BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 74–79 (1997)
ARTICLE NO. RC976222

Hypotonicity and Thrombin Activate Taurine Efflux in BC_3H1 and C_2C_{12} Myoblasts That Is Down Regulated during Differentiation

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Received January 20, 1997

The efflux of organic osmolytes such as taurine is an important mechanism by which cells regulate their volume. The effects of hypotonicity and thrombin on taurine efflux were studied in BC_3H1 and C_2C_{12} cells, two mouse myoblastic cell lines that can be induced to differentiate with serum deprivation. In proliferating cultures of both cell types preloaded with [^{3}H]taurine, exposure to 27% hypotonicity activated a 10- to 20-fold increase in [^{3}H]taurine efflux (J_{tau}). This effect was blocked by the Cl^- channel inhibitors NPPB and flufenamic acid. Thrombin and the thrombin receptor agonist SFLLRN also activated J_{tau} that was abolished by NPPB and flufenamic acid. Together, hypotonicity and thrombin synergistically activated J_{tau}. In differentiated myocytes, the effect of thrombin was abolished, while that of hypotonicity was significantly reduced. These results suggest that (i) hypotonicity and thrombin activate taurine-permeable anion channels in BC_3H1 and C_2C_{12} cells, and (ii) these anion channels may be involved in cell proliferation.

One of the mechanisms utilised by most mammalian cells to reduce their volume following swelling caused by hypotonic shock is the rapid efflux of organic osmolytes (1). These organic osmolytes can be found in high concentrations in the cytosol; they constitute a group of structurally dissimilar molecules such as amino acids, polyols, and methylamines, and they are represented in most studies by the amino acid taurine (2). Recently, it has been suggested that hypotonic swelling-activated efflux of organic osmolytes is mediated by volume-sensitive anion channels in some cell types (2).

In addition to hypotonicity, Cl^- channels can be activated by several chemical stimuli including endogenous agonists (3). The serine protease thrombin, in particular, has been shown to activate Cl^- channels in human megakaryocytes (4) and in *Xenopus* oocytes microinjected with mRNA from thrombin-responsive CCL39 cells (5), to stimulate ^{125}I efflux in endothelial cells (6), and to potentiate the effect of hypotonicity on Cl^- efflux in intestine 407 cells (7). These findings imply that thrombin may also affect organic osmolyte fluxes; however, no studies on the effects of thrombin on organic osmolyte fluxes have been reported to date.

The BC_3H1 and C_2C_{12} myogenic cell lines have been extensively used as models for the study of the process of muscle differentiation. Removal of serum from the growth medium causes proliferating BC_3H1 and C_2C_{12} cells to withdraw from the cell cycle and to differentiate to a non-proliferative state with muscle-specific properties (8–14). Using these two cell lines, we recently showed that volume-activated Cl^- currents are present in proliferating cells, but their expression is down regulated in differentiated cells (15). From this close correlation between volume-activated Cl^- currents and proliferation we hypothesised that these currents may be involved in cell proliferation.

No information is available on organic osmolyte efflux in BC_3H1 and C_2C_{12} cells and how it may be affected by the proliferative state of the cells. In the present study we report that in proliferating BC_3H1 and C_2C_{12} cells, hypotonicity and thrombin i) both activate [^{3}H]taurine efflux (J_{tau}) that is inhibited by anion channel blockers, and ii) together they have a synergistic effect on J_{tau}. Furthermore, we show that hypotonicity- and thrombin-activated J_{tau} is potently down regulated during muscle differentiation. These results suggest that volume- and agonist-sensitive anion channels mediate efflux of taurine in BC_3H1 and C_2C_{12} cells, and further support the idea that anion channels may be involved in cell proliferation.

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Abbreviations: HTS, 27% hypotonic solution; J_{tau}, [^{3}H]taurine efflux; FCS, fetal calf serum.

0006-291X/97 $25.00

MATERIALS AND METHODS

Materials. NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) was obtained from RBI (Natick, MA). Human α-thrombin was obtained from Calbiochem (La Jolla, CA) and it had a fibrinogen clotting activity of 2800 NIH units (U)/mg. Each unit was equivalent to a thrombin concentration of approximately 10 nM. The thrombin receptor-agonist peptide SFLLRN and the proteinase-activated receptor-2 (PAR-2) agonist peptide SLIGRL were purchased from Neosystem (Strasbourg, France). All cell culture solutions and reagents were supplied by Life Technologies (Gibco BRL, UK). All other chemicals were from Sigma.

Cell culture and myocyte differentiation. BC_3H1 cells, initially derived from a mouse neoplasm (11), were obtained from ATCC (Rockville, MD), and were grown in DMEM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/l streptomycin. C_2C_{12} cells, originally derived from injured thigh muscle of 2-month-old normal mice (12), were also supplied by ATCC, and were grown in the above DMEM with the addition of 0.5% chick embryo extract. Both cell types were cultured and passaged as previously described (15). Differentiation was induced by reducing FCS to 0.5% (BC_3H1 cells), or by switching to medium containing 1% horse serum and 0.5% insulin-transferrin-sodiumselenite, but no FCS (C_2C_{12} cells) (15).

Measurement of J_{tau}. Following trypsinization, 5000 cells/cm^2 were seeded on gelatin-coated wells of 12-well culture plates. Proliferating or differentiated monolayers of BC_3H1 or C_2C_{12} cells were incubated with 1 to 2 μCi of [^{3}H]taurine (Amersham, UK) at 37°C. After 3 to 6 h the plates were transferred to a Kreb's solution containing (in mM): NaCl 150, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10, pH 7.4 with NaOH, at 22°C. The osmolality of this solution was approximately 320 ±5 mOsm (isotonic solution), as measured with a vapour pressure osmometer (Wescor 5500, Schlag Instruments, Gladbach, Germany). A 27% hypotonic solution (HTS) was obtained by reducing NaCl concentration in the above solution to 90 mM. Cells were washed with isotonic solution 6 to 8 times over a 25-30 min period. The final wash was removed after 2 or 3 min and transferred directly to a vial containing scintillation liquid. This procedure was repeated throughout the duration of the experiment, with the isotonic solution being replaced with the appropriate isotonic or hypotonic solutions containing the compounds under study.

At the end of an experiment, cells were lysed with 0.5 N NaOH, and [^{3}H]taurine was counted in a liquid scintillation counter. The counts present in all collected fractions and those remaining in the well at the end of the experiment were added to obtain the total counts used for calculations. J_{tau} at each time point was calculated as fractional release, i.e., the radioactivity released in each fraction as a percentage of the total radioactivity present in the cells. Stimulated J_{tau} is expressed as fold increase over J_{tau} in corresponding control wells in the same plate kept in isotonic solution and without thrombin and treated identically (basal J_{tau}).

Pooled data are given as mean ± SEM. For calculating statistical significance, the Student's *t*-test was used (level of significance, $P < 0.05$).

RESULTS AND DISCUSSION

Hypotonicity Activates J_{tau}

Following a 3 to 6 h loading period with [^{3}H]taurine and repeated washing in isotonic solution, basal J_{tau} in both BC_3H1 and C_2C_{12} muscle cells was low and rather stable. Exposure to HTS significantly increased J_{tau} in both cell types. The time dependency of HTS-activated J_{tau} is shown in Fig. 1A. The stimulatory effect of HTS in both cell types reached a maximum at 6-8 min, and then it gradually declined in the continuing presence of the hypotonic stimulus (Fig. 1A). Moreover, HTS-activated J_{tau} in both cell types was significantly inhibited by the anion channel blockers, NPPB, 100 μM, and flufenamic acid 500 μM (Fig. 1B).

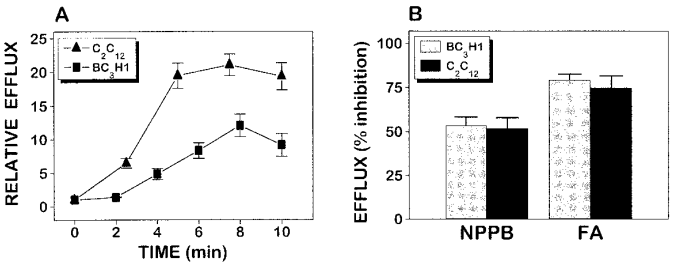

FIG. 1. (A) Effect of hypotonicity on J_{tau} in BC_3H1 and C_2C_{12} cells. At 0 min, cells were exposed to either isotonic solution or HTS, and J_{tau} was measured at regular intervals for up to 10 min. HTS-activated J_{tau} data are expressed as fold increase over basal J_{tau} at each time point. Values represent mean ± SEM from 3 experiments, each done in triplicate wells. (B) Inhibition of HTS-activated J_{tau} by anion channel blockers. Cells were exposed to HTS in the presence or absence of 100 μM NPPB or 500 μM flufenamic acid (FA). Data are expressed as percent inhibition of HTS-activated J_{tau} in the presence of a blocker. Values represent mean ± SEM from 4–7 wells for each condition from at least 2 different experiments.

Studies in several cell types (reviewed in (2)) have suggested that HTS-activated efflux of taurine and other organic osmolytes is mediated by anion channels. This appears to be the case also in BC_3H1 and C_2C_{12} cells, as HTS-activated J_{tau} was inhibited by two established anion channel blockers, NPPB and flufenamic acid. In a previous study, we showed that HTS activates Cl^- currents in BC_3H1 and C_2C_{12} cells, and these currents exhibit similar degrees of inhibition by the same doses of the two blockers (15). Taken together, these results suggest that Cl^- currents and J_{tau} activated by HTS in BC_3H1 and C_2C_{12} cells may be mediated by the same volume-sensitive anion channels.

Thrombin Activates J_{tau}

Thrombin, 1 U/ml, activated J_{tau} in BC_3H1 and C_2C_{12} muscle cells. This J_{tau} peaked at 2-4 min, and had returned to basal levels by 8-10 min (Fig.2A). In comparison with HTS-activated J_{tau}, the thrombin-activated J_{tau} had a small magnitude reaching a maximum of 2 fold increase in C_2C_{12} and a 3-4 fold increase in BC_3H1 cells (Fig.2A). A higher dose of thrombin (3 U/ml) did not activate significantly more efflux (data not shown). Thrombin-activated J_{tau} in BC_3H1 cells was abolished by NPPB, 100 μM, and flufenamic acid, 500 μM (Fig. 2B), suggesting that this efflux is mediated by an anion channel. In addition to thrombin, the peptide agonist of the thrombin receptor SFLLRN, 100 μM, also stimulated J_{tau} (Fig. 2B). Since the cloning of a G-protein-

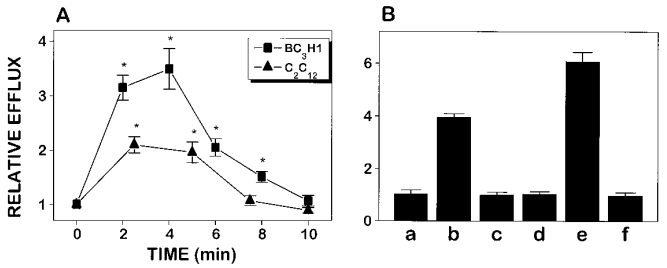

FIG. 2. (A) Effect of thrombin on J_{tau} in BC_3H1 and C_2C_{12} cells. At 0 min, cells were exposed to 1 U/ml thrombin, and J_{tau} was measured at regular intervals for up to 10 min. Thrombin-activated J_{tau} data are expressed as fold increase over basal J_{tau} at each time point. Values represent mean ± SEM from 3 experiments, each done in triplicate wells. (B) Properties of thrombin-activated J_{tau} in BC_3H1 cells. (a) Basal J_{tau}; (b-d) 3 U/ml thrombin-activated J_{tau} in the absence (b) and presence of 100 μM NPPB (c) or 500 μM flufenamic acid (d); (e) 100 μM thrombin receptor agonist peptide (SFLLRN)-activated J_{tau}, (f) 100 μM PAR-2 receptor agonist peptide (SLIGRL)-activated J_{tau}. Data are expressed as fold increase over basal J_{tau}. Values represent mean ± SEM from 4–6 wells for each condition from at least 2 different experiments.

coupled thrombin receptor and the discovery that it can be activated by synthetic peptides mimicking the amino acid sequence of the exposed amino terminus of the thrombin- cleaved receptor molecule (16), the ability of such peptides to reproduce an action of thrombin is taken as evidence that this particular action is mediated by the thrombin receptor (16,17). Remarkably, 100 μM of SLIGRL, a peptide agonist for the recently discovered proteinase-activated receptor (PAR-2) that is not activated by thrombin (18) was ineffective in activating J_{tau} (Fig. 2B). Similar results were obtained in C_2C_{12} cells (data not shown). Taken together, these results suggest that thrombin receptors are present in BC_3H1 and C_2C_{12} cells, and they are coupled to activation of a taurine-permeable anion channel.

We are aware of only two studies on the effects of thrombin in BC_3H1 cells (19,20), while to the best of our knowledge no such studies are available in C_2C_{12} cells. Our results provide indirect evidence that thrombin receptors i) are present in BC_3H1 and C_2C_{12} cells, and ii) they are coupled to taurine-permeable anion channels. As taurine permeation is a known property of the volume-sensitive Cl^- channel in several cell types (2), it is tempting to speculate that the effect of thrombin may result from an indirect effect through one of the many signalling pathways/cellular responses activated by thrombin (reviewed in 17) and ultimately leading to activation of the volume-sensitive Cl^- channel. Such cellular responses could include actin cytoskeletal rearrangement leading to changes in cell shape, or other effects resulting in changes in cell volume (see below). Alternatively, thrombin may activate an anion channel distinct from the volume-sensitive Cl^- channel, which is also permeable to taurine.

Hypotonicity and Thrombin Together Have a Strong Synergistic Effect on J_{tau}

Co-stimulation of BC_3H1 or C_2C_{12} cells with thrombin and HTS resulted in a several fold increase in J_{tau}. The combined effect of the two stimuli was significantly higher than the sum of their individual effects (Fig. 3). The kinetics of the combined effect were reminiscent of the effect of thrombin, as activation of J_{tau} developed fast, peaked at 4 to 5 min and subsequently declined, despite the continuing increase measured in parallel wells activated only by HTS. Similar results were obtained with both BC_3H1 (Fig. 3A) and C_2C_{12} cells (Fig. 3B).

As discussed earlier, thrombin on its own activates J_{tau} which might be mediated through either activation of the volume-sensitive Cl^- channels activated also by HTS, or activation of a distinct species of anion channels. Our findings that thrombin and HTS have synergistic effects on J_{tau} support the idea that both stimuli may act on the same channel molecule. However, this does not exclude the possibility that the effects of thrombin on basal and HTS-activated J_{tau} may result from two distinct actions of this agonist: activation of an anion channel by its own, and potentiation of the effect of HTS on the volume-activated Cl^- channel.

Tilly and coworkers have shown that increased tyrosine phosphorylation mediates potentiation of HTS-activated ion efflux through K^+ and Cl^- channels in Intestine 407 cells (7). More recently, the same investigators reported that osmotic swelling is accompanied by a rapid and transient reorganisation of the F-actin cytoskeleton, which involves the ras-related GTPase $p21^{rho}$ and coincides with tyrosine phosphorylation of p125 focal adhesion kinase ($p125^{FAK}$) (21). On the other side, several mitogens (including thrombin) have been shown to induce rapid reorganisation of the cellular F-actin pattern involving the $p21^{rho}$ signalling pathway

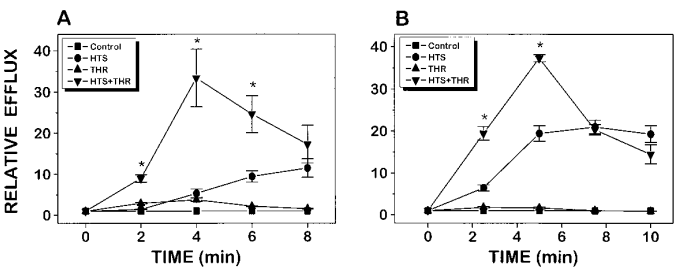

FIG. 3. Combined effect of HTS and thrombin on J_{tau} in (A) BC_3H1 and (B) C_2C_{12} cells. At 0 min, cells were exposed to either isotonic solution, 27% HTS, 1 U/ml thrombin, or both stimuli together, and J_{tau} was measured at regular intervals for up to 10 min. Stimulated J_{tau} data are expressed as fold increase over basal J_{tau} at each time point. Values represent mean ± SEM from 3 experiments, each done in triplicate wells. Asterisk (*) indicates values significantly ($p<0.01$) higher than the sum value of the separate effects of HTS and thrombin.

(22,23), and they also activate tyrosine phosphorylation of proteins associated with the cytoskeleton, including $p125^{FAK}$ (24). It is thus conceivable that stimulation by thrombin (and possibly other mitogens as well) of the volume-sensitive anion channel in BC_3H1 and C_2C_{12} cells may be mediated by tyrosine phoshorylation of $p125^{FAK}$ associated with focal adhesion complex formation and cytoskeletal changes. A report showing that the thrombin receptor induces tyrosine phosphorylation of several substrates in BC_3H1 cells fits well to this hypothesis (20).

Activated J_{tau} Is Down Regulated during Muscle Differentiation

In response to reduced serum conditions, BC_3H1 cells reversibly withdraw from the cell cycle and start expressing a variety of muscle-specific proteins such as skeletal and smooth muscle cell actins, creatine phosphokinase, and nicotinic acetylcholine receptor (8,9, 10,11). Similarly, following FCS withdrawal, C_2C_{12} cells start expressing several skeletal muscle characteristics, and eventually they commit to terminal, non-reversible differentiation by fusing to form polynucleated myocytes (12,13,14). Recently, we showed that volume-activated Cl^- currents are down-regulated in differentiated BC_3H1 and C_2C_{12} cells. As Cl^- currents and efflux of osmolytes both appear to be mediated by the same anion channels (2), we hypothesised that HTS-activated J_{tau} may be also down regulated in differentiated BC_3H1 and C_2C_{12} myocytes.

Following reduction of the serum content of the growth medium for BC_3H1 cells from 10% to 0.5%, proliferation stopped, and the cells gradually changed to a spindle-shaped non-fusing phenotype. At various days after serum reduction, basal, HTS- and thrombin-stimulated J_{tau} were measured. As it can been seen in Fig. 4, HTS-activated J_{tau} was significantly reduced already 4 days after serum deprivation and remained reduced even after 10 days (Fig. 4A). In control cells that had remained in normal serum conditions for more than 10 days, HTS could still elicit significant J_{tau} (Fig. 4A). Even more dramatic results were obtained with thrombin-activated J_{tau}, which was totally abolished in cells deprived from serum for 4 to 10 days, but remained present in control cells kept in serum-containing medium for similar periods of time (Fig. 4B). Finally, the synergistic effect of HTS and thrombin was also significantly down regulated in serum-deprived BC_3H1 cells (Fig. 4C). It is noteworthy that, upon re-introduction of serum and subsequent de-differentiation, the cells regained their proliferative, non-differentiated phenotype as well as their ability to fully respond to both HTS and thrombin (data not shown).

Similar results were obtained with C_2C_{12} cells. These cells stopped proliferating and gradually differentiated to a muscle-like phenotype after switching from a medium with 10% FCS to one with only 1% horse serum. In the differentiated myocytes, the stimulatory effect of HTS on J_{tau} alone or together with thrombin was significantly reduced, while the effect of thrombin alone was abolished (Fig. 5A-C).

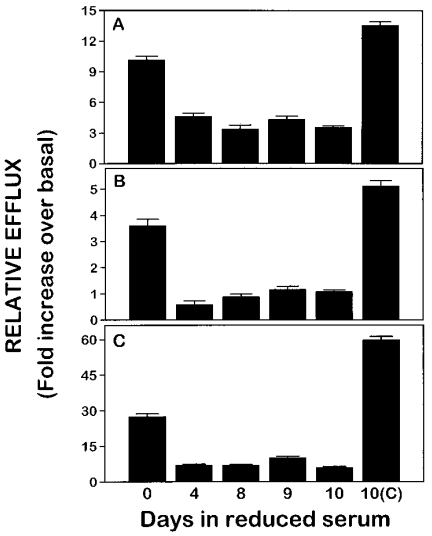

FIG. 4. Down regulation of HTS-activated J_{tau} in differentiated BC_3H1 cells. Two days after plating, one plate was immediately assayed (day 0 in graph), another was transferred to fresh normal medium (control plate), while 4 others were transferred to differentiation medium. At regular intervals (4 to 10 days), a plate was transferred at 22°C, and cells were stimulated for 5 min with either 27 % HTS (A) or 1 U/ml thrombin (B), or both stimuli together (C). J_{tau} was measured as described under Methods. The control plate was terminated on day 10 and processed identically. Stimulated J_{tau} data are expressed as fold increase over basal J_{tau}. Values represent mean ± SEM of triplicate determinations from one experiment out of 3 with similar results.

It is not clear from the present experiments whether the down regulation of HTS-activated J_{tau} in differentiated muscle cells is due to reduced expression of the channel protein mediating this efflux or to changes in the transduction mechanism linking the osmotic signal to the channel mediator. Several changes in ion channel expression have been documented during muscle differentiation, including changes in the expression pattern of voltage-sensitive K^+ channels (14), and increased expression of voltage-sensitive Ca^{2+} and Na^+ channels (9,13).

We recently found that both proliferating and differentiated BC_3H1 and C_2C_{12} cells swell to a similar extent in response to HTS (15), a fact that excludes the possibility that the down regulation of the volume-activated J_{tau} is due to the absence of cell swelling in the differentiated cells. Still, however, different responses to HTS-induced cell swelling between proliferating and differentiated cells might be due to structural differences between cells in the two states.

In primary cultures of skeletal muscle cells, it was recently shown that the thrombin receptor is present

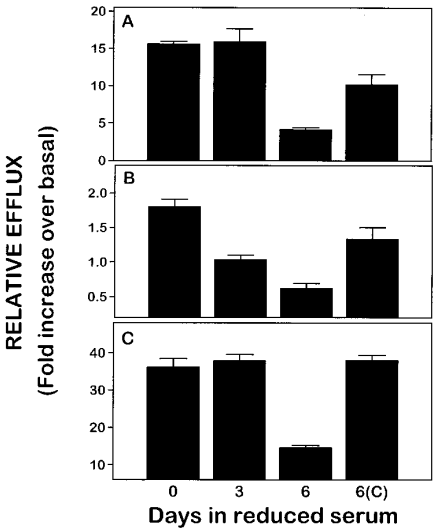

FIG. 5. Down regulation of HTS-activated J_{tau} in differentiated C_2C_{12} cells. Two days after plating, one plate was immediately assayed (day 0 in graph), another was transferred to fresh normal medium (control plate), while 2 others were transferred to differentiation medium. After 3 or 6 days, the plates were transferred at 22°C, and stimulated for 5 min with either 27% HTS (A) or 1 U/ml thrombin (B), or both stimuli together (C). J_{tau} was measured as described under Methods. The control plate was terminated on day 6 and processed identically. Stimulated J_{tau} data are expressed as fold increase over basal J_{tau}. Values represent mean ± SEM of triplicate determinations from one experiment out of 2 with similar results.

in proliferating myoblasts but disappears in fused myocytes (25). However, thrombin was able to stimulate proliferation in quiescent, differentiated BC_3H1 cells (19). In addition, the levels of J_{tau} obtained in differentiated BC_3H1 and C_2C_{12} cells in the presence of both HTS and thrombin were significantly higher than the levels obtained by HTS alone (Figures 4,5), suggesting that thrombin still exerts some effects on the differentiated cells. Thus, it is unlikely that the complete inhibition of thrombin-activated J_{tau} in myocytes is due to a loss of thrombin receptor expression upon differentiation.

The existence of a tight link between cell volume and cell proliferation has been proposed (26,27), and several studies have suggested that Cl^- channels may play a role in the regulation of cell proliferation (28-31). Accordingly, we recently showed that growth arrest and differentiation of BC_3H1 and C_2C_{12} cells is coupled with a down regulation of volume-activated Cl^- currents (15). The demonstration in the present study that efflux of the organic osmolyte taurine in BC_3H1 and C_2C_{12} cells is mediated by volume- and agonist-sensitive anion channels and is also down regulated during differentiation, further supports the idea that Cl^- channels may play a role in cell proliferation through regulation of cell volume.

In summary, we have shown that in BC_3H1 and C_2C_{12} muscle cell lines: i) HTS and thrombin activate J_{tau} that is mediated by one or more anion channels, ii) together these two stimuli have a synergistic effect, and iii) stimulated J_{tau} is significantly down regulated in non-proliferating myocytes. These results suggest that the efflux of osmoregulatory molecules in BC_3H1 and C_2C_{12} cells is mediated by anion channels and these channels are down regulated during muscle differentiation. They also provide support for the idea that myoblasts may require osmolyte-permeable anion channels to constantly adjust their volume during cell growth and division, a requirement that ceases to exist in non-proliferating myocytes.

ACKNOWLEDGMENTS

We thank Anja Florizoone and Marina Crabbé for expert technical assistance. V.G.M. was supported by a fellowship from the EU (Contract ERB-CHBG-CT94-0754).

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