Biochemical Pharmacology, Vol. 32, No. 2, pp. 249–252, 1983.
Printed in Great Britain.

0006-2952/83/020249-04 $03.00/0

INHIBITION OF TRANSMITTER RELEASE BY TI233,* A CALMODULIN ANTAGONIST, FROM CLONAL NEURAL CELLS AND A PRESUMED SITE OF ACTION

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(*Received* 27 *April* 1982; *accepted* 15 *July* 1982)

Abstract—Effects of TI233, a calmodulin antagonist, on transmitter release were studied using a clonal pheochromocytoma cell line (PC12h). TI233, at a concentration of 30 μM, completely suppressed the release of preloaded [^{3}H]NE and [^{3}H]DA. The 50% suppression dose was around 3 μM. TI233 did not inhibit the [^{3}H]NE release evoked by the calcium ionophore A23187. Electrophysiological examinations using a clonal neuroblastoma × glioma hybrid cell line (NG108–15) revealed that TI233 blocked the voltage-sensitive calcium channel of the membrane in the same concentration range. Thus it was suggested that TI233 inhibited transmitter release from neuronal cells by blocking the entry of calcium to the cytoplasm.

Calmodulin plays important roles in various calcium-dependent functions of eukaryotic cells as an intracellular calcium acceptor [1]. Within the nervous system calmodulin exists in synaptic regions and attention has been called to its role in neuronal functions [2]. Undoubtedly calcium ions are indispensable in neurotransmitter-releasing processes [3]. DeLorenzo *et al.* [4] showed that the calcium-induced norepinephrine release from rat brain synaptic vesicles was lost and restored by removal and addition of calmodulin, respectively. But it is still unclear whether the calcium acceptor in the transmitter-releasing processes is calmodulin or not.

Recently, various calmodulin antagonists, which bind to calmodulin and inhibit the calmodulin functions in the presence of calcium, have been used as ligands in affinity chromatography for calmodulin purification [5, 6] and as useful tools to examine the possibilities of calmodulin involvement in various cellular functions [7]. Hidaka *et al.* [8] showed that TI233, a drug originally developed as an antithrombotic [9], has calmodulin-antagonistic abilities and that it inhibited calcium/calmodulin-dependent enzyme activities (Ca/Mg ATPase, myosin light-chain kinase, phosphodiesterase). Maruyama‖ also showed that TI233 bound to calmodulin in a calcium-dependent manner.

In this study we examined the effects of TI233 on neuronal functions using a pheochromocytoma cell line and a neuroblastoma × glioma hybrid cell line. We found that TI233 blocked the voltage-dependent calcium channels and suppressed the high K^+-induced neurotransmitter release.

METHODS

Cells. PC12h cells, a clonal rat pheochromocytoma cell line, were kindly supplied by H. Hatanaka of our institute. This cell line is a subclone [10] of the PC12 line originally established by Greene and Tischler [11]. PC12h cells were plated on poly-D-lysine-coated 35 mm plastic dishes (Falcon) at a density of 1.5×10^6/dish, and were cultured for 1–2 days in 90% Dulbecco Modified Eagle's medium (DMEM) (Gibco, New York, NY) with 5% heat-inactivated horse serum (Gibco) and 5% precolostrum newborn calf serum (PNCS) (Mitsubishi-Kasei, Tokyo, Japan) at 37°. NG108–15 cells, a clonal neuroblastoma × glioma hybrid cell line [12–14], were kindly supplied by T. Amano of our institute. NG108–15 cells were plated on 35-mm plastic dishes at a density of 5×10^4/dish, and were cultured for 2 weeks in a medium containing 95% DMEM, 5% PNCS, 10^{-4} M hypoxanthine, 1.6×10^{-6} M thymidine and 1 mM dibutyryl cyclic AMP (Sigma, St. Louis, MO) purified by the method of Nelson *et al.* [14].

Transmitter release assays. The culture medium for PC12h cells was replaced with 1 ml of loading buffer composed of 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 5.5 mM glucose, 1.2 mM ascorbic acid and 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.3, adjusted with NaOH) supplemented with 0.25 μCi [^{3}H]norepinephrine (NE) or [^{3}H]dopamine (DA) (New England Nuclear; sp. acts for [^{3}H]NE and [^{3}H]DA were 44.7–46.5 and 22.9 μCi/mmole, respectively). After 60 min incubation at 37°, the loading buffer was replaced with a standard buffer ([^{3}H]NE or [^{3}H]DA removed from the loading buffer). After another 60 min incubation, the buffer was subsequently replaced 5 times with 1 ml of fresh standard buffer at 1-min intervals at 37°. Then the KCl concentration of the buffer was elevated to 51.4 mM. These buffers were stored in scintillation vials for radioactivity counting. At the

* TI233, N^2-dansyl-L-arginine-4-*t*-butylpiperazine amide.

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‖ M. Maruyama, manuscript submitted.

end of the experiment, the cells were collected with a silicon scraper and dissolved with 1.2 ml of 0.25 N NaOH (70°, 20 min). The radioactivity was counted on a liquid scintillation counter with a toluene/Triton scintillation cocktail. The transmitter release was indicated by the percentage of the released radioactivity compared to the retained radioactivity.

Experiments with a calcium ionophore were designed similarly, but 20 μM A23187 (Calbiochem-Behring, La Jolla, CA; diluted from a 5-mM stock solution dissolved in dimethylsulfoxide) was added instead of the elevated KCl. To see the effect of TI233 on transmitter release, 0–15 μM TI233 (Mitsubishi-Kasei) was included in the fifth and sixth buffers (i.e. 1 min pretreatment) or throughout the first to the sixth buffer (i.e. 5 min pretreatment).

Electrophysiology. A dish carrying NG108–15 cells was placed on a temperature-controlled (36 ± 1°) microscope stage after replacement of the culture medium with a recording buffer containing 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM HEPES–NaOH (pH 7.3) and 0–150 μM TI233. Under a phase-contrast view, the cell was impaled by a 3 M potassium acetate filled microelectrode connected to a standard amplifier–oscilloscope system. For quantification of the effect of TI233 on the sodium component of the action potential, the cell's membrane potential was hyperpolarized to −100 mV for 120 msec (by a current injected through a bridge circuit) and the anodal break amplitude and maximal rate of increase were measured. For the effect on the calcium component, the anodal break amplitude was measured as earlier in an Na-free recording buffer composed of 150 mM Tris–HCl (pH 7.3), 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 0–100 μM TI233. All measurements were carried out within 15–60 min of the TI233-containing buffer being introduced to the dish. Cells without stable resting potentials were discarded.

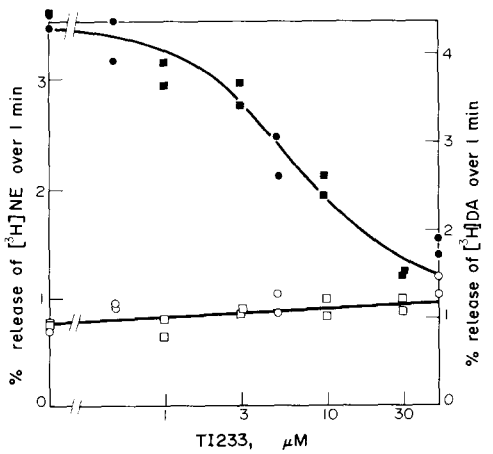

Fig. 1. Dose-dependent suppression of high K^+-induced [^{3}H]NE and [^{3}H]DA release by TI233 in PC12h cells. Releases under low K^- (5.4 mM) and high K^- (51.4 mM) conditions are indicated by open and closed symbols, respectively. Squares are for NE (left ordinate), circles for DA (right ordinate). The values are indicated by the percentages of the released radioactivities over 1 min compared to the retained radioactivity (see Methods).

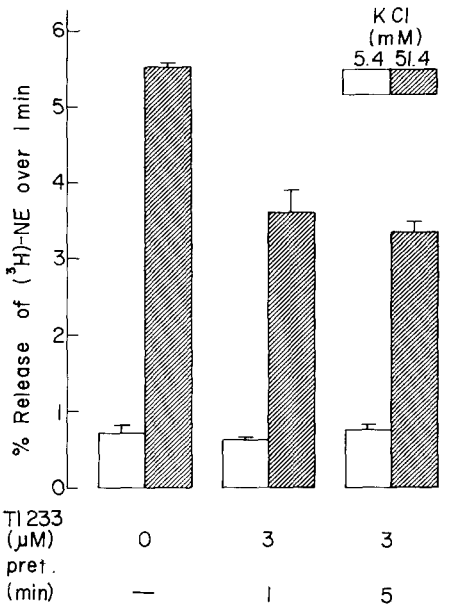

Fig. 2. Effect of pretreatment period with 3 μM of TI233 on high K^--induced [^{3}H]NE release from PC12h cells. Open and hatched columns represent the releases under low (5.4 mM) and high (51.4 mM) K^- conditions, respectively. Values are shown as means of duplicate assays.

RESULTS

Inhibition of catecholamine release by TI233

Figure 1 shows the effects of the various concentrations of TI233 on the catecholamine release from PC12h cells. The high K^+-induced release of the preloaded [^{3}H]NE and [^{3}H]DA was suppressed by

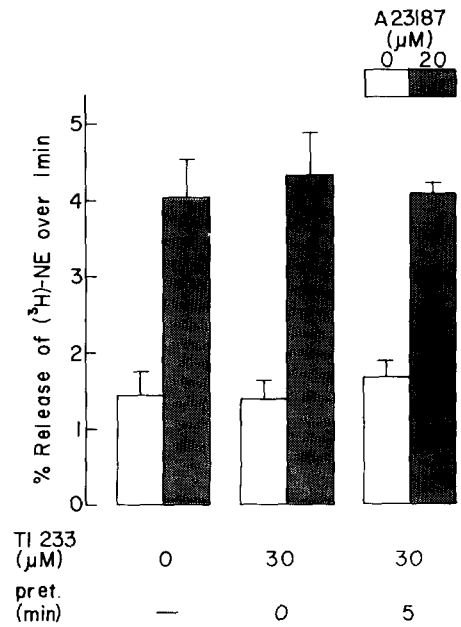

Fig. 3. Effect of 30 μM of TI233 on A23187-induced [^{3}H]NE release from PC12h cells. Open and shaded columns represent the releases before and after the addition of 20 μM A23187, respectively. Values are shown as means of duplicate assays.

TI233 (1–40 μM) in a dose-dependent manner. The suppression of the release appeared to be quite similar for [^{3}H]NE and [^{3}H]DA and their ID_{50}s were around 3 μM. On the other hand, TI233 did not affect the basal level of catecholamine release at K^+ concentration of 5.4 mM.

Figure 2 shows the effect of pretreatment of TI233 on the [^{3}H]NE release from PC12h cells. The full effect of TI233 was observed within 1 min of incubation, since an elongation of the pretreatment period from 1 to 5 min caused no further suppression of the [^{3}H]NE release. An elevation of external $CaCl_2$ from 1.8 to 10 mM scarcely altered the inhibitory effect of TI233 on the high K^+-induced [^{3}H]NE release (data not shown).

To know whether TI233 acted on the cell membrane or on the cytoplasmic components, the PC12h cells were exposed to the calcium ionophore A23187 which introduces calcium ions directly into the cytoplasm. As shown in Fig. 3, the per cent release of [^{3}H]NE from PC12h cells was increased from 1.4 to 4.2 by the addition of 20 μM A23187. TI233 did not suppress the A23187-induced [^{3}H]NE release even at 30 μM [a concentration sufficient to inhibit the high K^+-induced transmitter release (Fig. 1)]. The inhibitory effect of TI233 was not observed even after 5 min pretreatment.

Calcium channel blockage by TI233

A direct effect of TI233 on the cell membrane, suggested by the ionophore experiment, was supported by electrophysiological examinations. Because of the limited size of pheochromocytoma cells, the examination was carried out in neuroblastoma × glioma hybrid cells which are known to elicit the Na^+- and Ca^{2+}-dependent action potentials in response to electrical stimulation [12] (the top trace in Fig. 4C).

TI233 had no significant influence on the cell's resting potential and input resistance up to a concentration of 200 μM. Figure 4 shows the effects of TI233 on Na^+ and Ca^{2+} action potentials of NG108–15 cells. TI233 produced a dose-dependent inhibition of the Na^+ and Ca^{2+} spikes. The ID_{50} values for inhibition of the Na^+ spike amplitude and rate of increase were 25 and 10 μM, respectively (Fig. 4A). TI233 was more effective in reducing the Ca^{2+} conductance than the Na^+ conductance, because the ID_{50} value for inhibition of the Ca^{2+} spike amplitude was 3 μM, about one-eighth of that for the Na^+ spike amplitude (Fig. 4B). The ID_{50} values for inhibition of the Ca^{2+} spike were in good agreement with that for the catecholamine release from PC12h cells (Fig. 1). The inhibitory effect of TI233 were reversible and the Ca^{2+} component of the action potential reappeared after washing out TI233 (Fig. 4C, bottom trace).

DISCUSSION

Hikada *et al.* [8] showed that TI233 is one of the

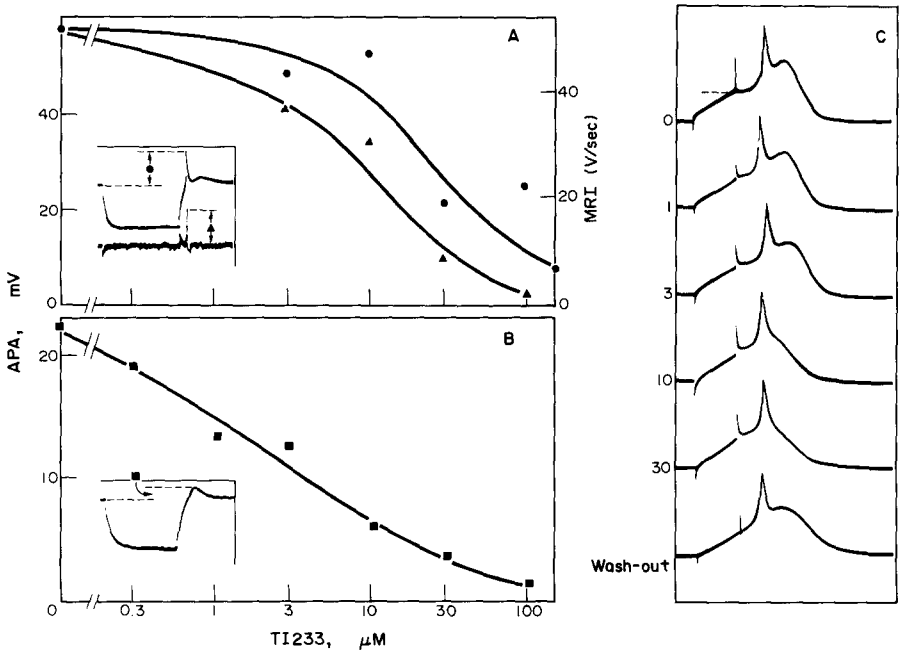

Fig. 4. Dose-dependent blockage of (A) Na and (B) Ca components of the action potential by TI233 in NG108–15 cells. Insets indicate methods of measurement; the membranes were brought to −100 mV by 120-msec current pulses. Action potential amplitudes (APA) (● for Na and ■ for Ca) and maximal rates of increase (MRIs) (▲) for Na are plotted (means of eight cells). The curves are fitted by eye. (C) Effect of increasing concentration of TI233 on the action potential in a single representative cell. Note the second peak (Ca component) was suppressed before the first peak (Na component) diminished. The bottom trace shows a partial recovery after a wash-out of TI233. The recording media containing TI233 were exchanged at 5-min intervals. The resting potential was held at −80 mV by a DC current and action potentials were triggered by 20-msec current pulses which raised the membrane to −50 mV (dashed line).

most efficient calmodulin antagonists as well as trifluoperazine (TFP), chloropromazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7). In the present study, we found that TI233 suppressed the high K^+-induced transmitter release in a dose-dependent manner but it had no effect on the A23187-induced transmitter release. We also found that TI233 at the same concentration suppressed the Ca^{2+} component of the action potential. Although TI233 also inhibits the Na^+ component of the action potential the effective dose of TI233 on the Na^+ component is higher than that on the Ca^{2+} component. From these results we concluded that TI233 suppressed the transmitter release by inhibition of the voltage-dependent calcium channels on the neuronal cell membrane.

The inhibitory effect of TI233 on transmitter release was unaltered by an elevation of the external $CaCl_2$ concentration. It is well known that polyvalent cations (such as Co^{2+}, Cd^{2+} and La^{3+}) and organic calcium channel blockers (such as verapamil and D600) inhibit the function of the calcium channel competitively with external Ca^{2+} [15, 16]. The mode of inhibition of the calcium channel by TI233 seemed different from that of calcium antagonists. A similar non-competitive mode of action was reported with W-7, another calmodulin antagonist, in vascular smooth muscle relaxation [16]. It is not clear whether this inhibition is due to a 'non-specific side effect' of the calmodulin antagonist, or due to the coupling of calmodulin with the voltage-sensitive calcium channels.

Undoubtedly calcium ions are indispensable in neurotransmitter- and hormone-releasing processes. But the mechanism of the action of calcium ions still remains unclear. DeLorenzo *et al.* [4] showed that the removal of an endogenous heat-stable protein fraction from a synaptic vesicle preparation abolishes calcium's effects on neurotransmitter release and protein phosphorylation in that preparation and that these effects on the vesicle system were restored by the addition of calmodulin. They suggested that calmodulin is involved in the norepinephrine-releasing processes. But there is little evidence whether the calcium acceptor in the excitation–secretion coupling is calmodulin or not. The involvement of calmodulin in excitation–secretion coupling is also suggested in pancreatic cells which release insulin in response to applied glucose or membrane depolarization [17]. Sugden *et al.* [18] showed that TFP inhibited the glucose-induced insulin secretion from pancreatic islet cells. But the site of action of the calmodulin antagonist is in doubt. Schubart *et al.* [19] suggested that TFP exerted its effect by blocking the activation of the calmodulin-dependent protein kinase in hamster insulinoma cells. Janjic *et al.* [20] claimed that TFP directly inhibited the calcium uptake system. In this study TI233 failed to suppress the calcium ionophore induced norepinephrine release. This meant that the primary site of action of TI233 in inhibiting the transmitter release was the calcium influx mechanism. But this did not necessarily mean the absence of calmodulin involvement in the exocytosis, since it is not clear in the present study that TI233 permeated the membrane to interfere with the possible cytoplasmic targets. Experiments are under way to examine the permeation through and the binding to the membrane of TI233, making use of the marked fluorescence of this drug.

Acknowledgements—We are grateful to Drs T. Amano and H. Hatanaka for their kind supplies of cells and continuous encouragement. Thanks are due to Dr Y. Kudo for encouraging discussions.

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