Cyclic AMP Effects on Cell-to-Cell Junctional Membrane Permeability during Adipocyte Differentiation of 3T3-L1 Fibroblasts

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ABSTRACT Mouse 3T3-L1 fibroblast cells, also known as preadipocytes, differentiate in vitro into adipocytes when treated with promoting agents and acquire numerous properties characteristic of mature fat cells. We studied junctional cell-to-cell communication by measuring the incidence of electrical coupling and transfer of carboxy-fluorescein among these cells. When 3T3-L1 cells were induced to differentiate into adipocytes, they lost virtually all cell-cell communication. Preadipocytes that remained nondifferentiated after the treatment maintained normal communication. Loss of communication in the adipocytes invariably coincided with appearance of lipid droplets and not with other phenotypic changes. In the differentiating cells, loss of cell-to-cell communication and lipid accumulation was prevented if dibutyryl cyclic AMP and caffeine were present in the culture medium. Addition of dibutyryl cyclic AMP and caffeine to already differentiated adipocytes resulted in loss of lipid and simultaneously improved junctional permeability. The results demonstrate that in the in vitro 3T3-L1 cell system, (*a*) cell-to-cell communication and lipid synthesis are intimately related during the adipose conversion and (*b*) cAMP affects the expression of the two phenotypes.

The in vitro conversion of 3T3-L1 fibroblasts into adipocytes has been recognized as a process of cellular differentiation that resembles the development of the mammalian adipose tissue in many respects (10, 11). Under appropriate culture conditions, the cells continue to accumulate lipid droplets that subsequently coalesce and ultimately occupy nearly the entire cell volume, except for a thin cytoplasmic rim and the flattened eccentric nucleus, characteristic of mature fat cells (12). Adneosine 3′,5′- monophosphate (cAMP) was shown to mediate the hormonal regulation of lipid metabolism in fat cells from rat epididymal fat pads (3). Moreover, dibutyryl cyclic AMP (dbcAMP)[1] and epinephrine greatly reduce lipid accumulation in 3T3-L1 adipocytes (10, 11). Underlying these effects is cAMP regulation of the expression of lipogenic enzymes (24).

cAMP also seems to play a role in the regulation of the permeability of cell-cell junctional membrane (15). It has been shown that elevation of intracellular cAMP concentration, by treatment with dbcAMP or agents known to increase the adenylate cyclase activity, leads to an increase in junctional membrane permeability and in the number of gap junction particles (1, 5, 7). Among such agents, catecholamines, in particular, were found to increase the junctional permeability (18). The gap junction is the membrane structure that presumably contains specialized cell-cell channels (4, 19, 26). These channels are direct hydrophilic pathways that interconnect cell interiors (14) and permit relatively unrestricted exchange of small molecules between cells (8, 9, 22).

In this paper we examined the junctional permeability during the 3T3-L1 preadipocyte conversion to adipocytes and the possible relationship between this permeability and cAMP. We found that the junctional permeability decreases during the conversion and that cAMP affects both the junctional permeability and lipid accumulation in a predictable and reversible manner in the differentiated adipocytes.

MATERIALS AND METHODS

Cell Culture and Growth Conditions

The mouse 3T3-L1 (Swiss Albino) fibroblast cell line was obtained from American Type Culture Collection (Rockville, MD). Stock cultures of cells

[1] *Abbreviation used in this paper:* dbcAMP, dibutyryl cyclic AMP; DEX, dexamethasone; FCS, fetal calf serum; INS, insulin; MIX, methyl-isobutylxanthine.

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were maintained in exponential growth by subculturing every 7 d. Experimental cultures were inoculated with 10^4 cells per square centimeter and grown to confluence (6–7 d) in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cells were fed three times weekly with 5 ml of medium per 60-mm culture dish and the cultures were maintained in a humidified 95% air and 5% CO_2 atomosphere at 37°C. Cultures were examined routinely and lipid synthesis was verified by staining cells with oil-red-O (10).

Induction of Adipocyte Conversion

Confluent cultures of 3T3-L1 cells were maintained on Dulbecco's modified Eagle's medium supplemented with 10% calf serum. We promoted differentiation by incubating confluent cultures with fresh medium containing 10% fetal bovine serum (FCS) supplemented with 0.5 mM methyl-isobutylxanthine (MIX), 0.25 μM dexamethasone (DEX), and 1 μg/ml insulin (INS) for 3 d. In some experiments the cultures were treated with dbcAMP (1 mM) and caffeine (1 mM) in an attempt to elevate the intracellular level of cAMP. Only cultures with fewer than 12 passages were used. Cultures maintained beyond the 12th passage were less efficient in adipose conversion.

Determination of Cell-to-Cell Junctional Permeability

ELECTRICAL COUPLING MEASUREMENTS: Contiguous cells, namely cells in contact as seen by phase-contrast microscopy, were randomly impaled with glass microelectrodes filled with 1 M KCl and with tip resistances of 100–120 MΩ. Each microelectrode was connected to a preamplifier (WP-I instruments, Inc., New Haven, CT., model M4A), which served the dual purpose of passing electric current and recording voltages. Square pulses of negative (hyperpolarizing) DC current of 50-ms duration and ~2.5 nA were microinjected into cell 1 (see Fig. 1) and, with a 100-ms delay, cell 2 once every 1.5 s. Traces of the resulting voltage displacements were displayed on a storage oscilloscope (Fig. 1, *C* and *F*). Measurements on a single culture plate were limited to 15 min and were performed in room air warmed to 30°–32°C. Incidence of electrical coupling was determined as the frequency of coupled cell pairs among the pairs tested. The limit of resolution was a coupling coefficient of 0.02 (16). Cell membrane potentials were routinely recorded and they ranged from 15 to 55 mV, typically ~35 mV. Impalements that resulted in membrane potentials of < 20 mV were rejected. The apparent input resistance of the cells ranged from 10 to 60 MΩ, typically ~30 MΩ. No significant differences were observed in the membrane potentials or in the input reistances of the preadipocytes and the adipocytes.

CELL-TO-CELL JUNCTIONAL TRANSFER OF CARBOXY-FLUORESCEIN: One of the electrodes (II) was also filled with carboxy-fluorescein (376,000 mol wt), a highly fluorescent hydrophilic dye that is junction permeant (see Fig. 1 *B*). In most cases the transfer time (the time from dye injection until its appearance in a first-order neighbor) was < 30 s (see also reference 8). We scored the incidence of transfering cell interfaces, namely the percentage of first-order neighbors in contact with the injected cell (as seen in phase-contrast microscopy) that exhibited fluorescence within 5 min after microinjection of the dye (as seen in dark-filled microscopy). This simple scoring method provides a sensitive and convenient index of relative changes in junctional permeability (6, 18). Cells that were presumed to be in contact on the basis of phase-contrast microscopy were without exception also electrically coupled in normal medium (see Table I), bolstering our confidence in the reliability of quantitation by this method. In addition, the loss of cellular fluorescence (which includes all losses; loss of the dye to the medium and by photobleaching) was measured on single cells using a photodiode system (5). The rates of fluorescence loss were not significantly different in the adipocytes and in the preadipocytes, excluding the possibility of a significant change in nonjunctional membrane permeability.

RESULTS

Adipocyte Differentiation

As previously reported (17, 20), the 3T3-L1 cells underwent rapid morphological changes when treated with agents promoting adipocyte differentiaion. These changes were associated with lipid synthesis and storage as seen in Fig. 1 *D*. We distinguish three stages in the in vitro adipose conversion. During stage one (preadipocyte population maintained on normal medium), the cell cultures were densely packed, stationary monolayers of flat cells. Stage two was promoted with the aid of MIX-DEX-FCS-INS. Within 4 h after the addition of the promoters, the cells became round and refractile and by 24 h numerous mitotic cells appeared. This cellular proliferation persisted for 3 d, approximately doubling the cell densities of the starting cultures (Fig. 1 *A*)(21).

Removal of MIX and DEX from the culture medium was the starting point for stage three of the adipocyte differentiation and marked the beginning of dramatic changes in the junctional membrane permeability described below. Interestingly, although during stage three FCS and INS were still present in the medium, the cellular proliferation ceased—even though both FCS and INS are known to be potent mitogenic factors. The most striking feature of stage three was the rapid appearance of cytoplasmic lipid droplets, in some instances as early as 6 h after the removal of MIX and DEX. By 24 h, 40–60% of the cells contained some lipid droplets. In the next 2 d the fraction of cells containing lipid reached 80–90% and it remained constant thereafter (Fig. 1 *D*). Accumulation of lipid by these cells continued unabated in the presence of calf serum alone. By day 8 they had become engorged with very large lipid droplets. The remaining 10–20% of the population were fibroblast-like cells that underwent no morphological changes and acquired no lipid droplets.

Junctional Permeability Changes.

There was no significant change in the incidence of the electrical coupling and fluorescence transfer of the preadipocytes during the treatment with the promoting agents (stage II)(Fig. 1, *A–C*), as evident from maximum incidence of electrical coupling present (100%) and relatively high incidence of carboxy-fluorescein transferring junctions (59–67%; Table I). In sharp contrast, the junctional membrane permeability changed dramatically in the third stage upon the removal of MIX and DEX from the culture medium. Within 24 h after removal of these agents, the incidence of communication fell: the junctional transfer of carboxy-fluorescein was zero in all the adipocytes tested and the incidence of the electrical coupling had fallen to 50% (Table I, stage III and Fig. 1, *D–F*). Over the next 48 h the coupling incidence leveled off at 6% with no further reduction in the incidence beyond the third day of stage three. Measurements performed 3 d later yielded very similar results indicating a stabilization at a very low level, which persisted even in the presence of normal medium (Table I). By contrast, the incidence of permeable junctions did not diminish amongst the preadipocytes still present that had not undergone adipose conversion and were easily identified by their fibroblast-like features. Junctional communication in these cells was not different from the preadipocytes in normal medium (Table I). Thus, treatment with the promoting agents per se was not sufficient cause to diminish the junctional membrane permeability of the cells.

cAMP Regulation of Junctional Membrane Permeability and Lipid Synthesis

Treatment of the preadipocytes with exogenous dbcAMP and caffeine prevented the loss of junctional permeability normally associated with adipose conversion. This went hand-in-hand with the prevention of lipid synthesis by the adipocytes. Washing away cAMP resulted in rapid reduction in the communication incidence to the levels exhibited in differentiating adipocytes, and at the same time lipid accumulation was resumed (Table II, experiment *a*).

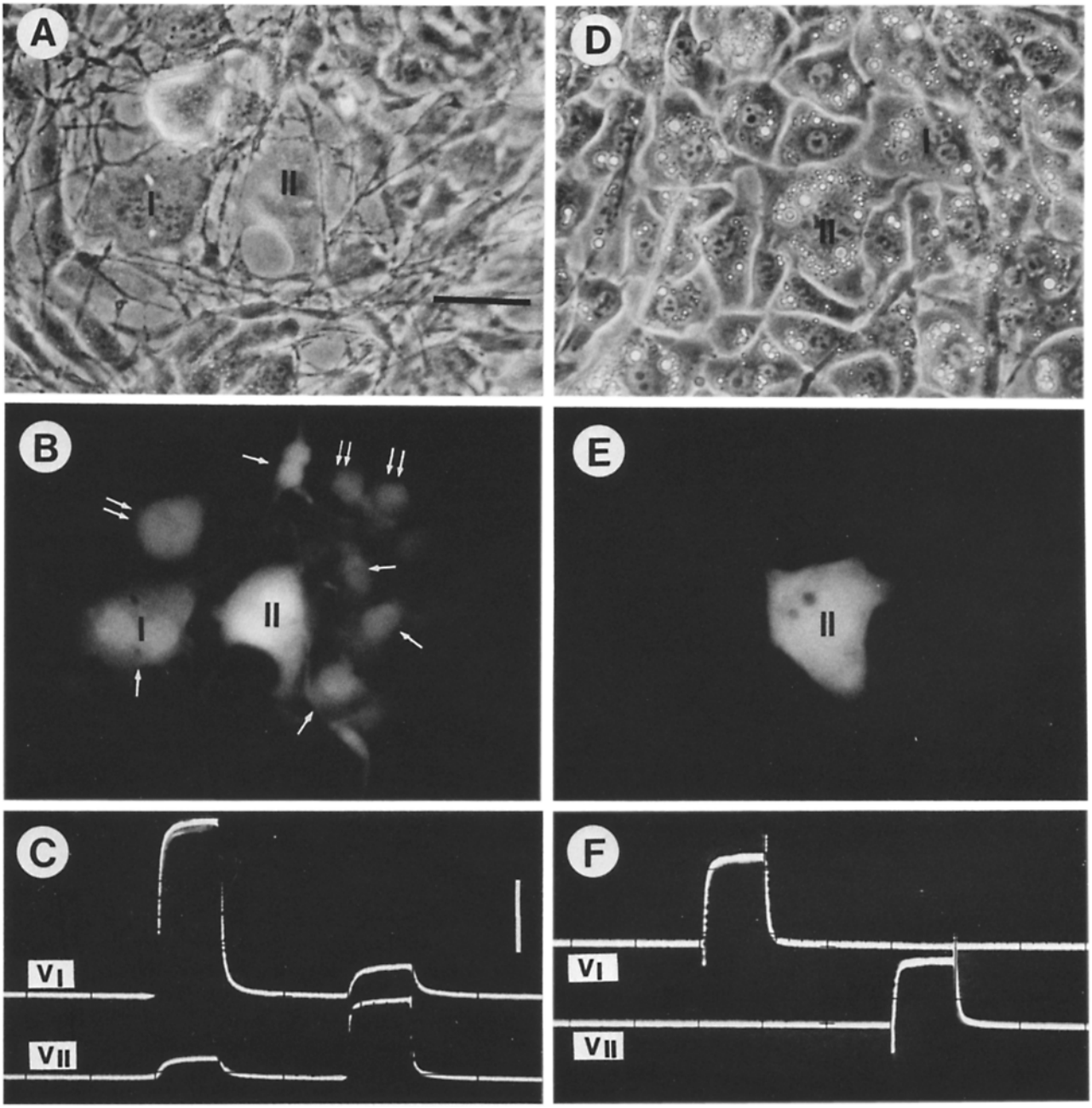

FIGURE 1 Cell-to-cell communication in differentiated 3T3-L1 adipocyte and 3T3-L1 preadipocyte. Negative (hyperpolarizing) DC current ($i = 2.5 \times 10^{-9}$ A) is pulsed into cell 1 and an equal current, with 100-ms delay, into cell 2. Simultaneously, the steady-state voltage displacements V_I and V_{II} are measured in both cells using a bridge circuit. Carboxy-fluorescein (contained in electrode II) is also iontophoretically microinjected into cell 2 and its fluorescence is viewed in 495 nm light and photographed 5 min after injection. *Left column* (*A–C*): 3T3-L1 preadipocytes in the presence of the promoting agents for 2 d (stage II, day 2; Table I). *Right column* (*D–F*): 3T3-L1 adipocytes (stage III, day 1; Table I). (*A* and *D*) Phase-contrast photomicrograph of cells probed for cell-to-cell communication. The adipocytes contain many lipid vesicles. Bar, 50 μm. (*B* and *E*) Dark-field fluorescent tracing of carboxy-fluorescein in 495 nm light. (*B*) The injected dye into cell 2 has spread to five first-order (single arrows) and three second-order (double arrows) neighbor cells. (*E*) No dye transfer is observed among the adipocytes. (*C* and *F*) Representative oscilloscope traces of voltage recordings, V_I and V_{II}, in the contiguous cell pairs 1 and 2. Current traces are not shown. (*C*) The steady-state voltage V_I is displaced momentarily during injection of current into cell 1. A fraction of the current has spread to neighboring cell 2 and displaced its membrane potential V_{II}. Injection of current into cell 2 causes shifts in V_I and V_{II} symmetric to those due to i_I. Traces shown in *F* indicate no passage of current between the adipose cell pair. Bar, 50 mV.

When dbcAMP and caffeine were added to already differentiated adipocyte cultures, within 24 h the cells lost virtually all lipid droplets and the incidence of electrical coupling increased to the 46% level. During the next 2 d the communication incidence continued to increase, reaching a maximum level (100%) on the third day (Table II, experiment *b*).

DISCUSSION

The major finding in this report is that during the in vitro differentiation of 3T3-L1 preadipocytes into adipocytes, the

TABLE I

Incidence of Cell-to-Cell Communication in 3T3-L1 Adipocytes and Preadipocytes

Treatment*	Time of measurement‡	Stage	Cell type	Communication incidence	
				Electrical coupling§	C-Fluorescein transfer‖
	d			%	
Normal medium		I	Preadipocyte	100 ± 0 (40,4)	60 ± 1 (90,12, 3)
MIX-DEX-FCS-INS	1	II	Preadipocyte¶	100 ± 0 (10,1)	62 ± 8 (23,4,1)
	2			100 ± 0 (30,3)	67 ± 2 (86,9,3)
	3			100 ± 0 (20,2)	59 ± 3 (43,6,2)
FCS-INS	1	III	Adipocyte**	50 ± 7 (46,4)	0 ± 0 (34,5,1)
	2			18 ± 5 (50,4)	0 ± 0 (39,6,1)
	3			6 ± 4 (34,5)	0 ± 0 (87,13,3)
	3		Preadipocyte‡‡	100 ± 0 (10,1)	63 ± 5 (28,4,1)
Normal medium	3	III	Adipocyte	8 ± 6 (24,3)	0 ± 0 (32,5,1)
	3		Preadipocyte	100 ± 0 (30,3)	65 ± 4 (37,5,1)

* Cells were grown to confluence in normal medium plus 10% bovine calf serum before treatment with the promoting agents. Cultures were then treated with MIX-DEX-FCS-INS for 3 d, at the end of which period MIX and DEX were removed but treatment with FCS and INS continued for an additional 3 d. Subsequently the cultures were maintained in normal medium.

‡ Time measured from onset of respective treatment.

§ Percentage of incidence of electrically coupled cell pairs (± SE) (microelectrodes were in contiguous cells). In parentheses: the number of cell pairs tested, the number of culture dishes examined.

‖ Percentage of carboxy-fluorescein (*c-fluorescein*) containing first-order neighbor cells, as evidenced by detectable permeable junctions within the first 5 min after injection (± SE). In parentheses: the total number of first-order junctions (neighbors) of the injected cells, the number of injections, the number of culture dishes examined.

¶ Preadipocytes in the presence of the promoting agents. These cells underwent morphological changes, but did not accumulate lipid.

** Up to 40% of cells contained small lipid droplets. By day 2 the percentage increased to >80%, and by day 3 up to 90% of cells had some lipid droplets.

‡‡ Cells present in the cultures treated with the promoting agents that did not undergo adipose conversion. These cells had the typical fibroblast-like appearance of preadipocytes.

TABLE II

Effects of dbcAMP and Caffeine on Cell-to-Cell Communication and Lipid Content

Experiment	Treatment*	Time of measurement‡	Lipid vesicles	Communication incidence
				Electrical coupling§
		d	*number, size*	%
a and *b*	Normal medium‖	0	None	100 ± 0 (30,3)
a and *b*	MIX-DEX-FCS-INS¶	3 (3)	None	100 ± 0 (20,2)
a	FCS-INS + dbcAMP + caffeine¶	1(4)	None	100 ± 0 (20,2)
a		2(5)	None	100 ± 0 (16,2)
a		3(6)	None	100 ± 0 (21,3)
a	FCS-INS**	1 (7)	Few, small	10 ± 6 (30,3)
a		2 (8)	Many, small	7 ± 5 (28,3)
a		3 (9)	Many, medium	8 ± 5 (26,3)
b	FCS-INS**	1 (4)	Few, small	9 ± 6 (22,2)
b		2 (5)	Many, small	12 ± 7 (25,3)
b		3 (6)	Many, medium	8 ± 6 (24,3)
b	FCS-INS + dbcAMP + caffeine¶	1 (7)	None	46 ± 10 (28,3)
b		2 (8)	None	79 ± 10 (19,2)
b		3 (9)	None	100 ± 0 (18,2)

* Cells were grown to confluence in normal medium. All cell cultures received MIX-DEX-FCS-INS on day 0. On day 3 MIX and DEX were removed and the cultures were subsequently maintained on FCS and INS for the remainder of the experiment. In addition, on day 3, the cultures were divided into two groups. The first group received dbcAMP and caffeine (1 mM of each) for the next 3 d (experiment *a*), whereas the second group received the same treatment 3 d later on day 7 (experiment *b*). Incidence of electrical coupling was determined throughout the experiment on a daily basis.

‡ Time measured from onset of respective treatment. The chronological time is given in parentheses starting with day 0.

§ Percentage of incidence of electrically coupled cell pairs (± SE). In parentheses: the number of cell pairs tested, the number of culture dishes examined.

‖ Cultures consisted of a monolayer of thin and flat cells.

¶ These cells appeared round and refractile.

** The cells were relatively flat and in good contact with each other. Some lipid vesicles appeared during the first day of this treatment and their size and frequency increased during subsequent days.

cells lose cell-to-cell communication. More than 90% of the adipocyte population have reduced junctional permeability during the first 3 d of the adipose conversion (Table I). Since the change in the junctional membrane permeability is intimately associated with the conversion of the preadipocytes into adipocytes, it may be an integral part of its differentiation program. Moreover, the observed loss of junctional permeability seems to be a relatively stable phenotypic change in the adipocyte, as is its capacity to synthesize lipids (10). The junctional permeability changes during the differentiation of the adipocyte did not correlate with changes in cell shape, nor was it a consequence of the changes observed in cell morphology. In fact, if anything, as seen in the phase-contrast microscope the cells appeared in better contact with each other during the critical period when the cells had lost communication (Fig. 1 and Table I). However, the reduction in

the junctional permeability invariably coincided with the onset of lipid synthesis (Table I). This suggests that the latter two phenotypes are intimately related devlopmentally in the 3T3-L1 adipocytes.

The junctional membrane permeability and the synthesis of lipids in the 3T3-L1 adipocyte are likely to be regulated by changes in the concentration of intracellular cAMP. Elevation of intracellular cAMP concentration, by the addition of exogenous dbcAMP, improved the junctional permeability and at the same time brought about a decrease in visible lipid droplets. Conversely, removal of the exogenous source of cAMP had the exact opposite effects: junctional permeability returned to the previous low levels and lipid synthesis was resumed (Table II). The ability of cAMP to regulate junctional permeability and to regulate junction formation has been demonstrated in various normal and tumor cell lines (1, 5), and the same mechanism for regulating junctional permeability may also exist in the adipocytes. However, the reduction in adipocyte junctional permeability suggests that the differentiation somehow may involve an abatement of the channel formation mechanism. This in turn could follow from a depression of cytoplasmic levels of cAMP. The latter suggestion is consistent with the finding that the cAMP content of untreated adipocytes is lower than that of the preadipocytes (20).

We do not know if the in vitro findings in the 3T3-L1 adipocytes represent the events in vivo. There is some evidence that fat cells from adipose tissue may have a low level of cell-to-cell communication. These studies, which were performed on newt fat body and mouse brown fat, have shown that although electrical coupling was present in these tissues, it was found with considerable difficulty, i.e., coupling could not be detected with every impalement (23). These findings were ascribed to possible injury during the preparation of the tissues. However, in the light of the findings reported here, we can not rule out the possibility that the findings were correct and therefore that the in vitro findings represent their in vivo counterpart.

The interesting possibility emerging from these observations is that the reduction of cell-to-cell communication may be a necessary event for the development of adipocyte function, which is the metabolism of lipids. Under basal conditions the adipocyte in situ accumulates lipids, whereas during conditions of stress it releases free fatty acids. The latter is, in the main, mediated by direct action of hormones, for example, epinephrine (11) and adrenocorticotropin (20), on the adipocytes. Furthermore, adipocyte differentiation results in increased sensitivity to β-adrenergic agonists and adrenocorticotropin (20). These hormones are potent lipolytic agents that effect breakdown of lipids in mature fat cells by increasing the intracellular cAMP levels (3). Finally, cAMP was shown to prevent lipid synthesis by reducing specifically the accumulation of mRNA for lipogenic enzymes (24). There is already convincing evidence that the junctional permeability of cells is enhanced by the action of hormones capable of interacting with mammalian cells in culture and elevating the intracellular cAMP concentration (18). All of this taken together suggests that the channels may be instrumental in adipose tissue responsiveness to the action of hormones. For example, increased cell-to-cell channel formation in response to lipolytic hormones may result in a more efficient tissue response, by permitting a more efficent spread of cAMP itself. Previous studies suggest that cAMP possibly may flow from one cell to its neighbors (2, 13, 25), and in addition, this molecule is certainly of a size one would expect to be admitted by the cell-to-cell channel (22).

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