**Ca\textsuperscript{2+}** causes active contraction of bile canaliculi: Direct evidence from microinjection studies

**(actin/calmodulin/cell motility/liver cell/intercellular communication)**

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**Abstract**

Cytoplasmic microinjection of Ca\textsuperscript{2+} triggers contraction of bile canaliculi in freshly isolated monolayer cultures of rat hepatocytes. Unseparated paired hepatocytes were used to demonstrate this motility-based phenomenon. Only one cell of the pair was injected, but fluorescence spread from the target cell to the opposite cell; also, the contractions were always uniform, equally involving both hepatocytes that form the canaliculus, indicating that communication exists between the cell pairs. Inhibitors of calmodulin and actin filaments, trifluoperazine and cytochalasin B, respectively, inhibited the Ca\textsuperscript{2+}-induced contractions. Hence, the mechanism of contraction has features in common with actin–myosin based cytoplasmic motility behavior found in other non-muscle cells.

Both actin and myosin are present in many non-muscle cells including hepatocytes (1–5). In the liver cell, actin is present throughout the cytoplasm but is most evident under the plasma membrane, especially in the region of bile canaliculi; this has been shown by many investigators (2, 6–9). It has been demonstrated recently that in non-muscle cells the mechanism of actin–myosin based contractile events is different from that observed in skeletal muscle; for instance, in non-muscle cells, contraction is myosin-based. Present evidence indicates that the initial event is an increase in intracellular calcium ion concentration from 0.1 to 1 μM; that calcium combines with calmodulin, forming calcium–calmodulin complexes, which are involved in the activation of inactive myosin light chain kinase; and results, finally, in myosin activation by actin. The precise details of this activation process are a matter of intense current research (for recent reviews, see refs. 10–12). Structurally, there are a number of close parallels between hepatic canaliculi and the intestinal brush border. The brush border is of special interest to this study, because like the bile canaliculi, it can be induced to move in vitro (13, 14). The biochemistry of the contraction mechanism in brush borders has been extensively studied (13–17) and present evidence is that, like other non-muscle cells, it is a myosin-based mechanism.

In the liver, this mechanism has not been studied. Indeed, it is only very recently that cytoplasmic motility events have been observed in liver cells (18). Cytoplasmic microinjection is a new technique, which permits the direct study of some aspects of hepatic cell function. In the present report, we compare spontaneous bile canalicular contractions with those that occur after intracellular microinjection of Ca\textsuperscript{2+} in isolated hepatocytes. The effects of inhibitors of actin (cytochalasin B) (19) and calmodulin (trifluoperazine) (20, 21) on the contractions are also reported.

**Materials and Methods**

**Experimental Materials.** Collagenase (type I) and trifluoperazine dihydrochloride were purchased from Sigma. Cytochalasin B was purchased from Aldrich.

**Measurement of Calcium.** Direct measurements of the ionized calcium concentration of the injected solutions and culture media were carried out using a Nova 2 ionized calcium analyzer (Nova Biochemical, Newton, MA) (22).

**Preparation of Primary Cultured Hepatocytes.** Monolayer cultures of rat hepatocytes were obtained from female Wistar strain rats (body weight, 200 g) by using a collagenase digestion technique (23, 24) described in detail previously (25). The cultured hepatocytes were maintained in L-15 medium, supplemented with 10% fetal bovine serum/10 mM Hepes, pH 7.2 penicillin (100 units/ml)/streptomycin (100 μg/ml). The culture medium containing 0.92 ± 0.01 (mean ± SEM) mM ionized calcium.

The cell-separation technique is designed to prepare isolated single hepatocytes, but in such preparations 10% of the cells remain in small incompletely separated clumps. Amongst them are cell pairs in which bile canaliculi are dilated and easily visible; these cell pairs were selected for evaluation.

Viability was assessed by trypan blue exclusion and by time-lapse cinemicrography as described (25). Other isolation, viability was 82%–93%. In the trifluoperazine and cytochalasin B experiments of the hepatocytes that were viable at the start of treatment, viability was 94%–100% at the end of treatment with trifluoperazine, and 95%–100% with cytochalasin B. There were no significant differences between controls (95%–100%) and these groups. Experiments were performed 4–8 hr after cell isolation.

**Microinjection Method.** Microinjections were done according to the modified methods of Stockem et al. (26) and Graessmann and Graessmann (27). Microspettes were prepared from glass capillaries (outer diameter, 2 mm) (W-P Instruments, New Haven, CT), using a micropipette puller (PG-1, Narishige Scientific Instruments, Tokyo, Japan). The diameter of the tip of the micropipette was 0.5–1.0 μm. After the capillary was pulled, the solution was delivered through its rear open end by means of a 20 GA Angiocath (Desseret, Sandy, UT). Then the micropipette was connected to the hub of the pipette holder (HI-1, Narishige), which was then attached to the micrometer. Microinjections were done using a micromanipulator (MP-1, Narishige) and a microinjector (IN-4A, Narishige) on the stage of an inverted microscope (Nikon, Tokyo, Japan) equipped with phase-contrast and fluorescence optics. The temperature was maintained at 37°C by using a plastic housing with an incubator (NP-2, Nikon).

In all instances, the dose of injected solution was ≈10% of the volume of the recipient cell (26). The microinjection technique itself sometimes caused cell damage, such as bleb formation; in all such cases, these experiments were rejected. The rate of successful microinjection was 40%–100% in the present study, and no significant difference in successful

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Bile canaliculi observed in normal, cytochalasin B-, and trifluoperazine-treated hepatocytes

### TABLE 1. Effects of Ca\(^{2+}\) on bile canicular contractions in normal, cytochalasin B-, and trifluoperazine-treated hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spontaneous contraction (non-injected)</th>
<th>Injected with standard calcium-free solution</th>
<th>Injected with standard calcium solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.8 ± 0.4 (14%)</td>
<td>3.0 ± 0.5</td>
<td>16.8 ± 0.4 (84%)</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>0.4 ± 0.4 (2%)</td>
<td>0.4 ± 0.2</td>
<td>1.6 ± 0.7 (8%)</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>1.2 ± 0.4 (6%)</td>
<td>1.0 ± 0.3</td>
<td>7.2 ± 0.9 (36%)</td>
</tr>
</tbody>
</table>

Each value indicates the number of bile canicular contractions during the 10-min observation period. Results are expressed as the mean ± SEM for five groups, each comprised of 20 bile canaliculi. Percentages are in brackets. Note that the microinjection with standard calcium solution causes bile canaliculi to contract (85% compared to 14%; P < 0.001). Calcium-triggered bile canicular contractions are inhibited by pretreatment with cytochalasin B (P < 0.001) and trifluoperazine (P < 0.01).

### RESULTS

The main results are summarized in Table 1, which compares spontaneous contractions with those occurring in cells microinjected with the standard calcium solutions. The table also summarizes the effects of the two inhibitors cytochalasin B and trifluoperazine.

**Spontaneous Bile Canalicular Contractions in Noninjected Cells.** In normal untreated hepatocytes, 2.8 ± 0.4 spontaneous contractions were seen in each of the five groups of 20 bile canaliculi observed over the 10-min experimental period
Fig. 1. Normal hepatocytes (no pretreatment) (×440.) (a) Open lumen of the bile canaliculus (arrow) can be seen between two (coupled) hepatocytes. Phase-contrast micrograph. (b) The micropipette, which contains the standard calcium solution, is penetrating the hepatocyte on the right. (c) The solution, which contains sodium fluorescein, has been microinjected into the hepatocyte. Note that only the target hepatocyte is visualized. Fluorescence micrograph. (d) Hepatocyte 10 min after the injection of the standard calcium solution. The lumen of the bile canaliculus has closed (i.e., canicular contraction has occurred). (e) Fluorescent dye has spread into the neighboring cell. Fluorescent intensity of the recipient cell is stronger than intensity of the neighboring cell.

below. Since the injected volume was ~10% of the cell volume, the results appear to be close to those reported by others, showing that intracellular Ca\(^{2+}\) of ~1 µM triggers contractions in other non-muscle cells, although exact intracellular Ca\(^{2+}\) contraction after the microinjection is not known (10, 11).

The effects of cytochalasin B and trifluoperazine on calcium-induced bile canicular contractions were dose dependent. The results are shown in Tables 2 and 3.

Cell–Cell Communication. In each instance, as shown in Figs. 1–4, after microinjection, the fluorescent marker spread into the neighboring noninjected hepatocyte of the cell pair. The incidence of spread of the marker to the opposite cell was as follows: using standard calcium solution, in the controls (no pretreatment), 16.6 ± 0.5 (83%); cytochalasin B, 18.0 ± 1.2 (90%); trifluoperazine, 16.8 ± 0.5 (84%); using standard calcium-free solution in the controls (no pretreatment), 17.2 ± 1.0 (86%); cytochalasin B, 17.6 ± 1.2 (88%); trifluoperazine, 17.2 ± 1.0 (86%). There is no statistical difference between these results. This spread of fluorescent marker is an indication of cell–cell communication.

Fig. 2. Normal hepatocytes (no pretreatment). (×440.) (a) Just prior to injection of the standard calcium-free solution. Open lumen of the bile canaliculus (arrow) can be seen between two hepatocytes. Phase-contrast micrograph. (b) Hepatocytes 10 min after the standard calcium-free solution. The bile canaliculus has not contracted. (c) Injected fluorescent dye has spread into the neighboring cell. Fluorescence micrograph.

Fig. 3. Hepatocytes pretreated with cytochalasin B. (×440.) (a) Just prior to injection of the standard calcium solution. Lumen of the bile canaliculus shows dilation (arrow). Phase-contrast micrograph. (b) Hepatocytes 10 min after injection of the standard calcium solution. The dilated bile canaliculus has not contracted. (c) Injected fluorescent dye has spread into the neighboring cell. Fluorescence micrograph.
DISCUSSION

Microinjection of cells is a powerful technique, because it overcomes the difficulty of the impermeability of the plasma membrane to the probes needed to study vital cell processes. The technique permits the direct study of an almost limitless number of agents in living cells and is an excellent tool for the evaluation of cytoplasmic motility events, such as bile canaliculur contraction. In the study reported here, it provides decisive evidence that microinjection of Ca^{2+} ions into the liver cell cytoplasm results in active bile canaliculur contraction in 84% of instances, compared to 14% in controls. Previous studies using time-lapse cinephotomicrography have shown that spontaneous canaliculur motility with opening and closing of canaliculi is intermittent (18, 25). The contractions may be related to bile secretion, because they are significantly increased by the addition of taurocholate, a choleretic bile acid, to the medium (29). It is not known if contractions occur in vivo, but they may be a mechanism for the forward movement of bile into the next upstream region of the canalicular network, hence facilitating bile flow. In previous work on spontaneous contractions, we have interpreted the closure of the canaliculus as an active process, because it appeared forceful, was inhibited by cytochalasin B (30), and was associated with the expulsion of material (canaliculus bile) from the lumen (25). However, an alternative possibility is that with bile secretion into the canaliculus, there is a buildup of pressure, which at a critical point results in forceful opening and then collapse of the lumen in a manner similar to a pressure valve. The finding that the microinjection of Ca^{2+} ions into liver cells results in the prompt contraction of bile canaliculi is strong evidence that the contracting phase (luminal closure) is an active process.

Regarding the mechanism, it is interesting that Ca^{2+} ions can trigger bile canaliculur contractions, because an increase in intracellular calcium ions is the basis of actomyosin-based intracellular motility events in other non-muscle cells (15, 17, 31, 32). Using presently available techniques, it is not possible to determine the exact intracellular free calcium ion concentration in the single cells injected or in the doublets before and after microinjection. Indirect measurements by others of free calcium ions in hepatocyte suspensions show this value to be ~0.1 μM (33). In the experiments reported here in which we microinjected 10 μM, 1 μM, and 10 nM Ca^{2+} solutions (buffered with EGTA), the results suggest that there is a threshold for contraction. The inhibitory action of trifluoperazine on the contraction process suggests that calmodulin is involved, as is also the case in other non-muscle cells (34). It is well known that calmodulin is an important regulator of many calcium-mediated cellular processes. Calmodulin has been found in most animals and plants, and calmodulin has many known functions, including activation of phosphodiesterase, adenylyl cyclase, Ca^{2+}-Mg^{2+} ATPase, myosin light-chain kinase, glycogen synthetase kinase, and cyclic nucleotide phosphodiesterase (34–36). Calmodulin apparently plays a role in processes such as prostaglandin synthesis, smooth muscle contraction, intestinal secretion, insulin secretion, disassembly of microtubules, and it may possibly be involved in ciliary motility and axonal flow (34, 36). In virtually every case, the phenothiazine antipsychotic drugs (including trifluoperazine) have been shown to inhibit these calmodulin-dependent enzyme activities and processes (36). Therefore, trifluoperazine has diverse effects, but an inhibition of bile canaliculur contractions would be expected, as was found, if the contraction process is calcium–calmodulin mediated.

In previous reports, it has been shown that the actin filament inhibitors cytochalasin B (and D) (30) and phalloidin (37) inhibit spontaneous canaliculur contractions. Here, we

Table 2. Dose–response effects of cytochalasin B and of trifluoperazine on spontaneous bile canaliculur contractions

<table>
<thead>
<tr>
<th>Cytochalasin B</th>
<th>0</th>
<th>5 μM</th>
<th>15 μM</th>
<th>50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of contractions</td>
<td>2.8 ± 0.4</td>
<td>3.2 ± 0.8</td>
<td>2.0 ± 0.6</td>
<td>0.4 ± 0.4*</td>
</tr>
<tr>
<td>(14%)(16%)</td>
<td>(10%)</td>
<td>(2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>0</td>
<td>5 μM</td>
<td>10 μM</td>
<td>20 μM</td>
</tr>
<tr>
<td>No. of contractions</td>
<td>2.8 ± 0.4</td>
<td>2.4 ± 0.4</td>
<td>2.0 ± 0.6</td>
<td>1.2 ± 0.4†</td>
</tr>
<tr>
<td>(14%)(12%)</td>
<td>(10%)</td>
<td>(6%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each number indicates mean ± SEM of five groups of 20 bile canaliculi.
*P < 0.01.
†P < 0.05.

Table 3. Dose–response effects of cytochalasin B and of trifluoperazine on Ca^{2+}-triggered bile canaliculur contractions

<table>
<thead>
<tr>
<th>Cytochalasin B</th>
<th>0</th>
<th>5 μM</th>
<th>15 μM</th>
<th>50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of contractions</td>
<td>16.8 ± 0.4</td>
<td>16.8 ± 1.2</td>
<td>12.0 ± 1.3*</td>
<td>1.6 ± 0.7†</td>
</tr>
<tr>
<td>(84%)(82%)</td>
<td>(66%)</td>
<td>(8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>0</td>
<td>5 μM</td>
<td>10 μM</td>
<td>20 μM</td>
</tr>
<tr>
<td>No. of contractions</td>
<td>16.8 ± 0.4</td>
<td>14.4 ± 0.4</td>
<td>11.6 ± 0.8*</td>
<td>7.2 ± 0.9†</td>
</tr>
<tr>
<td>(84%)(72%)</td>
<td>(58%)</td>
<td>(36%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each number indicates mean ± SEM of five groups of 20 bile canaliculi. Results were obtained using microinjections of the standard calcium solution.
*P < 0.05.
†P < 0.001.
report that cytochalasin B also inhibits calcium-triggered contractions, presumably because of the effects of cytochalasin B on actin filaments (19).

It is generally believed that the intercellular communication is regulated mainly by gap junctions and that high concentrations of intracellular Ca\(^{2+}\) (50–100 \(\mu M\)) disturb this communication (38). In our present results, it is shown that intercellular communication was well preserved in the primary cultured hepatocytes after the microinjection of 100 \(\mu M\) Ca\(^{2+}\). Although we did not measure the intracellular calcium concentration after the microinjection with calcium solution, it is not unreasonable to assume that the intracellular calcium concentration after the microinjection with 100 \(\mu M\) Ca\(^{2+}\) rapidly falls to levels lower than 50–100 \(\mu M\).

It is concluded from this study that calcium microinjection triggers bile canalicular contraction in vitro and that the mechanism involves calcium, calmodulin, and actin filaments. Many questions remain: for instance, do contractions occur in vivo and, if so, are they triggered by an increase in cytoplasmic free Ca\(^{2+}\) ions, and if this is the case, what is the physiological stimulus and mechanism of this increase in intracellular calcium? The answer to these and other questions must await further study.

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