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PERTUSSIS TOXIN PRETREATMENT ABOLISHES DIHYDROPYRIDINE INHIBITION OF CALCIUM FLUX IN THE 235-1 PITUITARY CELL LINE

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In the present study we used 235-1 cells, a prolactin secreting clone derived from a pituitary tumor. In these cells maitotoxin, a calcium channels activator, likely acting on voltage sensitive calcium channels, increases intracellular free calcium measured by Quin 2 tecnique. Maitotoxin stimulation of calcium flux was inhibited both by nicardipine and verapamil in a dose dependent manner. Pertussis toxin pretreatment does not modify maitotoxin activation of calcium channels, while completely abolishes nicardipine inhibition of maitotoxin induced voltage sensitive calcium channels activation, without affecting verapamil effect. These results suggest a possible involvement of a pertussis toxin sensitive G protein in dihydropyridine inhibition of voltage sensitive calcium channels.

Calcium mobilization across cell membrane is recognized to regulate numerous cell functions. The identification of voltage sensitive calcium channels (VSCC) in several tissue districts and their relation with cellularfunction is now established. Muscle cells, cardiac cells, neuronal cells as well as endocrine cells share VSCC (1,2,3,4,5). Voltage sensitive calcium channels are now classified in three different types: T channels, N channels and L channels (6). The latter mediate slowly activated, long lasting calcium current at strong depolarization and are sensitive to dihydropyridine (DHP) compounds (6). In neuronal tissue DHP molecules affect calcium movement with-

Abbreviations:
VSCC, voltage sensitive calcium channels ; DHP, dihydropyridine
PTX, pertussis toxin ; MTX, maitotoxin

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out modifying neurotransmitters release (7), while in endocrine tissue a direct correlation between VSCC, DHP action and both calcium mobilization and hormonal release has been established (4,5,8). Recently DHP receptor has been structurally characterized at least in some tissues (9). Immunoprecipitating assays in muscle cells have clearly shown a 170 KD polypeptide (10,11) structurally correlated to a Na^+ channel which contains the binding site for DHP.

Until now the biochemical events involved in the regulation of VSCC remain unknown even if there is evidence for a modulation by second messengers such as cyclic AMP or the products of polyphosphoinositide hydrolysis (12,13,14).

GTP binding proteins are fast coming to occupy a central role in coupling membrane events. Their initial role in coupling occupied receptors to adenylate cyclase system activation (15) is now expanded to the modulation of polyphosphoinositide hydrolysis (16,17) and more recently to the opening of ionic channels (14,18,19,20). Indeed with the aid of pertussis toxin (PTX), a G protein responsible of the opening of K^+ channels (Gk) has recently been purified (22) as well as a G protein that mediates neurotransmitters induced inhibition of VSCC (14). Thus the existence of an endogenous ligand that, upon binding to GTP, opens calcium channels in the endoplasmic reticulum has been proposed (23).

In the present study we tested the effect of PTX pretreatment on DHP inhibition of VSCC.

MATERIALS AND METHODS

- **Cells culture:** Here we used 235-1 cells, a clone derived from the 7315a, a transplantable pituitary tumor that secretes rat prolactin (24). The cells grow in culture flaskes or Petri dishes in RPMI 1640 medium containing 2.5% foetal calf serum and 7.5% horse serum (Flow), pennicillin G (100 U/ml) and streptomycin (1 ug/ml) (Squibb). 2-3 days before the experiment the cells, from a flask at 60-80% of confluency, were splitted and plated in Petri dishes. In the present study we use the cells from passsage 50 to passage 70.
- **Measurement of intracellular free Ca^{++}:** Cytosolic free calcium was measured

by Quin 2 tecnique (25). 235-1 cells were detached from the culture flaskes or Petri dishes by a rapid treatment with trypsin 0.25% (Flow) and then resuspended in 20 ml RPMI serum free (Flow). The cells were washed twice in RPMI serum free. For Quin 2 loading the cells were resuspended in RPMI serum free containing 0.5% bovine serum albumine (10.000.000 cells/ml). Aliquots of 20 mM Quin 2 acetoxymethylester (Amersham) in dimethylsulphoxide (Baker) were added in order to obtain a final concentration of 50 μM and cells suspension was incubated with gentle agitation at 37° C for 25 min. The cells were then diluted to approximately 1 million/ml by the addition of RPMI serum free and were further incubated at 37° C for 70 min. After the second incubation the cells were washed in RPMI serum free and finally resuspended in the same medium at the density of 3 millions/ml. Before fluorescence determination, one aliquot was centrifuged and resuspended in 2 ml of Hank's Balanced Salt Solution (GIBCO) plus $MgCl_2$ 1 mM (Baker), $CaCl_2$ 2mM (Fisher), BSA 0.2 mg/ml (SIGMA), NaH_2CO_3 0.35 mg/ml (GIBCO). The cells suspension was then placed in a quartz cuvette in a Perkin-Elmer spectrophotofluorimeter and continuosly gently stirred. Cytosolic calcium concentrations were calculated with the calibration procedure by Tsien et al. Each experiment was repeated at least three times.

- **Substances:** Nicardipine was solubilized in 20% methanol-80% distilled water as a stock solution of $2x10^{-2}$ M and then diluted in distilled water. 10 ul aliquots were added to the cells suspension from a stock solution 200 fold concentrated. The same procedure was followed to make up verapamil but it was solubilized in 50% methanol-50% distilled water. Maitotoxin was isolated and purified by Dr. T. Yasumoto (26). The concentration of maitotoxin, whose molecular weight has not yet been determined, was expressed gravimetrically $1x10^{-9}$=1 ng/ml; $5x10^{-9}$=5 ng/ml; $1x10^{-8}$=10 ng/ml. The compound was dissolved in 10% methanol-90% distilled water as a stock solution in a concentration of 0.1 mg/ml. The solution was further diluted (1:10) with distilled water and stocks were stored at -20° C until used.

RESULTS AND DISCUSSION

In the present study we used maitotoxin (MTX) a VSCC activator (27), calcium channels antagonists such as the DHP derivative nicardipine, the diphenylalchilamine derivative verapamil, and PTX.

Maitotoxin is a toxin, isolated and partially purified from a marine dinoflagellate Gamberidiscus Toxicus (26). Maitotoxin has positive inotropic effect, contracts smooth muscles (28), stimulates neurotransmitters release from PC12 pheocromocitoma cells (29) and neuroblastoma cells (27), increases intracellular Ca^{++}, cAMP levels and adenohypophyseal hormone release from primary culture of pituitary cells (30) and prolactin secreting GH3 clonal cells (8). Maitotoxin stimulates also calcium flux in PC12 cells (29), normal pituitary

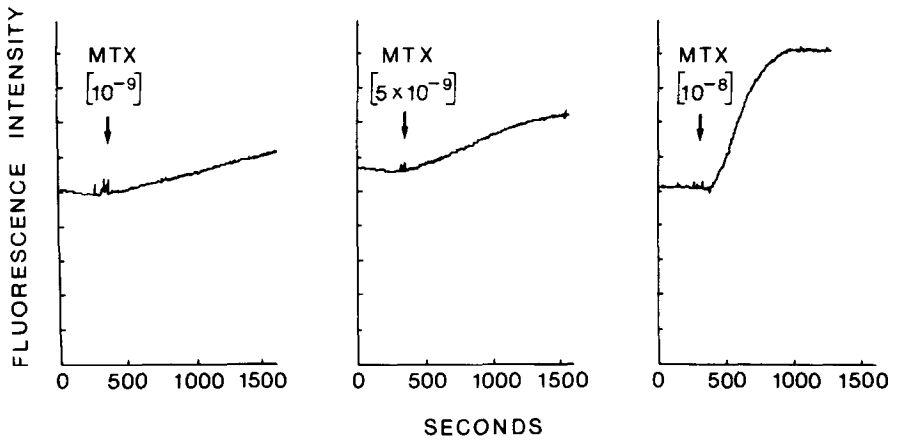

FIGURE 1

Maitotoxin stimulation of free intracellular Ca^{++} in 235-1 clonal cells measured with Quin 2 fluorescent probe. The initial concentration of cytosolic free Ca^{++} in the cells was 84-83-84 nM in the three experiments represented; after the addition of MTX $1x10^{-9}$ and MTX $5x10^{-9}$ the value was of 187 nM and 206 nM respectively; with MTX $1x10^{-8}$, intracellular calcium concentration rised up to 4168 nM.

cells (30), GH3 clone (8) and neuroblastoma cell line (27). These effects appear to take place through the activation of a calcium channel. Indeed Freedman et al. showed that MTX interacts with VSCC in cultured neuroblastoma cells (27). 235-1 clonal pituitary cells respond with enhanced prolactin secretion to stimuli that increase cellular cAMP (31,32) or calcium mobilization, such as MTX (32), moreover PTX pretreatment enhances PGE_1, cholera toxin and forskolin stimulation of cAMP production.

As shown in fig.1, MTX dose dependently enhanced free intracellular calcium measured with the fluorescent probe Quin 2 (25). Similarly the toxin enhanced the release of prolactin from 235-1 (data not presented), as already previously shown by Schettini et al. (32). The effect of MTX on free intracellular calcium measured with Quin 2 shows a quite rapid onset, reaching the maximal stimulation at 10 ng/ml of MTX. With this concentration the time dependent change of cytosolic free calcium resemble the modification of calcium fluxes induced by MTX in dispersed anterior pituitary cells in suspension (30) or neuroblastoma cell line (27) measured with $^{45}Ca^{++}$ radioisotope.

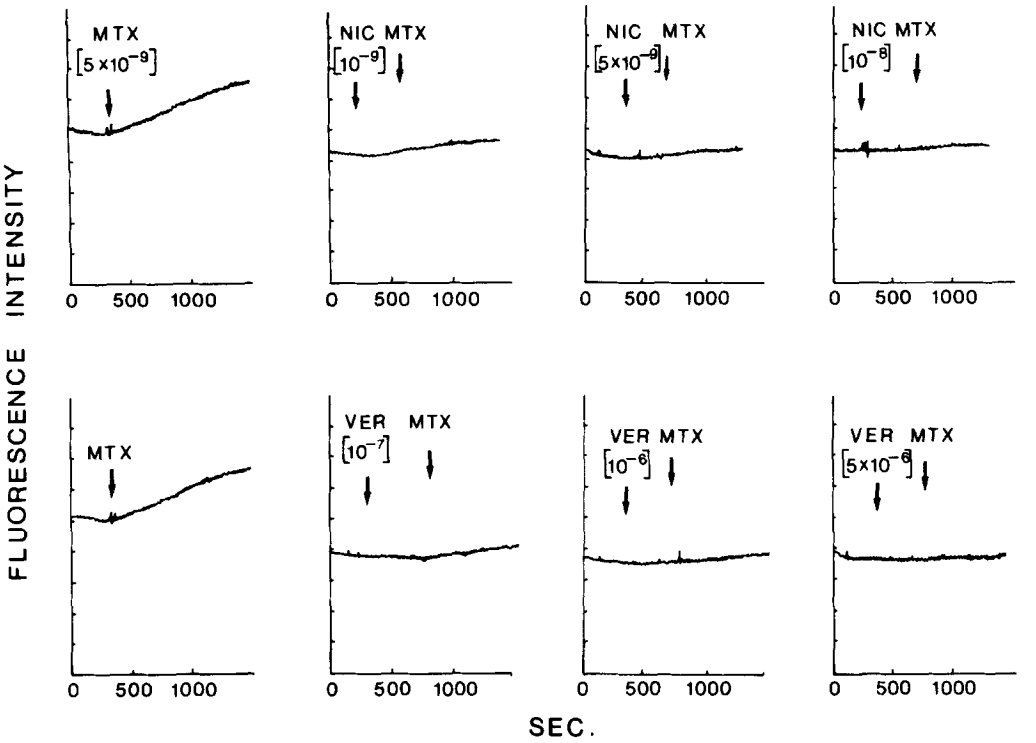

FIGURE 2

Effect of increasing concentrations of nicardipine and verapamil on MTX stimulation of intracellular Ca^{++}. As shown in the upper panel nicardipine inhibits MTX-induced increase of intracellular Ca^{++} concentrations; this effect was almost maximal at a nicardipine concentration of $5x10^{-9}$ M.

1) Basal Ca^{++}=83 nM; MTX ($5x10^{-9}$)=206 nM
2) Basal Ca^{++}=86 nM; Nic ($1x10^{-9}$)=80 nM; MTX ($5x10^{-9}$)+Nic ($1x10^{-9}$)=130 nM
3) Basal Ca^{++}=55 nM; Nic ($5x10^{-9}$)=52 nM; MTX ($5x10^{-9}$)+ Nic ($5x10^{-9}$)=73 nM
4) Basal Ca^{++}=76 nM; Nic ($1x10^{-8}$)=75 nM; MTX ($5x10^{-9}$)+Nic ($1x10^{-8}$)=84

Verapamil also reduces MTX effect on intracellular free calcium in a dose dependent manner:

5) Basal Ca^{++}=40 nM; MTX ($5x10^{-9}$)=206 nM
6) Basal Ca^{++}=79 nM; Ver ($1x10^{-7}$)=71 nM; MTX ($5x10^{-9}$)+Ver ($1x10^{-7}$)=122 nM
7) Basal Ca^{++}=61 nM; Ver ($1x10^{-6}$)= 56 nM; MTX ($5x10^{-9}$)+Ver ($1x10^{-6}$)=82
8) Basal Ca^{++}=58 nM; Ver ($5x10^{-6}$)=53 nM; MTX ($5x10^{-9}$)+Ver ($5x10^{-9}$)=63 nM

To check whether MTX action occurred through the activation of L type VSCC in these cells, we tested the effect of nicardipine and verapamil. Both agents dose dependently antagonized the MTX induced increase in free intracellular calcium (Fig.2). The effect of nicardipine was observed for nanomolar concentration which appears to be specific for VSCC as previously shown (27). Fur-

thermore MTX treatment did not affect DHP binding to specific receptors (27). Login et al. showed that the DHP compound nifedipine, inhibited $^{45}Ca^{++}$uptake induced by MTX in GH3 clonal cells (8). Verapamil antagonism of MTX action occurred for micromolar concentrations. Moreover nicardipine and verapamil did not antagonize the calcium influx stimulated by the ionophore ionomycin (data not presented). These results further support the evidence that MTX interacts with VSCC sensitive to DHP.

PTX, which ADP rybosilates GTP-binding protein Gi, Go, Gk (18,21,33), thereby preventing the agonist induced dissociation of the protein into active subunits, uncouples inhibitory receptors not only from the catalytic subunit of adenylate cyclase (15,21,33), as originally shown, but also impairs the coupling of occupied receptors with ionic channels (14,18,19,20). A GTP binding protein sensitive to PTX action has been shown to couple cardiac muscarinic receptors to K^+channels (19), serotonin and GABA receptors with the same K^+ channel in the hippocampus (34), norepinephrine and GABA B receptors to VSCC (14), somatostatinergic receptors in AtT20 clonal cells to VSCC (35). In our experiments PTX pretreatment abolishes nicardipine inhibition of MTX stimulated increase of free intracellular calcium, without affecting verapamil antagonism of MTX action(Fig.3). Moreover nicardipine did not inhibit basal or forskolin stimulated adenylate cyclase activity in 235-1 cell membranes(Basal=32.9±1.3;Nicardipine 10^{-6}=33.8±0.9;Forskolin 10^{-5}=127.8±3.5;Forskolin+Nic.=130.8±3.1 pmol cAMP/mg prot/min) showing that the DHP receptor is not coupled with adenylate cyclase enzyme. Whether the PTX substrate mediating the DHP inhibition of VSCC is a G protein structurally related to Gi or Go need to be clarified. However in 235-1 cells PTX ADP-rybosilated a G protein of apparent molecular weight of 39000 D (personal observation).

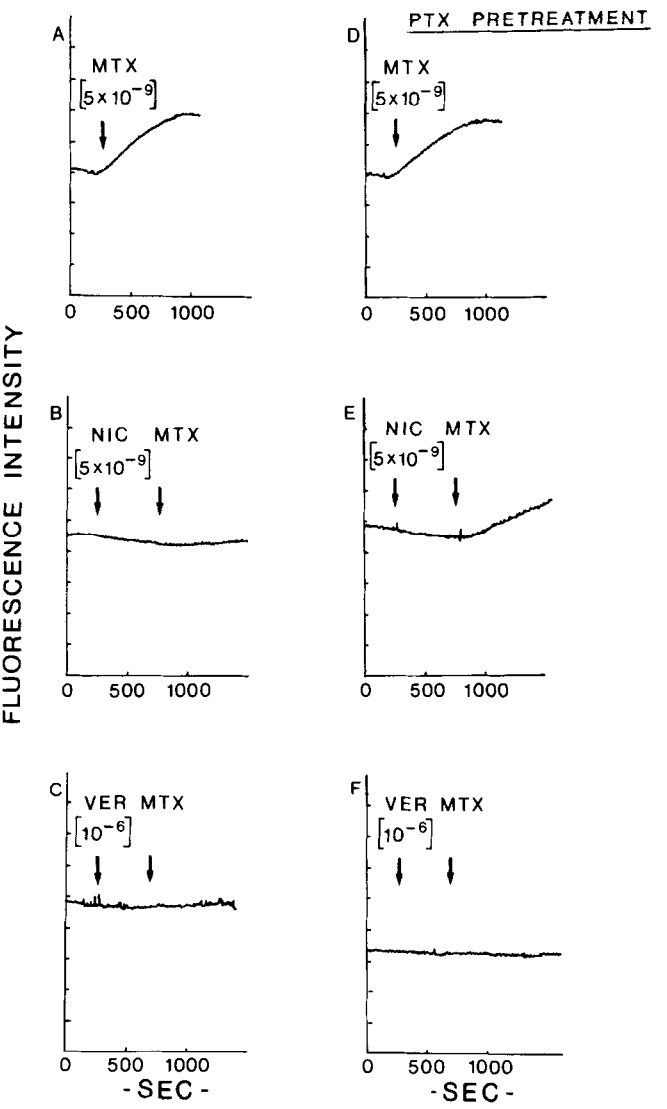

FIGURE 3

PTX pretreatment of 235-1 cells abolishes nicardipine but not verapamil inhibition of MTX stimulated increase in cytosolic free calcium. Before the experiment, growth medium was discarded and the cells were incubated at 37°C for 18 hours in RPMI serum free containing 150 ng/ml of PTX.
PTX was added to the medium just prior to use; the cells not treated with PTX were kept in the same condition and were incubated with RPMI serum free.
A) Basal Ca^{++}=93 nM; MTX ($5x10^{-9}$)=325 nM
B) Basal Ca^{++}=119 nM; Nic ($5x10^{-9}$)=101 nM; MTX ($5x10^{-9}$)+Nic ($5x10^{-9}$)=108 nM
C) Basal Ca^{++}=119 nM; Ver ($1x10^{-6}$)=112 nM; MTX ($5x10^{-9}$)+ Ver ($1x10^{-6}$)=119nM
D) Basal Ca^{++}=113 nM; MTX ($5x10^{-9}$)=294 nM
E) Basal Ca^{++}=97 nM; Nic ($5x10^{-9}$)=86 nM; MTX ($5x10^{-9}$)+Nic ($5x10^{-9}$)=195 nM
F) Basal Ca^{++}=83 nM; Ver ($1x10^{-6}$)=68 nM; MTX ($5x10^{-9}$)+Ver ($1x10^{-6}$)=83 nM

In conclusion these results suggest that a GTP binding protein could be involved in mediating the DHP inhibition of VSCC.

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