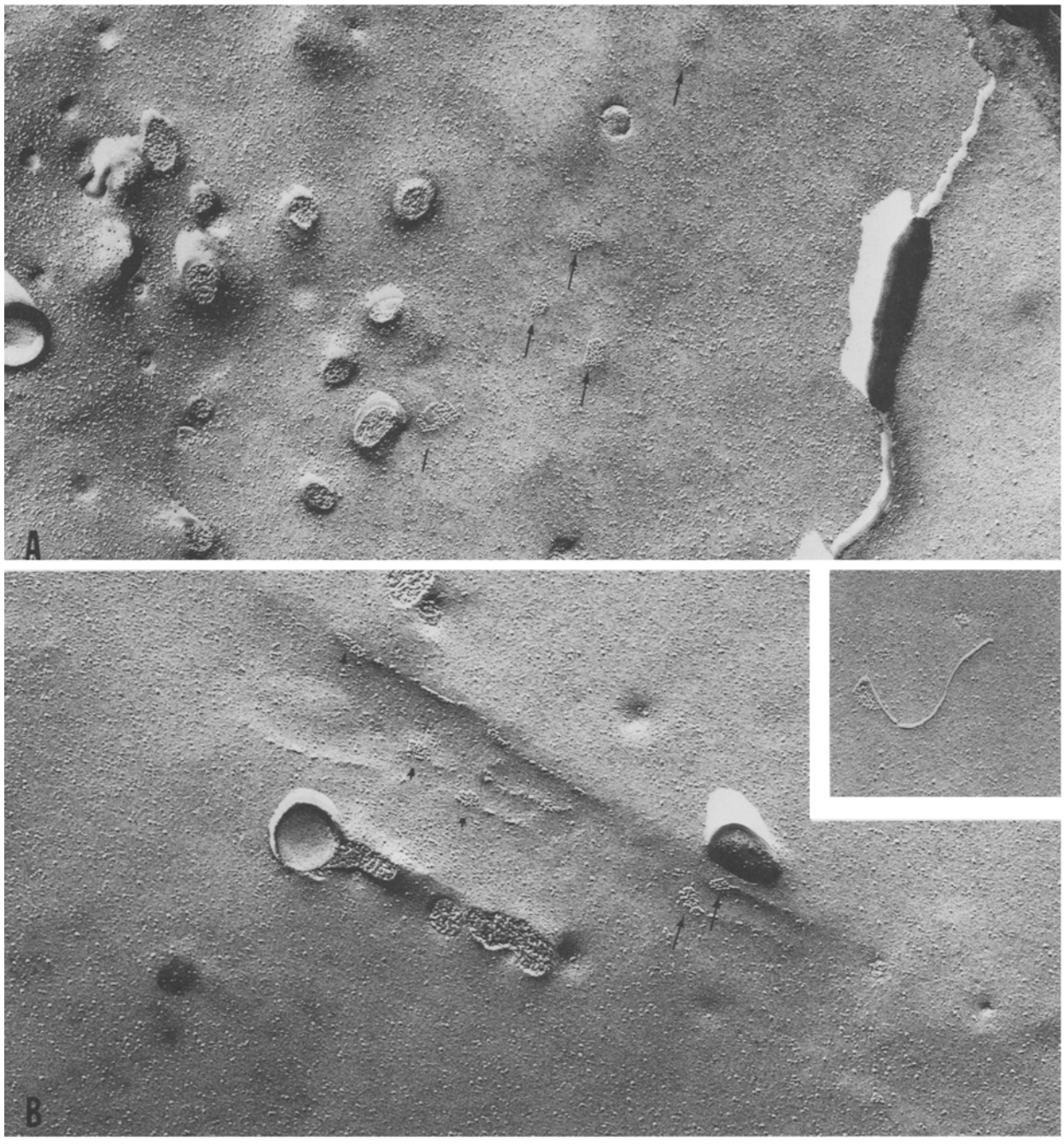

Fig. 7. Freeze-fracture electron micrographs (*A*) of C-6 and (*B*) WI-38 cells, hormone-treated. Typical clusters of gap-junctional membrane particles (arrows) are seen in both cell types. WI-38 cells also exhibit short and long (inset) tight junctional strands. The gap-junction frequency in these micrograph samples is much higher than average. 48,000 ×

The fractures went mainly along the cell surface membranes, thus offering a suitable material for quantitation of gap junction. We scanned a total of 58,500 μm^2 of C-6 cell membrane and 39,000 μm^2 of WI-38 membrane for each control and hormone-treated condition, and determined the frequency and the area of gap junction (the primary data) and the number of gap-junctional particles[3]. The last was calculated on the basis of the mean particle spacing in the clusters:

[3] It was necessary to scan such large areas because, as in other mammalian cell cultures (e.g., Flagg-Newton et al., 1981), the size and frequency of the gap junctions varied greatly in different cell surface samples.

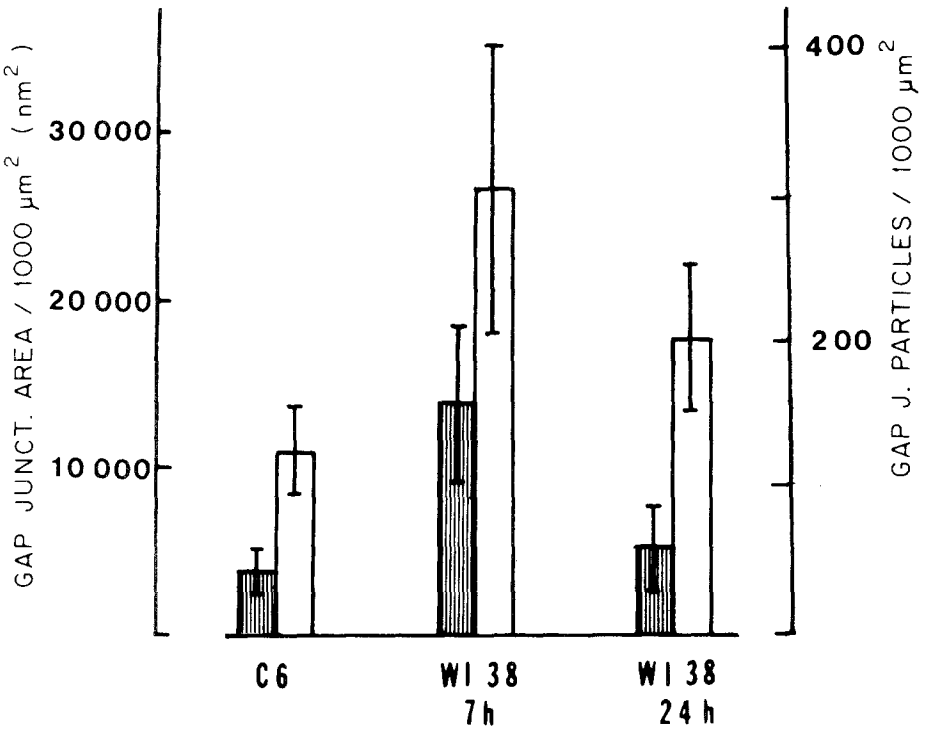

Fig. 8. Gap-junction area and particles in hormone-treated cells and controls (hatched). *Left ordinates:* total gap-junction area (nm^2) per 1,000 μm^2 of membrane area (the primary data). *Right ordinates:* the total number of gap-junction membrane particles for that area. Bars subtend the standard errors. *C-6 cells:* 7-hr treatment with 50 μM isoproterenol. Confidence level, *P*, control *vs.* treated <0.02. Same experiment as in Table 2*C* (electron microscopy and permeability determinations on parallel subcultures). The frequency distributions of the individual gap junction sizes in this experiment are given in Fig. 9 and the mean values in Table 5. *WI-38 cells:* 7 and 24-hr treatments with 17 μM prostaglandin E_1. *P*, control *vs.* treated $<0.2>0.1$ for 7 hr, and $<0.05>0.025$ for 24 hr. Frequency distributions of gap-junction sizes appear in Fig. 10 and mean sizes in Table 5. The 7-hr and 24-hr experiments were run on different batches of cells

9.3 ± 1.5 nm (SE; $n=50$ spacings in different randomly selected clusters) for C-6, and 9.3 ± 1.3 ($n=80$) for WI-38. Our identification criterion for gap junction was a minimum of 10 particles in a cluster[4]. For the electron microscopic studies, the cells were under the same experimental conditions as those on which the permeability studies were performed: in the case of C-6, the two studies were carried out in parallel, i.e., on parallel subcultures; in the case of WI-38, they were carried out on different batches of cells, at different times (but always control and tests for each study were on parallel subcultures).

C-6 Glioma Cells. The increase in junctional permeability went hand in hand with an increase in the number of gap-junctional particles in these catecholamine-sensitive cells. Thus, in series C (Table 2) the total number of gap-junctional particles per unit membrane area had about tripled after the 7-hr isoproterenol (50 μM) treatment (Fig. 8); it increased from 39 to 123 particles/1000 μm^2 (statistical confidence level, $P<0.02$). [Primary data; total gap junction area, $nm^2/1000\ \mu m^2$: control, $3{,}445.6 \pm 1{,}351.1$; treated, $10{,}674.6 \pm 2{,}588.7$ (SE).] This increase reflects an increase in the frequency of gap junction per unit area, mainly in frequency of the smaller gap junctions (clusters of up to 210 particles) (Fig. 9); the mean number of particles per cluster did not change significantly (Table 5).

WI-38 Lung Cells. Here again the increase in junctional permeability was parallelled by an increase in the number of gap-junctional particles. Within 24 hr of the treatment with 17 μM prostaglandin E_1, the number of gap junctional particles per unit membrane area had more than tripled (Fig. 8); it rose from 57.8 ± 30.5 to 200.1 ± 50.5 particles/1000 μm^2 ($P<0.05>0.025$). [Primary data, total gap junction area, $nm^2/1000\ \mu m^2$: control, $5{,}000.6 \pm 2{,}636.3$; treated, $17{,}310.2 \pm 4{,}371.6$ (SE).] This effect seems to represent an increase in both

[4] The criterion was set so high because clusters of up to eight membrane particles occurred on membrane areas that were evidently nonjunctional.

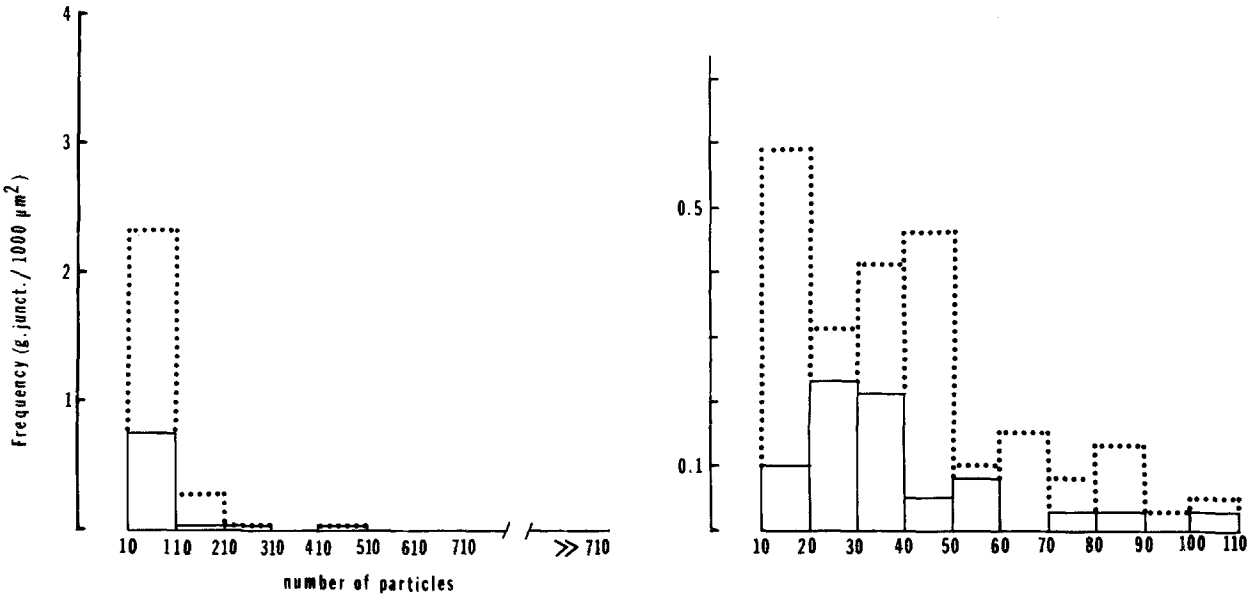

Fig. 9. Frequency distribution of gap-junction size. C-6 cells, experiment of Fig. 8. Control, solid line; hormone-treated, dotted line. Gap-junction size is given in number of particles; frequency is the number of particle clusters (gap junctions) per 1,000 μm² of cell membrane area. *Right:* the 10–110 size interval expanded

Table 5. Gap junction frequency and size

Condition	Mean gap junction frequency		Mean gap junction size		Membrane area scanned (μm²)
	Per 1,000 μm²	Per equivalent cell[a]	nm²	Number of particles	
C-6					
Control	0.56±0.14 (15)	1.15± 0.29	5,546.4±1,506.7 (32)	64±17	58,500
Isoproterenol	1.74±0.38 (15)	3.57± 0.78	4,809.5± 476.2 (102)	55± 5	58,500
WI-38					
Control	1.7 ±0.5 (10)	56.3 ±16.6	6,810.0± 949.2 (67)	79±11	39,000
PG-E_1[b], 7 hr	2.9 ±0.6 (10)	96.0 ±19.9	8,535.4±1,065.1 (114)	99±12	39,000
Control	0.7 ±0.4 (10)	23.2 ±13.2	6,512.9±1,327.2 (30)	75±15	39,000
PG-E_1, 24 hr	1.7 ±0.4 (10)	56.3 ±13.2	10,521.6±1,365.1 (66)	121±16	39,000

Means ±SE; in parenthesis, *n*, the number of unit squares (3,900 μm² each) or the number of gap junctions.
[a] Equivalent cell area: C-6, 1,025±56 (SE, $n=42$); WI-38, 16,548±1,957 ($n=24$).
[b] Prostaglandin E_1.
Confidence levels of differences (*P*) between control and hormone-treated conditions: Frequency: C-6, <0.01; WI-38, 7 hr, <0.3 >0.2; WI-38, 24 hr, <0.2 >0.1. Size: C-6, <0.6 >0.5; WI-38, 7 hr, <0.3 >0.2; WI-38, 24 hr, <0.1 >0.05.

the mean frequency of gap junction (including gap junction sizes larger than 710 particles) and the mean number of particles per gap junction (Table 5). By 7 hr, the effect was less pronounced and statistically less significant (Fig. 8, Table 5); yet the consistent increase of frequency of all gap junction sizes (Fig. 10) leaves little doubt that even then a junctional effect was present. (The 7-hr and 24-hr experiments were run on different batches of cells. Presumably because of that, the respective base (control) values were different; *see* Flagg-Newton & Loewenstein, 1981; Flagg-Newton et al., 1981).

We gained the impression that the tight-junctional strands became longer after the prostaglandin E_1 treatment. The total (cumulative) strand length per unit membrane area increased from 592.7 to 1025.4 nm/1000 μm². An effect of this kind has already been described for Cl-1D cell cultures stimulated with exogenous cAMP, where the increase started from a zero base (Azarnia et al., 1981). For the present cell system, however, the result is no more than an indication of tight-junctional

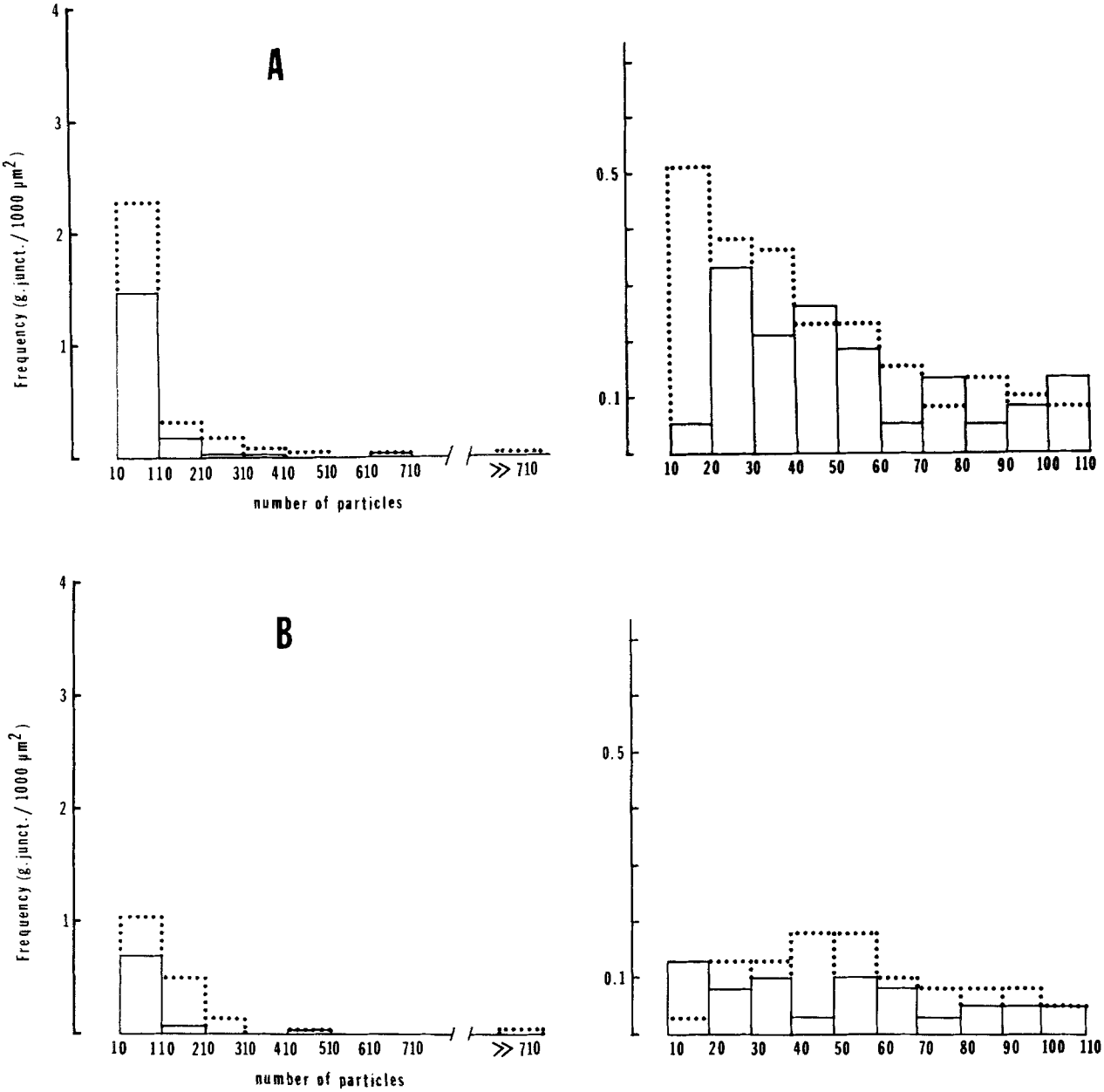

Fig. 10. Frequency distribution of gap junction size. WI-38 cells, experiments of Fig. 8. (*A*): 7-hr prostaglandin-E_1 treatment; (*B*): 24-hr treatment. *Right:* the 10–110 size interval expanded

growth. To establish this point, it is necessary to scan larger membrane areas than we did; tight junctions are more unevenly distributed over the cell membrane than gap junctions, and their individual variation in length is high.

Discussion

The present results show an enhancement of junctional permeability, P_j, by two hormones elevating the intracellular concentration of cAMP, $[cAMP]_i$. They conform to the simple upregulator scheme

$$\underset{(1)}{h+r} \rightarrow \underset{(2)}{[cAMP]_i\uparrow} \rightarrow \underset{(3)}{P_j\uparrow}$$

where h is the hormone, r, the adenylate cyclase activating receptor, and $[cAMP]_i$, the determinant of P_j (Loewenstein, 1981). Stages *1* and *2* occur within half an hour of hormone application. Stage *3* takes several hours. Since it is parallelled by a proliferation of gap-junctional particles, the enhancement of P_j is most simply explained by an

increase in the number of cell-to-cell channels. Such an increase may conceivably come about by a cAMP effect on the channel formation (or degradation) process. However, effects on unit-channel permeability are not excluded; the probes of the glutamic series are all of the same molecular width, and the fact that the wider fibrinopeptide and serum albumin molecules continue to be excluded by the channels after hormone treatment (p. 244) does not rule out finer channel gradations.

The question of the mode of action of cAMP in junctional permeability regulation has been discussed before (Flagg-Newton et al., 1981). Here we consider some physiological implications of such a regulation driven by hormones, on the assumption that what we found in culture has its counterparts in real tissues. On this assumption, the most obvious possibility is that hormones serve to regulate junctional communication in tissues. This kind of regulation would not be useful for situations demanding rapid cellular reaction – the change in P_j takes hours – but it may be adequate where the times of change of cellular states are that slow as, for example, in embryonic development and, indeed, in many kinds of hormone-dependent cellular differentiations, embryonic and adult. Since the communication regulation is finely graded, it would afford a range of control for cell interactions, going from graded to simple switching on or off, depending on the threshold concentration requirements (and losses) of the corresponding communication signals.

Further possibilities emerge as we view the findings in the broader context of an interaction between two basic forms of intercellular communication. On the hormonal form, the interaction confers potential for coordination of the cellular response. The enhancement of junctional permeability means an enhanced intercellular exchange of a range of cytoplasmic molecules, namely that of cell-to-cell channel permeants (inorganic ions, metabolites, high-energy phosphates, vitamins, etc.). Thus, at the very least, the interaction will foster tissue homeostasis in respect to these molecules. Although passive, such homeostasis constitutes a coordination of sorts. Besides, possibilities for more lively coordinations present themselves when one considers that among the junction-permeants are the second messengers of hormone action (cAMP and other nucleotides, for instance, probably fit through the cell-to-cell channel; Tsien & Weingart, 1976; Pitts & Simms, 1977; Lawrence, Beers & Gilula, 1978; Schwartzmann et al., 1981). Their enhanced intercellular flux would tend to promote propagation of hormonal responses in the connected cell community – a coordination involving cellular response amplification. Because P_j changes so slowly, this kind of coordination would require persistent or repeated hormone stimulation. [*See* Loewenstein (1981) for general conditions of response amplification.]

What are the counterparts in organs and tissues of our results in culture? There is as yet little to be said. There is evidence for a permeability increase in response to gonadotropin in frog oocyte/follicle cell junctions (Browne & Wiley, 1979). The rest are morphological hints: thyroid hormone-stimulated frog ependyma and estrogen-stimulated rat myometrium show proliferation of gap junction in the electron microscope (Decker, 1976; Dahl & Berger, 1978; Garfield, Kannan & Daniel, 1980; Garfield, Merret & Grover, 1980). Although this does not tell about junctional permeability (and it is not possible to infer that the particles represent patent channels), a cAMP-mediated enhancement of junctional permeability in these tissues seems a reasonable possibility in the light of the present results and of the knowledge that these hormones cause cAMP elevation elsewhere. There also is evidence for an action on junctional conductance by molting hormone in insect epidermis. But there the mechanism seems different; there are no reasons to believe that the action is mediated by cAMP (Caveney, 1978).

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