Trifluoperazine, a Calmodulin Antagonist, Inhibits Muscle Cell Fusion

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ABSTRACT We investigated the effect of trifluoperazine (TFP), a calmodulin antagonist, on the fusion of chick skeletal myoblasts in culture. TFP was found to inhibit myoblast fusion. This effect occurs at concentrations that have been reported to inhibit Ca\(^{2+}\)-calmodulin in vitro, and is reversed upon removal of TFP. In addition, other calmodulin antagonists, including chlorpromazine, \(N\)-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W7), and \(N\)-(6-aminohexyl)-1-naphthalene-sulfonamide (W5), inhibit fusion at doses that correspond closely to the antagonistic effects of these drugs on calmodulin. The expression of surface acetylcholine receptor, a characteristic aspect of muscle differentiation, is not impaired in TFP-arrested myoblasts. Myoblasts inhibited from fusion by 10 \(\mu\)M TFP display impaired alignment. In the presence of the Ca\(^{2+}\) ionophore A23187, the fusion block by 10 \(\mu\)M TFP is partially reversed and myoblast alignment is restored. The presence and distribution of calmodulin in both prefusional myoblasts and fused muscle cells was established by immunofluorescence. We observed an apparent redistribution of calmodulin staining that is temporally correlated with the onset of myoblast fusion. Our findings suggest a possible role for calmodulin in the regulation of myoblast fusion.

A characteristic event in the differentiation of skeletal muscle cells is the fusion of mononucleated myoblasts into multinucleated myotubes. Studies using cultured muscle cells have shown that Ca\(^{2+}\) plays an essential role in mediating myoblast fusion: reduction in Ca\(^{2+}\) concentration in the culture medium causes arrest of myoblast fusion, and fusion is rapidly renewed upon readdition of Ca\(^{2+}\) (8, 38, 39, 48, 49).

There is now substantial evidence that Ca\(^{2+}\) regulation in a variety of eukaryotic cells is mediated by the Ca\(^{2+}\)-binding protein calmodulin. Calmodulin has been implicated in the mediation of Ca\(^{2+}\) regulation of cyclic nucleotide metabolism, protein phosphorylation, microtubule assembly, cell motility, and Ca\(^{2+}\) flux across cell membranes (for reviews see references 3, 20, 29). The phenothiazine trifluoperazine (TFP) binds to the Ca\(^{2+}\)-activated form of calmodulin (23, 24) and inhibits its interaction with cellular target proteins (2, 24, 25, 50, 51). Consequently, TFP has been used as a pharmacological probe to study the participation of Ca\(^{2+}\)-calmodulin in the regulation of Ca\(^{2+}\)-dependent cellular events (5, 17, 33, 36, 37). Recently, TFP and phenothiazines closely related to TFP have been shown to interact with Ca\(^{2+}\)-binding proteins distinct from calmodulin (4, 30, 31).

In the present study we investigated the effects of TFP on the fusion of embryonic chick muscle cells in culture. We compared these effects with the action of other calmodulin inhibitors, as well as with the effects of Ca\(^{2+}\) depletion. This study constitutes an initial step toward elucidation of the possible role of Ca\(^{2+}\)-binding proteins in the regulation of myoblast fusion.

MATERIALS AND METHODS

Materials: TFP and chlorpromazine (CPZ) were a gift of the Smith Kline and French Laboratories, Philadelphia, PA. \(N\)-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) and \(N\)-(6-aminohexyl)-1-naphthalene-sulfonamide (W-5) were purchased from CAABCO, Inc. (Houston, TX). These drugs were stored dessicated in the dark, and dissolved immediately before use. The Ca\(^{2+}\) ionophore A23187 was obtained from Calbiochem-Behring Corp., San Diego, CA. A 20-mM stock solution was prepared in dimethylsulfoxide (DMSO; Fisher Scientific Co., Pittsburgh, PA), diluted to working concentration (10 \(\mu\)M) in growth medium, and added to cultures at a final concentration of 0.5 \(\mu\)M A23187 and 0.0025% DMSO. At this concentration DMSO had no effect of myoblast fusion. Sheep anticalmodulin IgG was purchased from ACHR, acetylcholine receptor; CPZ, chlorpromazine; TFP, trifluoperazine; W-5, \(N\)-(6-aminohexyl)-1-naphthalene-sulfonamide; W-7, \(N\)-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide.

Abbreviations used in this paper: AChR, acetylcholine receptor; CPZ, chlorpromazine; TFP, trifluoperazine; W-5, \(N\)-(6-aminohexyl)-1-naphthalene-sulfonamide; W-7, \(N\)-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide.

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CAABCO, Inc., and fluorescein-conjugated rabbit IgG to sheep IgG was purchased from Miles Laboratories Inc. (Elkart, IN). EGTA was purchased from Fisher Scientific Co. 125I-α-bungarotoxin (125I-Bgt) was purchased from New England Nuclear (Boston, MA). Other reagents were from Sigma Chemical Co. (St. Louis, MO). Tissue culture media were purchased from Gibco Laboratories (Grand Island, NY).

Cell Culture: Primary cultures of skeletal muscle cells were prepared from breast of 12-d old chick embryos as described (35, 38). Cells were plated on collagen-coated culture dishes at an initial density of 1.8 x 10^6 cells/60-mm culture dish. Cultures were grown in Dulbecco's modified Eagle's medium (DME), supplemented with 10% horse serum and 2% chick embryo extract at 37°C in an atmosphere of 92% air/8% CO2. Cultures were not fed after plating, and drugs were added directly to the growth medium.

Measurement of Cell Fusion: Cultures were rinsed twice with PBS and fixed for 5 min in methanol. After fixation, cells were stained with a 10% Giemsa solution (1:1 methanol/ glycerol) for 20 min and rinsed with water. Cell fusion was scored by light microscopy at a magnification of 250. Mononucleated myoblasts were distinguished from fibroblasts on the basis of morphological differences between these two cell types. Percentage fusion is defined as the ratio of the number of nuclei in myotubes to the total number of nuclei in both myoblasts and myotubes. For this purpose, cells containing three or more nuclei are considered myotubes. Each value represents the average of at least 10 randomly selected fields with a total number of nuclei >500. The variance between counts from different fields did not exceed 10% of the average value. Cell protein was determined by the method of Lowry et al. (27).

Measurement of Acetylcholine Receptor (AChR): AChR on the surface of intact muscle cells was measured by the specific binding of 125I-Bgt as described previously (38). After two washes with DME, cultures were incubated with 125I-Bgt (10 nM) in 1 ml DME containing 1 mg/ml BSA for 1 h at 37°C. Unbound toxin was removed by five washes with DME and cells were suspended in 1 N NaOH containing 1% Triton X-100. Radioactivity was determined by gamma spectroscopy. Nonspecific binding, established in replicate cultures in the presence of the competitive ligand decamethonium (10 μM), was subtracted, and it accounted for <10% of total labeling.

Indirect Immunofluorescence: Cultures were fixed with methanol for 10 min at -20°C. Prefixation with 3.7% formaldehyde yielded equivalent results. The fixed cells were rinsed with PBS and incubated with sheep anti-rat testis calmodulin (100 μg/ml in PBS containing 1% BSA) for 45 min at 37°C. The cells were then rinsed five times for 10 min in PBS and incubated with preadsorbed fluorescein isothiocyanate (FITC) rabbit anti-sheep antibodies (1:200 dilution in PBS containing 1% BSA) for 45 min at 37°C. The cells were washed five times with PBS and once with water, and were mounted with Aqua-Mount (Lerner Laboratories, New Haven, CT). FITC rabbit anti-sheep antibodies were preadsorbed on methanol-fixed muscle cell monolayers. Control staining, carried out using second antibody alone, yielded very low background fluorescence. Fluorescence was observed with a Zeiss fluorescence microscope equipped with epi-illumination and with the appropriate fluorescein excitation and emission filters. Images were photographed on Kodak Tri-X film (Eastman Kodak Co., Rochester, NY) at 1100 ASA.

RESULTS

Under the culture conditions used in this study, myoblasts undergo rapid fusion during the second day post-plating (Fig. 1). Synchronous fusion of aligned, bipolar myoblasts is initiated at ~24 h after plating and is essentially complete by 54 h post-plating. Addition of TFP to cultures, at 24 h after plating, inhibits subsequent fusion (Fig. 1). As can be seen, this effect is dose dependent; inhibition is significant at concentrations as low as 5 μM TFP and is essentially total at 10 μM TFP. The low concentrations of TFP that are effective in eliciting inhibition of myoblast fusion are similar to the TFP concentrations reported to inhibit calmodulin activity in vitro (ID50 = 10 μM; reference 50).

To establish further whether the TFP inhibition of fusion is consistent with the effect of this drug on calmodulin, we compared the effect of TFP with that of a related phenothiazine, CPZ, as well as with those of W-5 and W-7, calmodulin antagonists that are structurally unrelated to phenothiazines. The effects of various concentrations of CPZ on myoblast fusion are shown in Fig. 2. The percentage of fusion is decreased by CPZ in a dose-dependent manner, first detectable at 15 μM and attaining a maximum at 30 μM. In comparison, an approximately threefold-lower concentration of TFP is required for achieving an equivalent degree of fusion inhibition. A similar relationship has been reported for the
The relative potencies of CPZ and TFP in the inhibition of calmodulin in vitro (50). W-7, a nonphenothiazine inhibitor of calmodulin (13, 14), blocks myoblast fusion (Fig. 2). The W-7 concentration required for 50% inhibition of fusion is ~75 μM. A related sulfonamide derivative, W-5, is a less potent inhibitor of calmodulin (13). W-5 inhibits muscle fusion as well, but, as shown in Fig. 2, at significantly higher concentrations than W-7.

To test whether the inhibitory effect of TFP on myoblast fusion is reversible, TFP was added to cells at 24 h postplating, for 30 h. The drug-containing medium was then replaced by conditioned medium, and fusion was monitored during the subsequent 48 h. As shown in Fig. 3, the fusion block induced by the various concentrations of TFP is reversible. The extent of fusion increases gradually after TFP removal and reaches approximately control levels after 48 h. In comparison, the block of fusion was maintained in the presence of TFP (10 μM) during this interval (Fig. 3).

The requirement of the process of muscle fusion for extracellular calcium (8, 38, 39, 48) has been illustrated by the effectiveness of the calcium chelator EGTA in arresting myoblast fusion (39). As shown in Fig. 4 B, EGTA-arrested myoblasts display a characteristic elongated bipolar shape (see also reference 39), which is distinct from the morphology of prefusional myoblasts (Fig. 4 A) or fusion-arrested myoblasts obtained by various other means, such as treatment with bromodeoxyuridine, cycloheximide (18), a prostaglandin antagonist (7), and an inhibitor of protein glycosylation (34). As shown in Fig. 4, B and C, the appearance of cells inhibited from fusing by TFP is very similar to that of EGTA-arrested myoblasts. Myoblasts that are fusion-blocked with W-5, W-7, or CPZ closely resemble the TFP-arrested cells (results not shown). Untreated cultures of the same age are fully fused (Fig. 4 D).

A characteristic aspect of muscle differentiation is the expression of AChR on the cell surface (19). As shown in Fig. 5, the block of fusion induced by TFP does not impair the appearance of AChR. In both TFP-blocked and control cultures, the amounts of surface AChR increase sharply at similar rates during the second day in culture. In this respect, TFP treatment resembles Ca2+ depletion by EGTA (Fig. 5) or by replacement of growth medium with Ca2+-free medium (38).

![Figure 3](image-url) Figure 3. Reversibility of the effect of TFP on myoblast fusion. Cultures were treated with TFP at 24 h postplating for a 30-h interval. After this period, the drug-containing medium was replaced by conditioned medium, and percentage fusion was determined at the indicated times. Values shown are the averages of three determinations. Bars represent the range. Dashed line indicates percentage fusion in unreversed cultures that were exposed continuously to 10 μM TFP during this time interval. Amounts of protein per culture dish, determined in both control and reversed cultures, were similar.

![Figure 4](image-url) Figure 4. Effects of TFP and EGTA on myoblast fusion. (A) Prefusional myoblasts at 24 h postplating. (B) EGTA-treated cultures 54 h postplating; EGTA (1.8 mM) was added at 24 h postplating. (C) TFP-treated cultures 54 h postplating; TFP (7.5 μM) was added at 24 h postplating. (D) Control cultures at 54 h postplating. Cultures were fixed and stained as described under Materials and Methods. Note the morphological similarity between EGTA treated cells and TFP-treated cells, and the distinct morphology displayed by prefusional myoblasts. Bar, 50 μm. X 350.

![Figure 5](image-url) Figure 5. Appearance of surface AChR in control cultures (○), EGTA-treated cultures (■), and TFP-treated cultures (▲). EGTA (1.8 mM) and TFP (10 μM) were added to cultures at 24 h postplating. Surface AChR was measured by the specific binding of 125I-Bgt as described under Materials and Methods. Amount of protein per culture dish was determined in replicate cultures. Data points represent averages of triplicate measurements.
which inhibits myoblast fusion but allows the unimpaired expression of surface AChR.

To test whether a rise in intracellular Ca²⁺ will affect the TFP-induced inhibition of muscle fusion, we used the Ca²⁺ ionophore A23187, which has been shown to increase membrane permeability for this cation (46). Myoblasts were treated with the ionophore (0.5 μM) at 25 h postplating, 1 h after the addition of TFP. As shown in Fig. 6A, the inhibition of myoblast fusion caused by treatment with 10 μM TFP is clearly attenuated in the presence of A23187. The reversal by the Ca²⁺ ionophore is incomplete, allowing fusion to reach levels comparable to those observed in cells treated with 5 μM TFP. In comparison, the ionophore has no effect on the inhibition of muscle fusion by 5 μM TFP. As can be seen in Fig. 6, B–D, the inhibition of fusion by 10 μM TFP is characterized by reduced alignment of myoblasts (Fig. 6C), as compared with the inhibition obtained with 5 μM TFP (Fig. 6B). In the presence of the ionophore, cultures treated with 10 μM TFP display enhanced alignment (Fig. 6D) similar to that observed in cultures treated with 5 μM TFP (Fig. 6B).

To establish the presence and distribution of calmodulin, we used immunofluorescence microscopy, using anticalmodulin antibody. The immunofluorescent staining pattern of calmodulin in prefusional myoblasts (24 h postplating), fusing myoblasts, and fused myotubes is shown in Fig. 7. Appreciable staining of muscle cells is observed at all stages of development. Prefusional myoblasts display a diffuse staining pattern, with a somewhat higher intensity in perinuclear regions. In addition, a proportion of these myoblasts exhibits localized regions of intense staining at the cell tips (Fig. 7A, lower panel). This staining pattern appears to be transient, and shortly after onset of fusion, a uniform staining is again observed (Fig. 7B). The pattern of calmodulin staining is not affected by TFP treatment (results not shown).

**DISCUSSION**

In the present study we have investigated the effects of several pharmacological agents known to inhibit calmodulin. We have found that the phenothiazines TFP and CPZ, as well as the sulfonamide derivatives W-5 and W-7, inhibit myoblast fusion at doses that correspond closely to the doses reported to inhibit the activity of Ca²⁺-calmodulin in vitro (13, 14, 50).

TFP and other phenothiazines are known to have hydrophobic properties (47) that may result in membrane-perturbing effects (41, 43, 44). Several considerations argue against the possibility that the inhibitory effects of TFP on muscle fusion result from its membrane-perturbing properties. First, we observed that the phenothiazine CPZ, although it possesses hydrophobic properties similar to those of TFP, is less effective than TFP in blocking myoblast fusion. Second, we found that two other calmodulin inhibitors, W-5 and W-7, which are structurally unrelated to phenothiazines, inhibit myoblast fusion. Third, our observation that elevation of intracellular Ca²⁺ concentration by the Ca²⁺ ionophore A23187 diminishes the TFP-induced fusion block is consistent with an intracellular site of action for TFP.

The essential role of Ca²⁺ in mediating muscle fusion is well documented (8, 38, 39, 48, 49). Several aspects of the TFP-induced inhibition of muscle fusion support the possibility that TFP interferes with Ca²⁺-dependent mechanisms regulating fusion. TFP-arrested myoblasts display a unique bipolar elongated morphology which is similar to that of myoblasts prevented from fusing by Ca²⁺ deprivation (Fig. 4; see also reference 39). Furthermore, as in the case of EGTA-arrested myoblasts, the fusion block by TFP does not impair the elaboration of surface AChR. In addition, the inhibitory effect of TFP can be partially overcome in the presence of the Ca²⁺ ionophore A23187. This observation is consistent with the possibility that the TFP-induced block of muscle fusion is associated with impaired availability of cellular Ca²⁺. This interpretation is supported by a previous finding which suggested that an increase in intracellular Ca²⁺ constituted an essential step in myoblast fusion (8). Furthermore, TFP has been shown to inhibit Ca²⁺ transport processes in erythrocytes (15, 25, 45) and lymphocytes (26). The ability of the Ca²⁺ ionophore to reverse the inhibition by TFP of Ca²⁺-mediated processes has been reported previously (11).

It is noteworthy that the ionophore is effective in reversing the fusion block only to ~60% of the control levels. Conse-
FIGURE 7 Immunofluorescence staining of calmodulin in muscle cells. (a) Prefusional myoblasts 24 h postplating. Note the elevated staining at the tip of the prefusional myoblast shown in the lower panel. (b) Myoblasts 28 h postplating, shortly after onset of fusion. (c) Fused myotubes 96 h postplating. Cultures used for staining of fused myotubes were treated with cytosine arabinoside (10 μM) at 48 h postplating for 2 d to minimize fibroblast proliferation. Fixation and staining procedures are described under Materials and Methods. Bar, 20 μm. × 725.

quentely, the reversal of the fusion block is apparent only at maximal levels of fusion inhibition obtained with 10 μM TFP and is not detected under conditions of partial fusion block induced by 5 μM TFP (Fig. 6). Several studies have noted that fusion of cultured myoblasts is preceded by alignment of mononucleated cells (8, 21, 22, 32). Various procedures that cause fusion-arrest result in impaired alignment (16, 39, 48), whereas others do not (1, 16, 32), which indicates that myoblast alignment and fusion constitute distinct steps in the formation of multinucleated myotubes. Of relevance is our observation that the alignment of fusion-blocked myoblasts is impaired at the higher TFP concentration used, but appears to be unaffected at lower concentrations of the phenothiazine. Moreover, the impairment of myoblast alignment by 10 μM TFP is to a large extent prevented in the presence of the Ca2+ ionophore. These results indicate that a component of the TFP block is associated with the Ca2+ requirement of myoblast alignment. This component can be overcome by the Ca2+ ionophore, and presumably reflects an inhibition by TFP of a Ca2+ entry mechanism. An additional component of the TFP block cannot be overcome by the ionophore and is apparently associated with the interference of TFP with subsequent steps, independent of Ca2+ entry, that mediate muscle fusion.

The phenothiazine TFP has been shown to bind tightly to purified Ca2+-calmodulin (23, 24) and to inhibit calmodulin activity by preventing its interaction with receptor enzymes (2, 25, 51). Recently, TFP and other phenothiazines have also been shown to interact with Ca2+-binding proteins distinct from calmodulin (4, 30, 31). We have found that muscle fusion is inhibited by the phenothiazines TFP and CPZ, as well as by W-7 and W-5, calmodulin antagonists that are structurally unrelated to the phenothiazines. These findings are consistent with the possibility that TFP exerts its inhibitory effect on myoblast fusion by interfering with either calmodulin or pharmacologically closely related Ca2+-binding protein(s). The capacity of the various pharmacological agents used in this study to inhibit myoblast fusion, at doses that in each case closely correspond to their antagonistic effect on calmodulin activity in vitro, further suggests that the inhibition of muscle fusion results primarily from inhibition of Ca2+-calmodulin.

What role may calmodulin play in the regulation of muscle fusion? Calmodulin has been shown to activate at least two processes responsible for controlling intracellular concentrations of Ca2+: the Ca2+ pumps of the plasma membrane and the sarcoplasmic reticulum (for review see reference 20). The sensitivity of the fusion process to TFP and the ability to partially overcome the inhibitory effect of TFP by increasing Ca2+ entry to cells may reflect the participation of calmodulin in the regulation of Ca2+ transport mechanisms required for fusion. Based on our observations that the reversal of TFP fusion block by ionophore-induced Ca2+ entry is incomplete, it appears likely that the transduction of the Ca2+ requirement for muscle fusion involves an additional calmodulin-mediated regulatory mechanism.

Ultrastructural studies of muscle fusion have indicated that the formation of particle-depleted plasma membrane domains is an essential step in myoblast fusion (19). In view of the increasing evidence for the role of cytoskeleton in modulating cell-surface organization (40), the changes in plasma membrane properties that accompany fusion may be regulated by...
cytoskeletal rearrangement. Recent findings which indicate that localized changes in the organization of cytoskeletal components subjacent to the plasma membrane at the fusion tips of myoblasts are relevant (10). The potential role of calmodulin in regulating cytoskeletal organization is suggested by the observations that calmodulin mediates the calcium-dependent assembly-disassembly of microtubules (28) and that this protein activates a specific myosin light-chain kinase, which in turn regulates the activity of the myosin associated with microfilaments (6). Therefore, the participation of calmodulin in the process of muscle fusion may involve changes in cytoskeletal organization, which in turn alter surface membrane properties. The elevated staining of calmodulin at the tips of fusion-competent myoblasts, observed in the present study, appears to be spatially correlated with areas of specialized cytoskeletal organization observed previously (10). Moreover, the expression of both properties is transient and occurs at the onset of muscle fusion. This correlation is consistent with the possibility that calmodulin may play a role in the production of localized changes in cell-surface organization compatible with myoblast fusion.

The fusion of mononucleated myoblasts to form multinucleated myotubes is a striking aspect of muscle differentiation. Numerous studies have shown fusion of muscle cells to be a multistep process involving a variety of coordinated changes in cell-surface structural and functional properties (12, 16, 18, 19, 42). The requirement for Ca\textsuperscript{2+} of the process of muscle fusion has been known for more than a decade. However, the mechanisms by which Ca\textsuperscript{2+} exerts its essential role in fusion of myoblasts are relevant (10). The potential role of calmodulin in regulating cytoskeletal organization is suggested by recent findings which indicate that calmodulin activates a specific myosin light-chain kinase, which in turn regulates the activity of the myosin associated with microfilaments (6). Therefore, the participation of calmodulin in the process of muscle fusion may involve changes in cytoskeletal organization, which in turn alter surface membrane properties. The elevated staining of calmodulin at the tips of fusion-competent myoblasts, observed in the present study, appears to be spatially correlated with areas of specialized cytoskeletal organization observed previously (10). Moreover, the expression of both properties is transient and occurs at the onset of muscle fusion. This correlation is consistent with the possibility that calmodulin may play a role in the production of localized changes in cell-surface organization compatible with myoblast fusion.

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