Effects of calmodulin and protein kinase C modulators on transient Ca^{2+} increase and capacitative Ca^{2+} entry in human platelets: Relevant to pathophysiology of bipolar disorder

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Abstract:

Disturbed intracellular calcium (Ca^{2+}) homeostasis has been implicated in bipolar disorder, which mechanisms may be involved in the dysregulation of protein kinase C (PKC) and calmodulin systems. In this study, we investigated a transient intracellular Ca^{2+} increase induced by thapsigargin, a inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase pump (SERCA), and a capacitative Ca^{2+} entry followed by addition of extracellular Ca^{2+}, in the presence or absence of PKC/calmodulin modulators in the platelets of healthy subjects in order to elucidate the role of SERCA in Ca^{2+} homeostasis and to assess how both PKC and calmodulin systems regulate the two Ca^{2+} responses. Moreover, we also examined the thapsigargin-elicited transient Ca^{2+} increase and capacitative Ca^{2+} entry in patients with mood disorders. PKC and calmodulin systems have opposite regulatory effects on the transient Ca^{2+} increase and capacitative Ca^{2+} entry in the platelets of normal subjects. The inhibitory effect of PKC activation on capacitative Ca^{2+} entry is significantly increased and the stimulatory effect of PKC inhibition is significantly decreased in bipolar disorder compared to major depressive disorder and normal controls. These results suggest the possibility that increased PKC activity may activate the inhibitory effect of capacitative Ca^{2+} entry in bipolar disorder. However, this is a preliminary study using a small sample, thus further studies are needed to examine the PKC and calmodulin modulators on the capacitative Ca^{2+} entry in a larger sample.

Key words: Bipolar disorder; Calcium; Calmodulin; Platelet; Protein kinase C; Thapsigargin

Abbreviations: Ca^{2+}; intracellular calcium, ER; endoplasmic reticulum, 5-HT; serotonin, MLCK; myosin light chain kinase, PKC; protein kinase C, PRP; platelet rich plasma, SERCA;

sarco/endoplasmic reticulum Ca^{2+}-ATPase pump, TG; thapsigargin

Introductions

It has been suggested that intracellular calcium (Ca^{2+}) mobilization is dysregulated in the platelets of bipolar disorders. We have reported that serotonin (5-HT)- or thrombin-induced intraplatelet Ca^{2+} mobilization is enhanced in unmedicated patients with bipolar disorder (Kusumi et al. 1992; 1994; Suzuki et al. 2001). Other researchers have indicated similar findings (Dubovsky et al. 1991; Okamoto et al. 1995). Moreover, the 5-HT-stimulated Ca^{2+} response is significantly attenuated in the presence of staurosporine, a protein kinase C (PKC) inhibitor, in normal controls and major depressive disorder, whereas the inhibitory effect of staurosporine is not observed in bipolar disorder (Suzuki et al. 2003). This finding suggests increased PKC activity in bipolar disorder as shown by other investigators (Friedman et al. 1993; Wang and Friedman 1996; Pandey et al. 1998; Wang et al. 1999). Meanwhile, in the presence of ML-9, a myosin light chain kinase (MLCK) inhibitor, both 5-HT- and thrombin-induced Ca^{2+} response are enhanced in the platelets of normal subjects (Suzuki et al. 2004). This result suggests that the enhanced Ca^{2+} response observed in bipolar disorder may be relevant to decreased function of MLCK, a Ca^{2+}/calmodulin-dependent enzyme. These findings indicate that the dysregulation of Ca^{2+} mobilization in bipolar disorder may be closely related to PKC and calmodulin systems.

It is well established that depletion of intracellular stores of Ca^{2+} can activate Ca^{2+} influx across the plasma membrane, a process known as ‘capacitative Ca^{2+} entry’ (Putney 1986). It can be switched on by variety of stimuli such as Ca^{2+} mobilizing agonists, inositol 1,4,5-triphosphate, Ca^{2+} ionophore ionomycin or inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) thapsigargin (Berridge 1995). Pathogenic mutations of ATP2A2 gene at chromosome

12q which encodes SERCA has recently been identified in Darier's disease, a dominant skin disorder, which often comorbid with bipolar disorders (Craddock et al. 1994). Findings from a recent linkage study suggest that this chromosomal region may contain a susceptibility gene for bipolar disorders (Dawson et al. 1995). Moreover, DNA microarray analysis of lymphoblastoid cells derived from pairs of twins discordant with respect to bipolar disorder, suggested that a polymorphism of XBP1, a pivotal gene in the endoplasmic reticulum (ER) stress response elicited by thapsigargin, might contribute to the genetic risk factor for bipolar disorder (Kakiuchi et al. 2003).

In this study, we investigated the effects of thapsigargin on intracellular Ca^{2+} concentration in the presence or absence of PKC/calmodulin modulators in the platelets of healthy subjects in order to elucidate the role of SERCA in Ca^{2+} homeostasis and to assess how both PKC and calmodulin systems regulate the thapsigargin-induced Ca^{2+} mobilization. Moreover, we also preliminarily examined the thapsigargin-elicited transient Ca^{2+} increase and capacitative Ca^{2+} entry in patients with mood disorder.

Methods

Subjects

For basic study, subjects were 6 healthy male volunteers (29-43 years old), free of psychiatric and physical illness. For patient study, subjects were 7 patients with bipolar disorder (euthymic or depressive), 10 patients with major depressive disorder (all depressive) and 17 sex- and age-matched control diagnosed by two psychiatrists using DSM-IV criteria. The patients were

drug-naïve except 3 bipolar patients treated with lithium (1 case), levothyroxine (2 cases), cabergoline (2 cases), risperidone (1 case), olanzapine (1 case) and quetiapine (1 case). Written informed consent was obtained from all participants prior to blood sampling, which was approved by the Institutional Review Board in Hokkaido University Graduate School of Medicine. For at least 4 weeks before blood sampling, all subjects had not received any calcium channel blocking medication, acetylsalicylic acid or non-steroidal anti-inflammatory drugs.

Measurement of intracellular Ca concentration

The isolation of platelets and the measurement of intracellular Ca^{2+} concentration were performed as described previously (Kusumi et al. 1991a; 1991b). Briefly, platelet rich plasma (PRP) was prepared by centrifugation at 200 x g for 10 min at room temperature. After incubation with 4 μM fura-2/acetoxymethyl ester for 15 min, platelets were isolated from the PRP by further centrifugation at 600 x g for 15 min. The platelet pellet was suspended in Krebs-Ringer HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 6 mM glucose, 10mM HEPES, pH 7.4). Fluorescence was measured on a Hitachi F-2000 fluorometer with excitation at 340 and 380 nm, and with emission at 510 nm using a Ca^{2+}-dye dissociation constant of 224 nM. Intracellular Ca^{2+} concentrations were calculated from the ratio of fluorescence intensities at two excitation wavelengths in the platelet samples according to the method of Grynkiewicz et al. (1985). Ca^{2+} increase represents that initial peak minus resting level.

***Basic study I*; Measurement of transient Ca^{2+} increase and capacitative Ca^{2+} entry in the**

platelets of normal controls

In nominal Ca^{2+}-free buffer, we measured a transient Ca^{2+} increase by adding various concentrations (1nM-10μM) of thapsigargin to the platelet suspension. After intracellular Ca^{2+} concentration reached a steady state, we measured a capacitative Ca^{2+} entry by restoring extracellular Ca^{2+} (1mM $CaCl_2$).

***Basic study II*; Effects of PKC and calmodulin modulators on transient Ca^{2+} increase and capacitative Ca^{2+} entry in the platelets of normal control**

Either W-7 (1-100 μM), a calmodulin antagonist, PMA (0.1-10 nM), a PKC activator or bisindolylmaleimide II (10-1000 nM), a PKC inhibitor was pre-incubated at 37 C˚ for 4 min prior to thapsigargin stimulation. The effects of PKC and calmodulin modulators on a transient Ca^{2+} increase and capacitative Ca^{2+} entry were assessed by the same method as in the basic I study using 30nM thapsigargin and 1mM $CaCl_2$.

Patient Study

In the absence or presence of the PKC and calmodulin modulators mentioned above, we compared the transient Ca^{2+} increase and the capacitative Ca^{2+} entry among patients with bipolar disorder and major depressive disorder, and normal controls.

Statistical analysis

Values were expressed as the mean±SEM. Statistical evaluation was performed using

repeated-measures analysis of variance followed by a Dunnett's test. Differences were considered statistically significant at $P < 0.05$.

Results

For basic study, in nominal Ca^{2+}-free buffer, thapsigargin (1nM-10μM) induced a transient Ca^{2+} increase in a concentration-dependent manner in the platelets of healthy subjects. Its EC_{50} is 65 nM (Fig 1a). In the presence of extracellular Ca^{2+} (1mM $CaCl_2$), thapsigargin (1μM) induced a long lasting increase in intracellular Ca^{2+} concentration (Fig 1b). Thapsigargin (10-100 nM) enhanced dose-dependently the Ca^{2+} response to the addition of 1mM $CaCl_2$ (Fig 1c). In the presence of 30 μM and more concentrations of W-7, both the transient Ca^{2+} increase induced by 30 nM thapsigargin (Fig 2a) and the capacitative Ca^{2+} entry by the addition of 1 mM $CaCl_2$ (Fig 2b) were significantly attenuated in normal controls. Pre-treatments with 3-10 nM PMA significantly inhibited the transient Ca^{2+} increase (Fig 3a) and 1-10 nM PMA also significantly reduced the capacitative Ca^{2+} entry (Fig 3b). On the other hand, bisindolylmaleimide II significantly enhanced both the transient Ca^{2+} increase at 100 nM – 1 μM (Fig 4a) and the capacitative Ca^{2+} entry at 500 nM – 1 μM (Fig 4b). Each PKC or calmodulin modulator was effective within the range of concentrations that have a specific action in intact platelets (Kaibuchi et al. 1982; Nishikawa et al. 1980; Toullec et al. 1991). For capacitative Ca^{2+} entry, similar results were obtained by the addition of each PKC or calmodulin modulator after thapsigargin stimulation (data not shown).

For patient study, there was no significant difference in either transient Ca^{2+} increase or

capacitative Ca^{2+} entry among patients with bipolar disorder and major depressive disorder, and normal controls (Fig 5a and 5b). Next, we examined the effects of PKC and calmodulin modulators on the two Ca^{2+} responses among the three groups by comparing percentage of Ca^{2+} increase which calculates as follows; Ca^{2+} increase with pre-treatment / Ca^{2+} increase without pre-treatment x100 (%). No significant difference in the effect of PKC or calmodulin modulators on the transient Ca^{2+} increase was found between patients with bipolar disorder and major depressive disorder, and normal controls (Fig 6a). Although there was no significant difference in the effect of W-7 (49.0% for bipolar disorder, 57.3% for major depressive disorder, 51.1% for normal control), capacitative Ca^{2+} entry was significantly inhibited by PMA in bipolar disorder compared to normal controls (Fig 6b; 24.6% for bipolar disorder, 53.1% for major depressive disorder, 60.6% for normal control, $p < 0.05$). The enhancement of capacitative Ca^{2+} entry by bisindolylmaleimide II was significantly lower in bipolar disorder than in major depressive disorder and normal control (Fig 6b; 106.0% for bipolar disorder, 127.8% for major depressive disorder, 137.6% for normal control, $p < 0.05$). For bipolar patients, there is no significant difference in capacitative Ca^{2+} entry pretreated with PMA or bisindolylmaleimide II between medicated and non-medicated groups (data not shown) although it is a preliminary analysis based on a small sample.

Discussion

The present results suggested that PKC activator negatively, and PKC inhibitor positively regulated the Ca^{2+} leakage from internal Ca^{2+} store that follows the inhibition of the SERCA, and

the capacitative Ca^{2+} entry induced by the depletion of internal Ca^{2+} stores in human platelets. In contrast with PKC inhibitor, a calmodulin antagonist caused inhibitory effects on both the transient Ca^{2+} increase and capacitative Ca^{2+} entry. Taken together, these results may indicate the possibility that PKC and calmodulin systems have opposite regulatory effects on the two Ca^{2+} responses.

Recently several reports indicate that capacitative Ca^{2+} entry has been found in CNS (Grudt et al. 1996; Lo et al. 2002) and may have a role in synaptic plasticity (Baba et al. 2003). In platelets, it is known that PKC activates Ca^{2+} entry into ER through increasing the Vmax of SERCA (Tao et al. 1992). Therefore, the present results that Ca^{2+} efflux from ER decreases by PKC activation and increases by PKC inhibition, may possibly be due to this direct effect of PKC on SERCA. For calmodulin systems, similar finding was reported on the effect of calmodulin inhibitor upon capacitative Ca^{2+} entry in cultured human mesangial cells (Mene et al. 1996). However, to our knowledge, this is the first report to examine the effect of calmodulin modulator on capacitative Ca^{2+} entry in platelets. It is possible that SERCA itself may be directly regulated by calmodulin in the same way as PKC system.

There have been a few reports to examine whether SERCA-related Ca^{2+} mobilization have some role in the pathphysiology of bipolar disorder. Hough et al.(1999) reported that the platelets / lymphocytes of bipolar patients responded to thapsigargin with greater intracellular Ca^{2+} increases than did control subjects. However, the thapsigargin-induced Ca^{2+} influx was measured in the Ca^{2+}-containing buffer, thus it is uncertain for the transient Ca^{2+} increase and capacitative Ca^{2+} entry in their study. Another difference in methodology is the concentration of

thapsigargin (30 nM in our study vs. 1 μM in Hough's study). It is shown that there are two subfamilies of SERCA, SERCA2b and SERCA3 in platelets (Kovacs et al. 2001). SERCA2b is sensitive to low concentration (10 nM) of thapsigargin, while SERCA3 is sensitive to higher concentration (100-200 nM) (Cavallini et al. 1995). Kato et al.(2003) showed that 10 μM thapsigargin-induced cytosolic Ca^{2+} response was significantly higher in the transformed lymphoblastoid cells from patients with bipolar disorder only in the presence of extracellular Ca^{2+}.

The present preliminary patient study shows that the inhibitory effect of PKC activation on capacitative Ca^{2+} entry is significantly increased and the stimulatory effect of PKC inhibition is significantly decreased in patients with bipolar disorder compared to patients with major depressive disorder and normal controls. However, there is no significant difference in effect of calmodulin inhibitor on the capacitative Ca^{2+} entry, or effect of PKC or calmodulin modulators on the transient Ca^{2+} increase among the three groups. These results suggest the possibility that increased PKC activity may activate the inhibitory effect of capacitative Ca^{2+} entry in bipolar disorder. Actually, there have been several reports showing that the PKC activity is enhanced in the postmortem brain and platelets of patients with bipolar disorder (Friedman et al. 1993; Wang et al. 1996; Wang et al. 1999).

As mentioned in the introduction, Kakiuchi et al.(2003) reported that a polymorphism in the promoter region of XBP1, a pivotal gene in the ER stress response elicited by thapsigargin etc., increased the risk of bipolar disorder. Although there have been no report showing a direct connection between the XBP1 polymorphism and SERCA function or clear evidence of altered SERCA function in bipolar disorder, the present study suggests the possibility that decreased

function of SERCA may be involved in the pathophysiology of bipolar disorder. The molecular basis of capacitative Ca^{2+} entry remains elusive, but transient receptor potential channel families have been put forward as possible candidates. Especially, transient receptor potential Ca^{2+} permeable channel melastatin type 2 (TRPM2) is one of candidates in regards to the etiology of bipolar disorder (McQuillin et al. 2006; Xu et al. 2006). It may be worth examining the relationship between these SNPs and capacitative Ca^{2+} entry in bipolar disorder.

In conclusion, PKC and calmodulin systems have opposite regulatory effects on the transient Ca^{2+} increase and capacitative Ca^{2+} entry in the platelets of normal subjects. The inhibitory effect of PKC activation on capacitative Ca^{2+} entry is significantly increased and the stimulatory effect of PKC inhibition is significantly decreased in bipolar disorder compared to major depressive disorder and normal controls. These results suggest the possibility that the increased activity of PKC may activate the inhibitory effect of capacitative Ca^{2+} entry in bipolar disorder. However, this study is preliminary using a small sample, thus further studies are needed to examine the PKC and calmodulin modulators on the capacitative Ca^{2+} entry in a larger sample.

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Figure legends

Fig 1. Effect of Ca^{2+}-ATPase inhibitor thapsigargin on platelet intracellular Ca^{2+} mobilization.

(a) In normal Ca^{2+}-free buffer, thapsigargin (TG) stimulated a transient increase in intracellular Ca^{2+} concentration by releasing from endoplasmic reticulum Ca^{2+} stores. Columns are the mean±SEM. (N= 6).

(b) In the presence of extracellular Ca^{2+}(1mM $CaCl_2$), TG (1μM) stimulated a lasting increase in intracellular Ca^{2+} concentration. Each point is the mean±SEM. (N=4).

(c) After a transient Ca^{2+} increase stimulated by TG in normal Ca^{2+}-free buffer, $CaCl_2$ (1mM) was added to the buffer, which induced a capacitative Ca^{2+} entry. Columns are the mean±SEM. (N=5.)

Fig 2. Effect of W-7 on (a) transient Ca^{2+} increase and (b) capacitative Ca^{2+} entry

W-7 was added 4 min prior to 30 nM thapsigargin stimulation. One factor repeated ANOVA was performed to analyze the each effect.

** $p< 0.01$ compared with control (C: without W-7) ; by Dunnett's test (N=4).

Fig 3. Effect of PMA on (a) transient Ca^{2+} increase and (b) capacitative Ca^{2+} entry

PMA was added 4 min prior to 30 nM thapsigargin stimulation. One factor repeated ANOVA was performed to analyze the each effect.

* $p< 0.05$, ** $p< 0.01$ compared with control (C: without PMA) ; by Dunnett's test (N=4).

Fig 4. Effect of bisindolylmaleimide II (BIS) on (a) transient Ca^{2+} increase and (b) capacitative Ca^{2+} entry

BIS was added 4 min prior to 30 nM thapsigargin stimulation. One factor repeated ANOVA was performed to analyze the each effect.

* $p < 0.05$, compared with control (C: without BIS) ; by Dunnett's test (N=6).

Fig 5. (a) Transient Ca^{2+} increase and (b) capacitative Ca^{2+} entry in patients with bipolar disorder and major depressive disorder , and control subjects

Fig 6. Effects of W-7, PMA or bisindolylmaleimide II (BIS) on (a) transient Ca^{2+} increase and (b) capacitative Ca^{2+} entry in patients with bipolar disorder and major depressive disorder, and normal controls.

Data are shown by percent of Ca^{2+} increase which calculates as follows; Ca^{2+} increase with pre-treatment / Ca^{2+} increase without pre-treatment x100 (%). TG: thapsigargin

* $p < 0.05$, compared with control subjects ; by Dunnett's test.

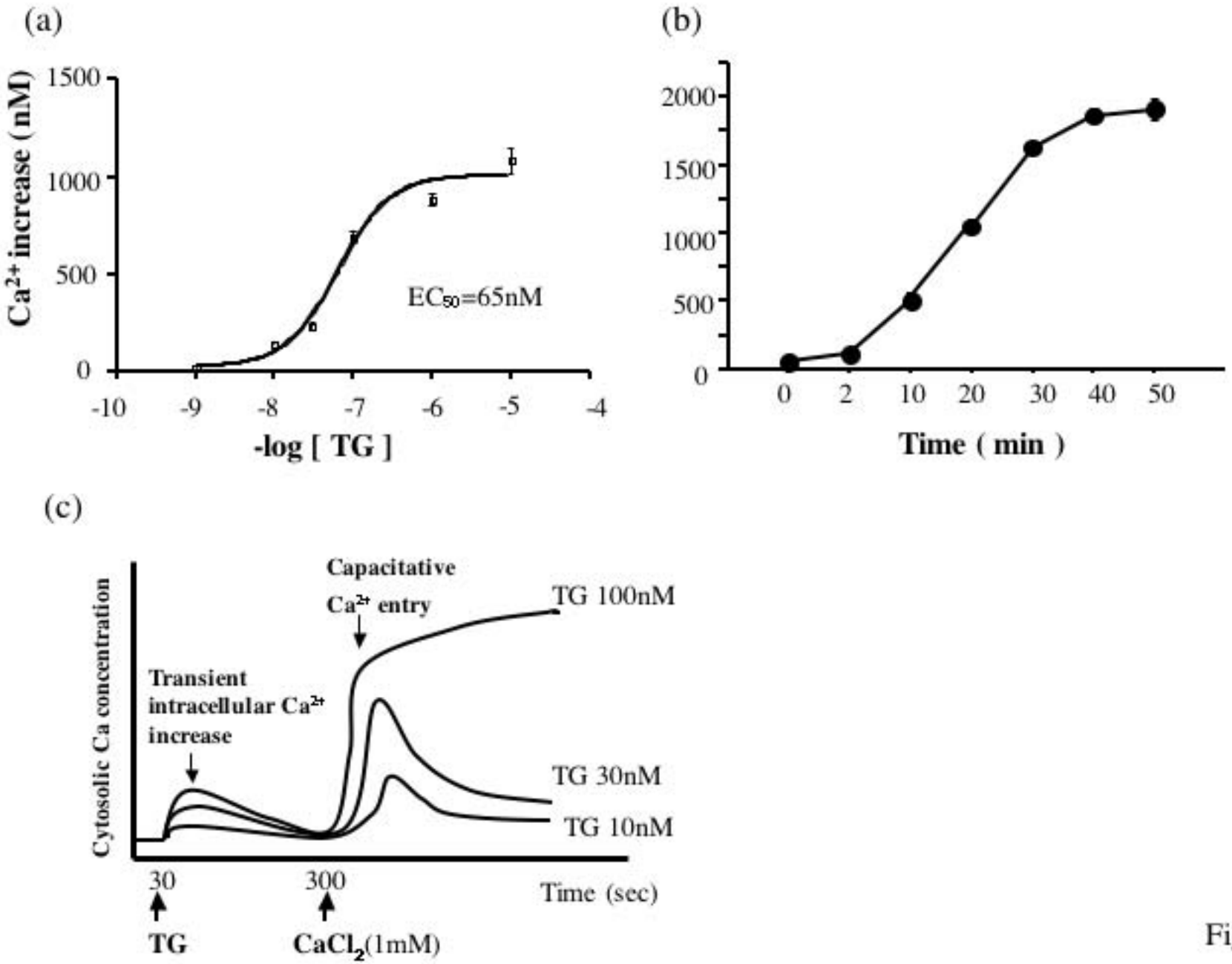
(a)
1500
1000
500
0
Ca2+ increase (nM)
EC50=65nM
-10
-9
-8
-7
-6
-5
-4
-log [TG]
(b)
2000
1500
1000
500
0
0
2
10
20
30
40
50
Time (min)
(c)
Capacitative
Ca2+ entry
TG 100nM
Transient
intracellular Ca2+
increase
TG 30nM
TG 10nM
Cytosolic Ca concentration
30
300
Time (sec)
TG
CaCl2(1mM)

Fig. 1

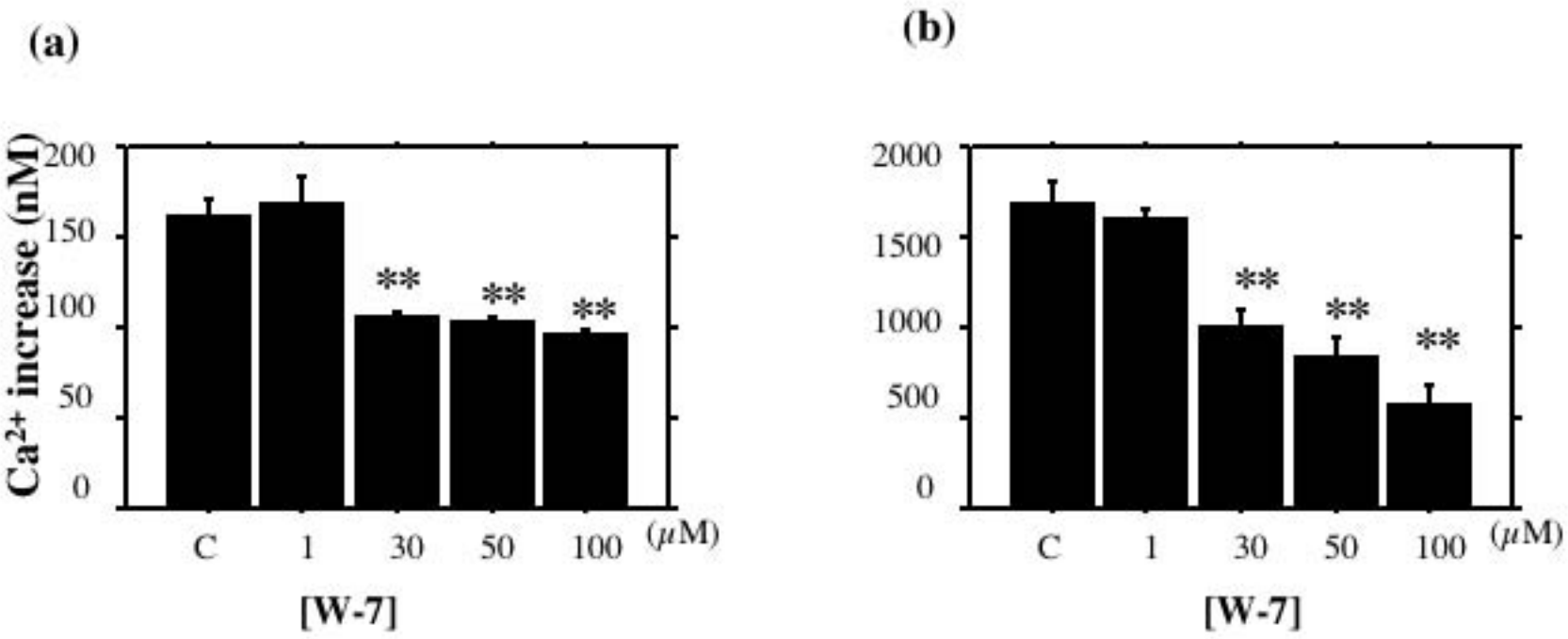
(a)
Ca2+ increase (nM)
200
150
100
50
0
**
**
**
C
1
30
50
100
(μM)
[W-7]
(b)
2000
1500
1000
500
0
**
**
**
C
1
30
50
100
(μM)
[W-7]

Fig. 2

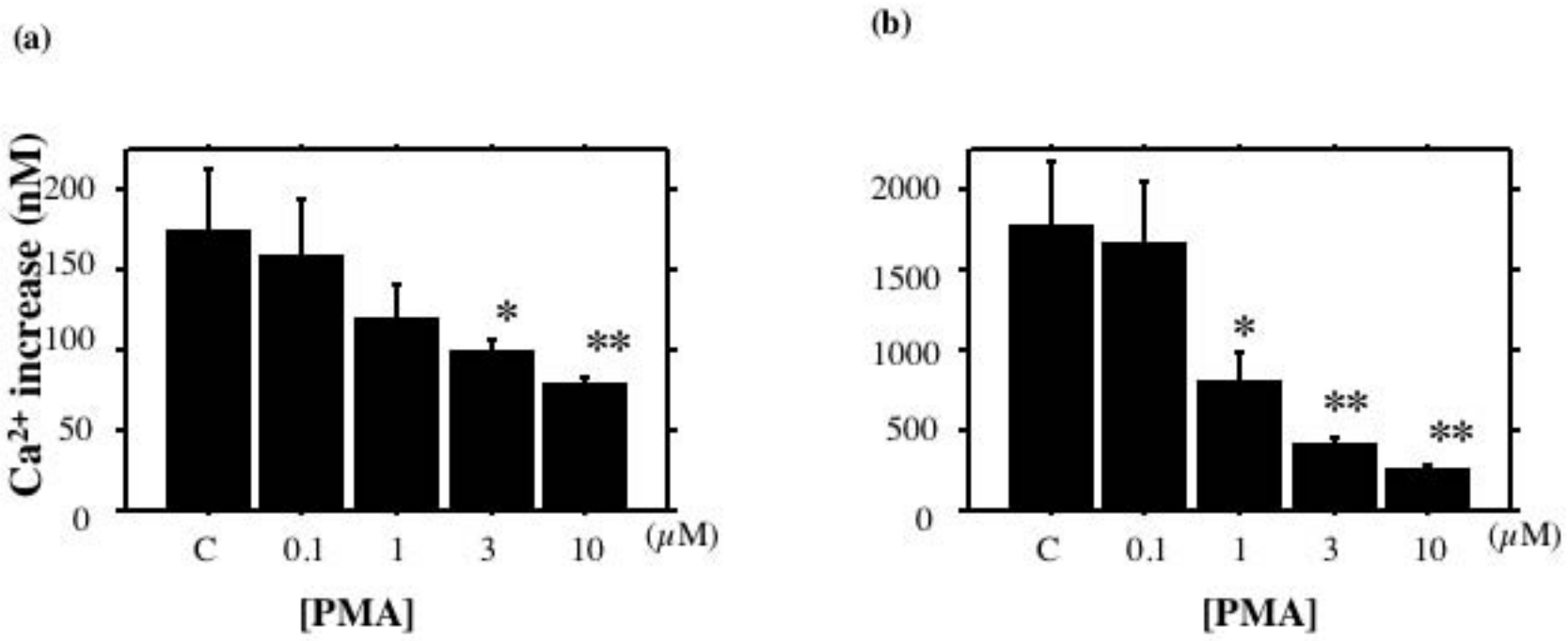
(a)
Ca2+ increase (nM)
200
150
100
50
0
C
0.1
1
3
10
(μM)
[PMA]
*
**
(b)
2000
1500
1000
500
0
C
0.1
1
3
10
(μM)
[PMA]
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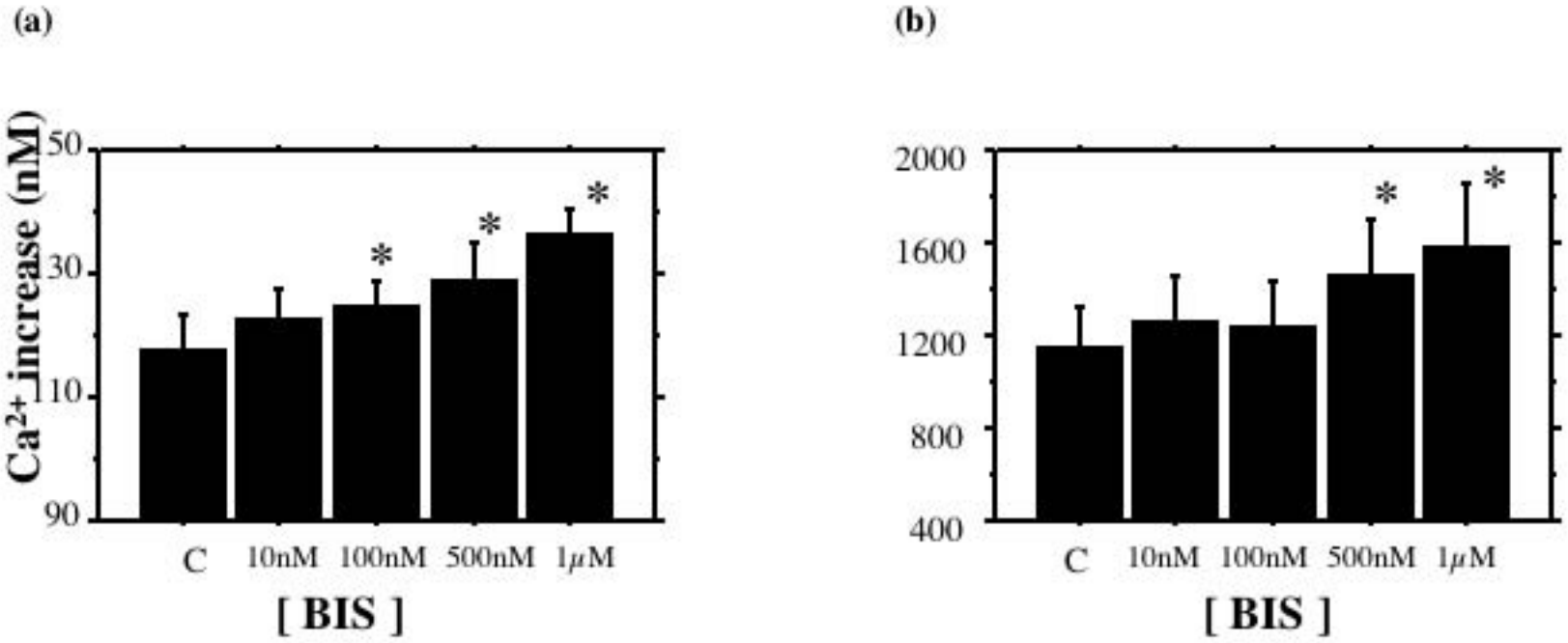
Fig. 3


(a)
Ca2+ increase (nM)
50
30
10
90
C
10nM
100nM
500nM
1μM
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(b)
2000
1600
1200
800
400
C
10nM
100nM
500nM
1μM
[BIS]

Fig. 4

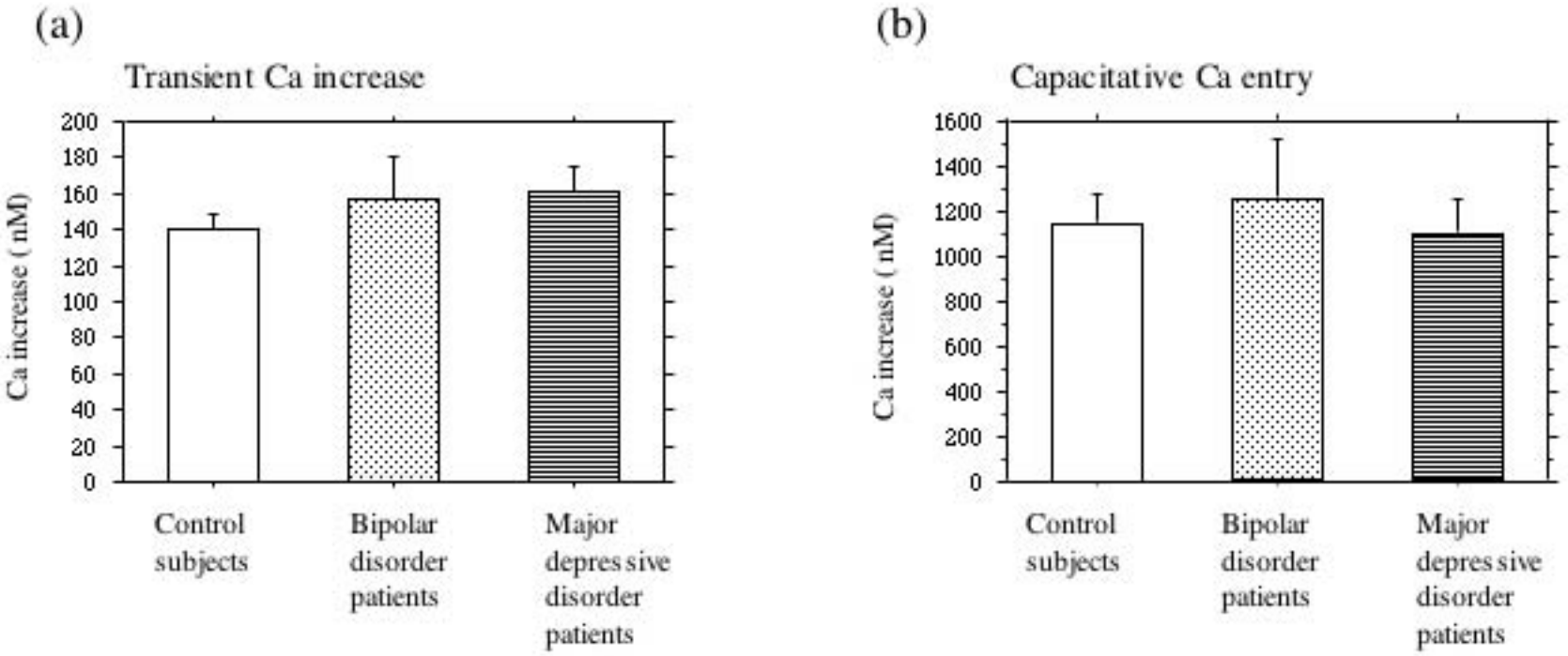
(a)
Transient Ca increase
Ca increase (nM)
200
180
160
140
120
100
80
60
40
20
0
Control subjects
Bipolar disorder patients
Major depressive disorder patients
(b)
Capacitative Ca entry
Ca increase (nM)
1600
1400
1200
1000
800
600
400
200
0
Control subjects
Bipolar disorder patients
Major depressive disorder patients

Fig. 5

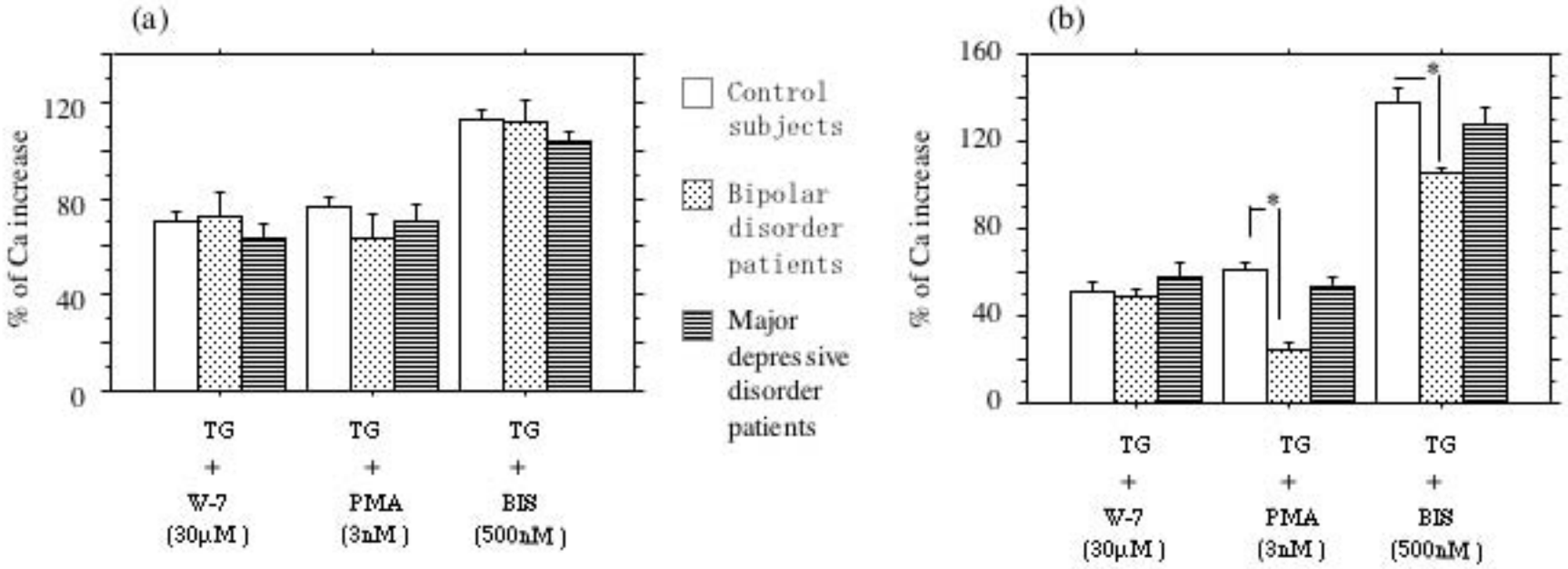
(a)
% of Ca increase
0
40
80
120
TG + W-7 (30μM)
TG + PMA (3nM)
TG + BIS (500nM)
Control subjects
Bipolar disorder patients
Major depressive disorder patients
(b)
% of Ca increase
0
40
80
120
160
*
*
TG + W-7 (30μM)
TG + PMA (3nM)
TG + BIS (500nM)

Fig. 6