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Amplification of depolarization-induced and ryanodine-sensitive cytosolic Ca^{2+} elevation by synthetic carbocyclic analogues of cyclic ADP-ribose and their antagonistic effects in NG108-15 neuronal cells

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Running title: carbocyclic cADPRs and $[Ca^{2+}]_i$ increases

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; RyR, ryanodine receptor; CICR, Ca^{2+}-induced Ca^{2+} release; cADPR, cyclic ADP-ribose; cADPcR, cyclic ADP-carbocyclic ribose; IDPcR, cyclic IDP-carbocyclic-ribose; 8-Cl-cADPcR, 8-chloro-cADPcR; 8-Br-cIDPcR, 8-bromo-cIDPcR; VACCs, voltage-activated Ca^{2+} channels; fura-2 AM, fura-2 acetoxymethylester.

Abstract

We synthesized analogues modified in the ribose unit (ribose linked to N1 of adenine) of cyclic ADP-ribose (cADPR), a Ca^{2+}-mobilizing second messenger. The biological activities of these analogues were determined in NG108-15 neuroblastoma x glioma hybrid cells that were pre-loaded with fura-2 AM and subjected to whole-cell patch-clamp. Application of the hydrolysis-resistant cyclic ADP-carbocyclic-ribose (cADPcR) through patch pipettes potentiated elevation of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) at the depolarized membrane potential. The increase in $[Ca^{2+}]_i$ evoked upon sustained membrane depolarization was significantly larger in cADPcR-infused cells than in non-infused cells, and its degree was equivalent to or significantly greater than that induced by cADPR or β-NAD^+. 8-chloro-cyclic ADP-carbocyclic-ribose (8-Cl-cADPcR) and two inosine congeners (cyclic IDP-carbocyclic-ribose and 8-bromo-cyclic IDP-carbocyclic-ribose) did not induce effects similar to those of cADPcR or cADPR. Instead, 8-Cl-cADPcR together with cADPR or cADPcR caused inhibition of the depolarization-induced $[Ca^{2+}]_i$ increase as

compared with either of cADPR or cADPcR alone. These results demonstrated that our cADPR analogues have agonistic or antagonistic effects on the depolarization-induced $[Ca^{2+}]_i$ increase, and suggested the presence of functional reciprocal coupling between RyRs and VACCs *via* cADPR in mammalian neuronal cells.

Keywords: cyclic ADP-ribose, cytoplasmic free Ca^{2+} concentration, β-NAD^+, ryanodine receptor, neuroblastoma NG108-15 cells.

Introduction

In neuronal cells, Ca^{2+} entry through voltage-activated Ca^{2+} channels (VACCs) causes Ca^{2+} release from intracellular Ca^{2+} stores by activation of ryanodine-sensitive Ca^{2+} pools and subsequent elevation of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Berridge 1998; Lee 2001; Galione and Churchill 2002). The mechanism by which increased $[Ca^{2+}]_i$ induces further Ca^{2+} release from ryanodine receptor (RyR) Ca^{2+}-release channels is called Ca^{2+}-induced Ca^{2+} release (CICR) (Endo 1977; Lee 1997). Cyclic ADP-ribose (cADPR) may be a second messenger or endogenous modulator of CICR, acting on specific subtypes of RyRs (Lee 1997 and 2001; Galione and Churchill 2002; Higashida *et al.* 2001a,b): The cardiac and brain isoforms of type-2 RyRs and brain forms of type-3 RyRs are activated by cADPR (Sonnleitner *et al.* 1998, Kunerth *et al.* 2004). Experiments with islet microsomes suggested that cADPR binds to FKBP12.6 on the RyRs, and the subsequent dissociation of FKBP12.6 from RyRs changes channel kinetics to release Ca^{2+} (Noguchi *et al.* 1997). The role of the cADPR-FKBP12.6-RyR2 complex in excitation-contraction coupling was also

proposed in smooth muscle cells (Wang *et al.* 2004). However, the mechanism of action of cADPR on RyRs in neurons is not fully understood.

The use of specific agonists and/or antagonists provides a straightforward approach to decipher the intricate cellular mechanisms involving cADPR. Several partial agonists, hydrolysis-resistant agonists or antagonists have been described (Walseth and Lee 2002; Huang *et al*. 2002). These agents were generated by enzymatic conversion from NAD^+ analogs, which were characterized using the Ca^{2+} signaling system in invertebrate oocytes (Bailey *et al.* 1996; Bailey *et al.* 1997; Sethi *et al.* 1997; Ashamu *et al.* 1997). It was shown that some of non-hydrolyzable mimics of cADPR have more potent Ca^{2+}-releasing activity than cADPR (Zhang *et al.* 1996; Wong *et al.* 1999). In addition, it was confirmed that the inosine congener of cADPR, cIDPR has Ca^{2+}-releasing activity in intact T cells using the membrane-permeable form (Gu *et al.* 2004; Kunerth *et al.* 2004).

Recently, we performed total-synthesis of cyclic ADP-carbocyclic ribose (cADPcR) (Shuto *et al.* 2001), its inosine congener (cyclic IDP-carbocyclic-ribose, cIDPcR) (Shuto *et al.* 1988), 8-chloro-cADPcR (8-Cl-cADPcR), and 8-bromo-cIDPcR

(8-Br-cIDPcR) (Fukuoka *et al.* 2000). These agents are hydrolysis-resistant mimics of cADPR with the oxygen atom in the N-1-ribose ring being replaced with a methylene group. Among these agents, cADPcR was shown to release Ca^{2+} more effectively than cADPR in sea urchin eggs (Shuto *et al.* 2001; Shuto *et al.* 2003), whereas an identical effect of cADPcR was not found in mammalian T cells (Guse *et al.* 2002). Alterations at the 8-position of the adenine ring of cADPcR markedly altered the Ca^{2+} release activity, which decreased efficiency for Ca^{2+} release or developed antagonistic activity (Shuto *et al.* 2003).

In mammalian neurons, Ca^{2+} entry through N- and/or L-type VACCs facilitates the action of RyR Ca^{2+}-release channels, known as the orthograde signal (Hua *et al.* 1996). This signal is enhanced in the presence of cADPR (Hua *et al.* 1996; Empson and Galione 1997; Hashii *et al.* 2000). Depolarization-induced Ca^{2+} entry augmented in the presence of cADPR, is inhibited by ryanodine in neurons (Hashii *et al.* 2000). Thus, we assumed that cADPR-activated RyRs and VACCs are linked in a retrograde fashion (Chavis *et al.* 1996; Nakai *et al.* 1996). However, more solid evidence is required to support this hypothesis.

Therefore, we examined reciprocal functional coupling between ryanodine receptors and VACCs in voltage-clamped NG108-15 cells in the presence or absence of cADPR and synthetic cADPR analogues. We also examined whether cADPcR has an effect in mammalian neuronal cells.

Materials and Methods

Materials

cADPcR, cIDPcR, and 8-Br-cIDPcR were prepared as described previously (Shuto *et al.* 1988; Fukuoka et *al.* 2000), and synthesis of 8-Cl-cADPcR was performed as described (Shuto *et al.* 2003).

Cell Culture

NG108-15 neuroblastoma x glioma hybrid cells were cultured at 37˚C in 90 % air and 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal calf serum, 100 μM hypoxanthine, 0.1 μM aminopterin, and 16 μM thymidine (Higashida *et al.* 1990). Cells were differentiated in DMEM supplemented with hypoxanthine, thymidine, 1 % FCS and 0.25 mM dibutyryl cAMP for 7 days as described previously (Higashida *et al.* 1990).

Measurement of $[Ca^{2+}]_i$

Concentrations of $[Ca^{2+}]_i$ were determined microspectrofluorometrically using fura-2 in differentiated NG108-15 cells cultured on polylysine-coated glass coverslips. The cells were loaded with fura-2 using 5 μM fura-2 acetoxymethylester (fura-2 AM). Fluorescence was measured at 36°C at determined sites through a pinhole (10 - 20 μm in diameter) with alternating excitation wavelengths of 340 and 380 nm (Grynkiewicz *et al.* 1985) using a Ca^{2+} microspectrofluorometric system (OSP-3 model, Olympus, Tokyo, Japan) (Hashii *et al.* 2000).

Patch voltage-clamp and drug-application

Fura-2 AM-loaded NG108-15 cells were patch voltage-clamped in the whole-cell configuration as described previously (Higashida *et al.* 1990). NG108-15 cells were superfused at 36°C with a bath solution of the following composition (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 20; Hepes, 20, pH 7.3, buffered with NaOH. Patch electrodes contained the following solution (in mM): KCl, 150; $MgCl_2$, 1; Na_2ATP, 1; fura-2, 0.1; Hepes, 10, pH 7.2, buffered with KOH. The electrode

resistance was 8 - 16 MΩ. About 90 s after touching the cells, the electrode tip was sealed to the cell body by suction (seal resistance >1 GΩ), and the membrane in the patch electrode was disrupted by administration of negative potentials. After establishing a whole-cell recording, the cell was voltage-clamped with a single-electrode voltage-clamp amplifier (Axoclamp 2A, Axon Instruments Inc., Foster City, CA) in switching mode operation at about 1 - 3 kHz. $[Ca^{2+}]_i$ increases were measured during depolarization maintained for 1.6 – 25 min (Hashii *et al.* 2000). Intracellular recording medium containing cADPR (10 μM), ADPR (10 and 300 μM), β-NAD^+ (100 μM), α-NAD^+ (100 μM), cADPcR (10 nM - 100 μM), 8-Cl-cADPcR (10 μM), cIDPcR (10 μM), 8-Br-cIDPcR (10 μM), ruthenium red (10 μM) and 8-Br-cADPcR (10 μM) was applied by diffusion into the cytoplasm from the patch pipette.

Sources of compounds

cADPR was purchased from Yamasa Shoyu (Choshi, Japan). Fura-2 and fura-2 AM were purchased from Dojindo Laboratories (Kumamoto, Japan). Ryanodine, ADPR, 8-Br-cADPR and ruthenium red were purchased from Sigma Chemical Co. (St Louis, MO). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

Results

NG108-15 cells loaded with fura-2 AM were sealed with patch pipettes in the presence of 2 mM extracellular Ca^{2+}, and then membrane potential and $[Ca^{2+}]_i$ were measured. The average resting membrane potential was –45 ± 3.5 mV (Mean ± S.E.M.) (n=12). The $[Ca^{2+}]_i$ levels 1 min after application by diffusion through patch pipettes filled with 10 μM cADPcR, cIDPcR, 8-Cl-cADPcR, or 8-Br-clDPcR (Fig. 1a-c) were unchanged or increased slightly (not statistically significant): values were 110 ± 4.7, 107 ± 2.1, 100 ± 2.2, and 95 ± 7.2% of the pre-injection level, respectively (n=3). Therefore, our results indicated that the cADPR analogues themselves did not evoke any apparent changes in $[Ca^{2+}]_i$, similarly to previous reports regarding cADPR in sympathetic neurons (Hua *et al.* 1996) and in NG108-15 cells (Hashii *et al.* 2000).

We then examined the role of cADPR analogues in $[Ca^{2+}]_i$ elevation at constant depolarization, in which $[Ca^{2+}]_i$ increased due to Ca^{2+} influx through VACCs (Fig. 1d-f). In control cells, $[Ca^{2+}]_i$ was increased by depolarization from -40 to -20 mV with an average peak value of 178 ± 15% (at the initial peak) and 130 ± 13% (after 5 min) of the

pre-depolarization level (n=3) (Fig. 1j). In cells injected with 10 μM cADPcR, the same membrane depolarization induced significantly greater and persistent increases in $[Ca^{2+}]_i$ (Fig. 1d): the average $[Ca^{2+}]_i$ level was 318 ± 17% (at the initial peak) and 246 ± 9.2% (after 5 min) of the pre-depolarization level (n=4). The increase by cADPcR was equivalent to or significantly greater than that evoked by 10 μM cADPR (Fig. 1g). In contrast, 10 μM 8-Cl-cADPcR or 10 μM cIDPcR showed slight and statistically non-significant potentiating effects on the depolarization-induced $[Ca^{2+}]_i$ rise (Fig. 1e and f): the average $[Ca^{2+}]_i$ levels at the initial peak points were 204 ± 24% and 228 ± 13%, respectively. Intracellularly infused β-NAD^+, the precursor of cADPR, did not have the immediate significant effect, but continuously enhanced the depolarization-induced increase in $[Ca^{2+}]_i$ (Fig. 1h): the average $[Ca^{2+}]_i$ level was 214 ± 4% (at the initial peak) and 172 ± 4% (after 5 min) of the pre-depolarization level (n=4). While α-NAD^+, an inactive analogue of β-NAD^+ (Clapper *et al.* 1987; Lee and Aarhus 1991), did not show an identical potentiating effect (Fig. 1i): the average $[Ca^{2+}]_i$ level was 186 ± 6% (at the initial peak) and 140 ± 13% (after 5 min) of the pre-depolarization level (n=4). These results are in agreement with the previous report

in the sea urchin egg (Sethi *et al.* 1996), and suggest that β-NAD^+-induced potentiation of $[Ca^{2+}]_i$ elevation is mediated by its conversion to cADPR by the intrinsic ADP-ribosyl cyclase in NG108-15 cells (Higashida *et al.* 2000b; Hashii *et al.* 2000). Activation of the RyR by caffeine and ryanodine (Sitsapesan *et al.* 1995) also transiently potentiated depolarization-induced $[Ca^{2+}]_i$ increases (Fig. 1k and l), suggesting that the potentiating effects of cADPR and its analogues are mediated through stimulation of the ryanodine receptor. cADPcR-elicited potentiation of depolarization-induced $[Ca^{2+}]_i$ increases was concentration-dependent in the tested range between 0.01 – 10 μM. On the other hand, 8-Cl-cADPcR, cIDPcR, or 8-Br-clDPcR at concentrations below 1 μM did not show apparent potentiating effects (Fig. 2).

Fig. 3a shows an example of the marked increase in $[Ca^{2+}]_i$ evoked by stimulation to seven different voltage steps from –40 mV to +30 mV in increments of 10 mV in cADPcR-infused cells. The increase was maximal at depolarization to –20 or -10 mV, decreasing with further depolarization. Fig. 4a shows the average $[Ca^{2+}]_i$ plotted as a function of holding potentials. The level of $[Ca^{2+}]_i$ at -10 mV was 279 ± 21% (n =4) of the

value at -40 mV in cADPcR-injected cells, while its equivalent in control cells was 156 ± 11% (*n* = 4). Analyses were performed using the same protocol for 8-Cl-cADPcR, cIDPcR, 8-Br-clDPcR, and results were shown in Figs. 3b, c and 4a. In cells infused with 8-Cl-cADPcR, cIDPcR, or 8-Br-cIDPcR, the depolarization-induced increase was slightly, but not significantly, potentiated in this rank order, peaking at voltage steps to –10 mV, similarly to the observations in control cells: the corresponding levels of $[Ca^{2+}]_i$ at -10 mV were 211 ± 26%, 198 ± 26%, and 173% ± 8%, respectively (n=4).

In cADPcR-infused cells, the depolarization-induced $[Ca^{2+}]_i$ increase was abolished by treatment with nifedipine as an L-type VACC antagonist (Figs. 3d and 4b), and with ruthenium red as an antagonist of the RyR (Figs. 3e and 4b): with these compounds, the $[Ca^{2+}]_i$ levels at -10 mV were 108 ± 8% and 123 ± 12%, respectively (n=4). These results suggest that cADPcR amplified CICR triggered by the activation of VACCs, in the same manner as the action of cADPR in neuronal cells (Hua *et al.* 1996; Empson and Galione 1997; Hashii *et al.* 2000).

cADPR is hydrolyzed to ADP-ribose (ADPR) by cADPR hydrolases such as CD38 in neurons (Higashida *et al.* 2001b). It was recently demonstrated that free ADPR gates the calcium-permeable cation channels (LTRPC2/TrpC7) (Perraud *et al.* 2001; Kuhn and Luckhoff 2004), which is expressed at high levels in neurons (Perraud *et al.* 2001). This finding prompted us to examine the involvement of ADPR in our system**.** ADPR slightly potentiated the depolarization-induced increase in $[Ca^{2+}]_i$, although the effect was not statistically significant: The level of $[Ca^{2+}]_i$ at -10 mV was 168 ± 12% of that at -40 mV (n = 5). ADPR had no effect on hyperpolarization-activated Ca^{2+} influx in NG108-15 cells (Fig. 3g and 4c). Thus, no relationship was found between ADPR and CICR in NG108-15 cells.

The interfering effects of 8-Cl-cADPcR and 8-Br-cADPR were examined in co-infused cells, as several cADPR analogues modified at the 8-position of the adenine group have antagonistic activities against cADPR-induced Ca^{2+} release (Walseth and Lee 1993). Depolarization-induced increases in $[Ca^{2+}]_i$ were significantly decreased by co-injection of 8-Cl-cADPcR or 8-Br-cADPR from the potentiated level by cADPcR alone

(Fig. 5a and c). In addition, the depolarization-induced increases in $[Ca^{2+}]_i$ were significantly decreased by co-injection of 8-Cl-cADPcR or 8-Br-cADPR from the potentiated level by cADPR alone (Fig. 5b and c).

Discussion

The present results show that a stable analogue of cADPR, cADPcR, applied through a patch pipette did not trigger the $[Ca^{2+}]_i$ increase by itself under the clamp conditions used in the present study, but potentiated $[Ca^{2+}]_i$ increases induced by activation of VACCs, similarly to the endogenous ligand, cADPR (Sitsapesan *et al.* 1995). Thus, we concluded that cADPcR is an agonist and that 8-Cl-cADPcR is an antagonist of the ryanodine-involving reaction in mammalian neuronal cells. The results of the present study indicate that the NG108-15 cell system is unique and useful for characterizing analogues, although the structure-activity relationships of cADPR analogues have not been fully characterized, in comparison with that of sea urchin egg microsomes or T-lymphocytes (Guse *et al.* 1999 and 2002; Walseth and Lee 1993 and 2002).

The data for holding membrane potential - $[Ca^{2+}]_i$ relationship in Figs. 3 and 4 indicated that cADPcR-induced potentiation was maximal at –20 mV. On the other hand, cADPR and β-NAD^+ augmented depolarization-induced $[Ca^{2+}]_i$ elevation due to the activation of L-type VACCs, peaking at –10 or 0 mV (Hashii *et al.* 2000). Thus, the peak

induced by cADPcR was shifted to the left by more than –10 mV. This indicates that L-type VACCs are considerably sensitized by cADPcR, and suggests that cADPcR is more active than cADPR. However, although it is less likely, the present study did not exclude the possibility that the cADPcR-induced potentiation of Ca^{2+} increases is due to the recruitment of different types of VACCs, such as T- or N-types found in NG108-15 cells (Higashida *et al.* 1990).

The cADPcR-induced augmentation in rodent NG108-15 cells is similar to that in sea urchin eggs (Shuto *et al.* 2001). However, injection of cADPcR itself can induce increases in $[Ca^{2+}]_i$ in sea urchin eggs, but not in NG108-15 cells. This is not surprising because cADPR can act as a direct ligand to induce $[Ca^{2+}]_i$ increases in sea urchin eggs, while in mammalian neurons, cADPR is not a direct ligand, but is an indirect modulator of Ca^{2+} increases (Hua *et al.* 1996; Empson and Galione 1997; Hashii *et al.* 2000). The increase induced by the derivative was 5 to 7-fold greater than that induced by native cADPR in the sea urchin, although it was less than 2-fold in mammalian neurons. Thus, the peak level of cADPcR-evoked Ca^{2+} concentrations reached 3 μM in sea urchin eggs (Shuto

et al. 2001), whereas it reached only 200 – 300 nM in the present study. These observations indicate that the non-hydrolytic property of cADPcR is effective in neuronal cells. Ca^{2+} release in permeabilized T cells was also directly activated by cADPcR, but was much less effective than that by cADPR (Guse *et al.* 1999 and 2002). The discrepancy in the sensitivity of cADPcR between different cell types may be explained in part by the diversity of cADPR binding proteins.

We demonstrated an antagonistic effect of 8-Cl-cADPcR against both cADPcR and cADPR (Fig. 5). Generally, substitution with amino (8-NH_2), azido (8-N_3), or bromo (8-Br) groups at the 8-position of the adenine ring produces an antagonistic effect on cADPR-induced Ca^{2+} release in various biological systems (Walseth and Lee 2002), including sea urchin eggs (Walseth and Lee 1993) and T lymphocytes (Guse *et al.* 1999 and 2002). This is also true of cADPcR with 8-Cl substitution. Our findings in Fig. 5 suggest that cADPcR acts on the same site as cADPR, and that 8-Cl-cADPcR competes for the cADPR/cADPcR binding site. The antagonistic activity of 8-Br-cADPR against cADPcR-induced Ca^{2+} release, as shown in Fig. 5, support this idea. Although not

significant, 8-Cl-cADPcR seemed to have slight agonistic activity (Figs. 1,2, and 4). Further studies are required to determine if 8-Cl-cADPcR behaves as a partial agonist as described previously in the sea urchin (Shuto *et al.* 2003).

Another stable mimic, cIDPcR, differs structurally from cADPcR in that the adenine ring has been replaced by hypoxanthine linked to the "Northern ribose" *via* N-1 (Fukuoka *et al.* 2000). As the conformation of cIDPcR was considered to be similar to that of cADPcR, cIDPcR should play a role in Ca^{2+} mobilization. It was recently reported that stable cIDPR mimics have strong Ca^{2+} release activity in rat brain microsomes and intact HeLa cells (Huang *et al.* 2002). However, cIDPcR did not cause such strong amplification in NG108-15 cells as far as we tested at 10μM. As cIDPR analogues are anticipated as therapeutic agents (Huang *et al.* 2002), it is necessary to define the efficiency of other stable cIDPR mimics in this neuronal system.

In conclusion, cADPcR, a stable mimic of cADPR, enhances the depolarization-induced Ca^{2+} increase that is antagonized by co-injection with 8-Cl-cADPcR.

These results indicated the presence of functional reciprocal coupling between RyRs and VACCs *via* cADPR, which was revealed by cADPcR and antagonized by 8-Cl-cADPcR.

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Figure legends

Fig. 1. Structures of cADPR and its analogues, and the effects of cADPcR on membrane depolarization-evoked $[Ca^{2+}]_i$ increases in patch-clamped NG108-15 cells.

Structures of cADPR (a), cADPcR (b), 8-Cl-cADPcR (b), cIDPcR (c), and 8-Br-cIDPcR(c) are shown. (d-i) Traces show $[Ca^{2+}]_i$ changes in fura 2-loaded cells infused with test compounds. At about 2 min before the beginning of each trace, cell membranes were ruptured with pipettes filled with the indicated concentrations of cADPcR, 8-Cl-cADPcR, cIDPcR, cADPR, β-NAD^+, and α-NAD^+. Then, the membranes were voltage-clamped at -40 mV, followed by depolarization to -20 mV at time zero. (j-l) Traces show $[Ca^{2+}]_i$ changes in control cells without drugs (j), those treated with 25 μl of 10 mM caffeine applied extracellularly (k), and those treated with 25 μl of 200 μM ryanodine applied extracellularly (l); the arrow indicates the time of application.

Fig. 2. Action of various concentrations of cADPR analogues in NG108-15 cells.

(a) Cells were infused with the indicated concentrations of cADPcR, 8-Cl-cADPcR, cIDPcR, and 8-Br-cIDPcR, and then membrane potentials were depolarized from -40 mV to -20 mV at time zero, as shown in Fig. 1d-l. (b) Concentration-response curves for the action of cADPR analogues. Cells were infused with the indicated concentrations of cADPR analogues, 10 μM cADPR (●), or without drugs (○). Each plot indicate $[Ca^{2+}]_i$ levels at peak after depolarized to -20 mV. $[Ca^{2+}]_i$ levels are represented as percentages of $[Ca^{2+}]_i$ relative to that at -40 mV. Each symbol shows mean ± S.E.M. of 3 - 6 experiments. *, Significantly different from drug-free control value ($P<0.05$).

Fig. 3. Effects of cADPR analogues, co-application of various inhibitors, and ADPR on $[Ca^{2+}]_i$ at various holding membrane potentials in NG108-15 cells.

Time courses of holding membrane potentials are shown at the top of each panel. Traces in the middle show fluorescence-ratio signals for $[Ca^{2+}]_i$. The lowest squares indicate the duration of whole-cell mode, with intracellular injection of 10 μM of cADPcR through the patch pipette (a), 10 μM cIDPcR (b), 10 μM 8-Br-cIDPcR (c), 10 μM cADPcR with

extracellular application of 10 μM nifedipine (d), 10 μM of cADPcR infused with 10 μM ruthenium red (e), without treatment (f), or 300 μM ADPR through the patch pipette (g). Bar, 100 s.

Fig. 4. Effects of cADPR analogues, nifedipine, ruthenium red, and ADPR on $[Ca^{2+}]_i$ at various holding membrane potentials in NG108-15 cells.

Average $[Ca^{2+}]_i$ as a function of holding membrane potential was measured as described in the legend to Fig. 3. The $[Ca^{2+}]_i$ levels were determined 100 s after stepping to the specified potential, and are expressed as percentages of the values at -40 mV. (a) The $[Ca^{2+}]_i$ levels are shown for cells infused with 10 μM cADPcR (□), 10 μM 8-Cl-cADPcR (■), 10 μM cIDPcR (Δ), 10 μM 8-Br-cIDPcR (····), or non-infused control cells (○). Values in cells exposed to cADPcR from -30 to +30 mV were significantly higher than those in control cells at $P<0.05$. Each point shows mean±S.E.M. of 3 - 4 cells. (b) The $[Ca^{2+}]_i$ levels shown for cells infused with 10 μM cADPcR (□), cADPcR +10 μM ruthenium red (■), or cADPcR +10 μM extracellular nifedipine (····), or non-infused control cells (○). (c) The

$[Ca^{2+}]_i$ levels are shown for cells infused with 300 μM ADPR (■), 10 μM ADPR (----), or non-infused control cells (○).

Fig. 5. Inhibitory effects of 8-Cl-cADPcR and 8-Br-cADPR on $[Ca^{2+}]_i$ increases evoked by membrane depolarization in NG108-15 cells infused with cADPcR or cADPR.

(a, b) Traces show $[Ca^{2+}]_i$ changes in fura 2-loaded cells. At about 2min before the beginning of each trace, cell membranes were ruptured with patch pipettes containing (a) 10 μM cADPcR, 10 μM cADPcR+10 μM 8-Cl-cADPcR, 10 μM cADPcR+10 μM 8-Br-cADPR, or (b) 10 μM cADPR, 10 μM cADPR+10 μM 8-Cl-cADPcR, or 10 μM cADPR+10 μM 8-Br-cADPR. Membranes were voltage-clamped at -40 mV, followed by depolarization to -20 mV at time zero, as shown in Fig. 1. (c) $[Ca^{2+}]_i$ levels were plotted at the peak after depolarization to -20 mV, and are given as percentages of that at -40 mV. Each symbol is mean ± S.E.M. of 3 - 4 experiments. *, Values in cells

pretreated with 8-Cl-cADPcR or 8-Br-cADPR were significantly lower than those in cells treated with cADPcR or cADPR alone at $p<0.05$.

Fig. 1 Hashii et al.

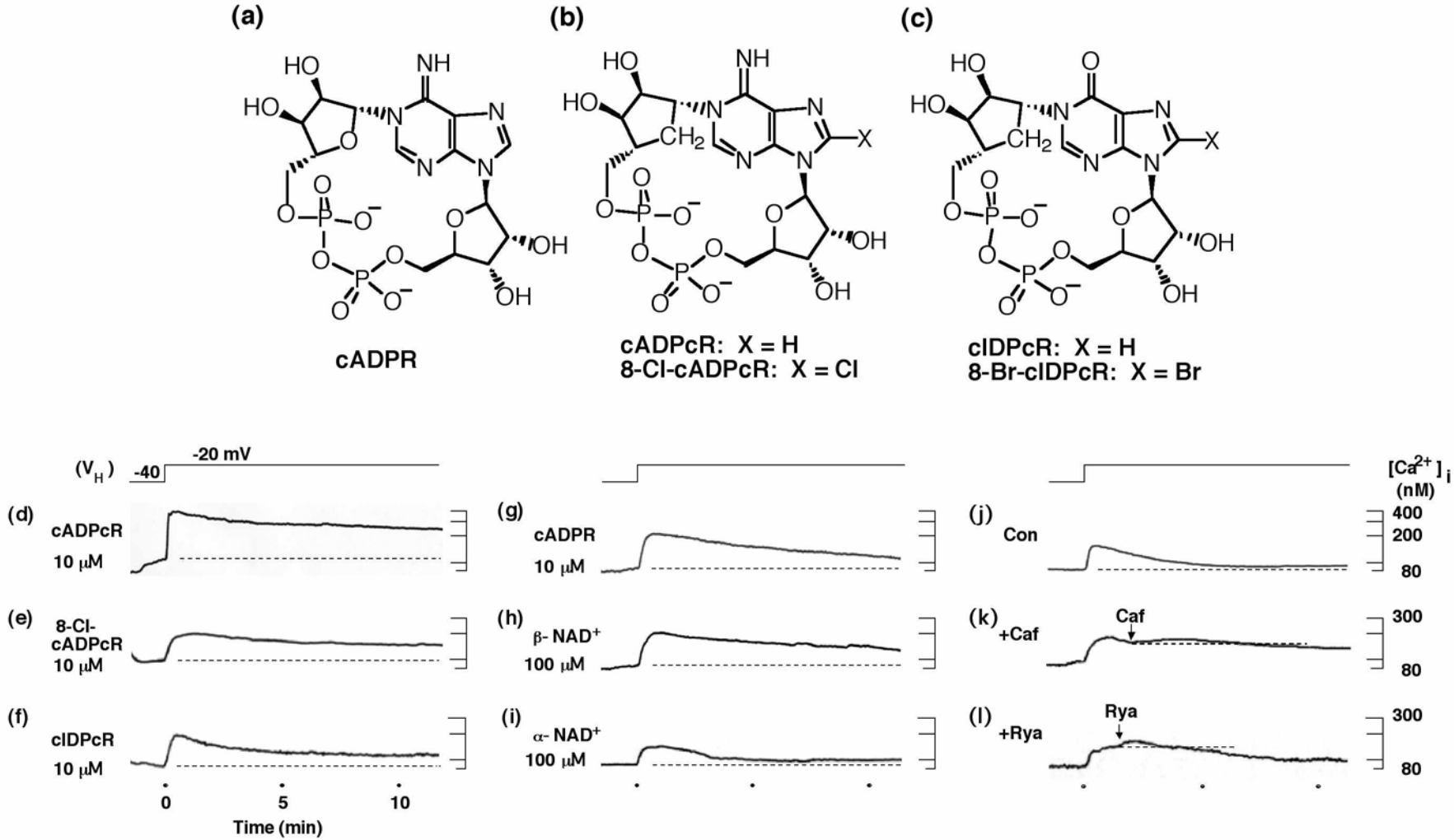
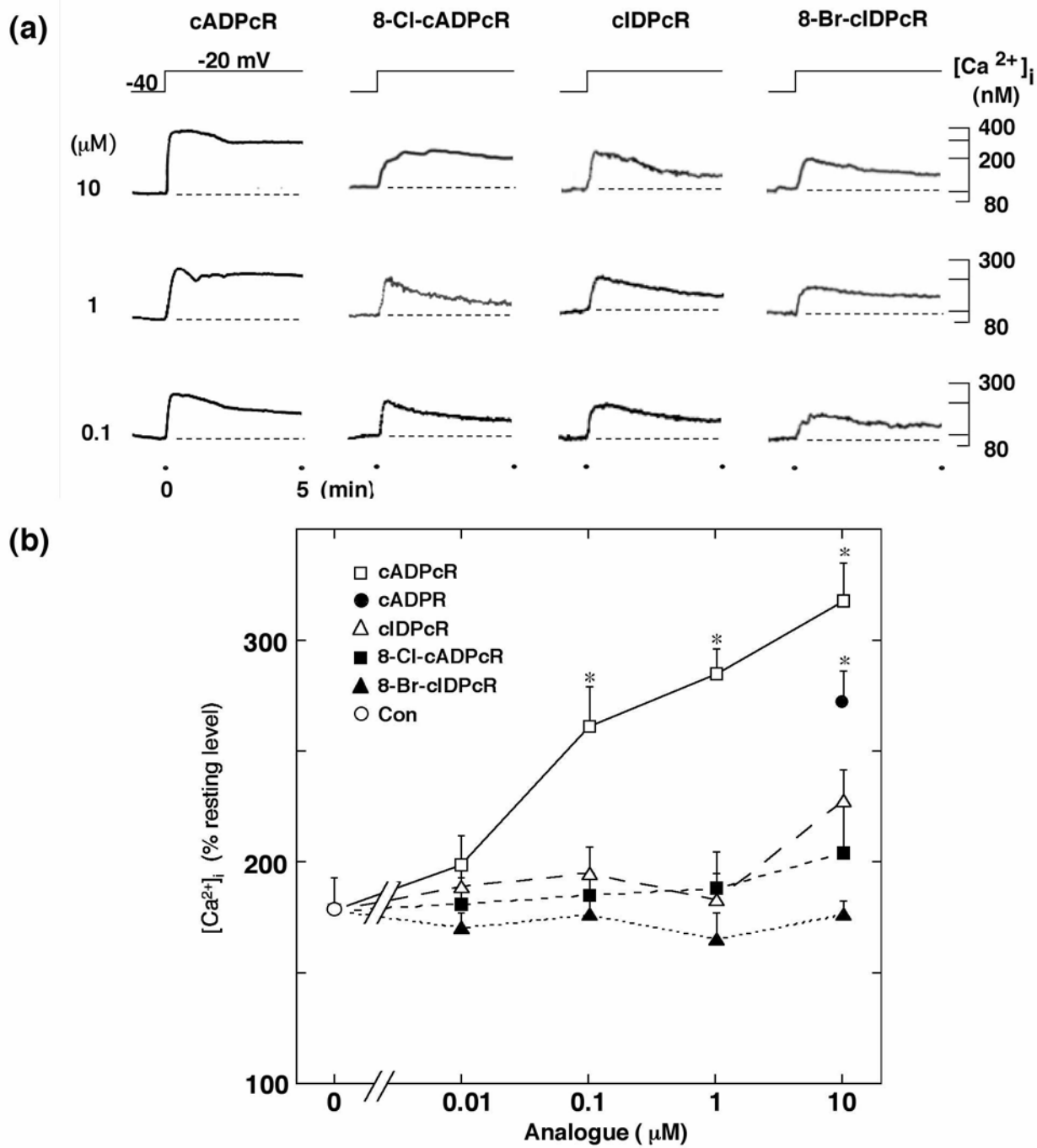


Fig. 2 Hashii et al.



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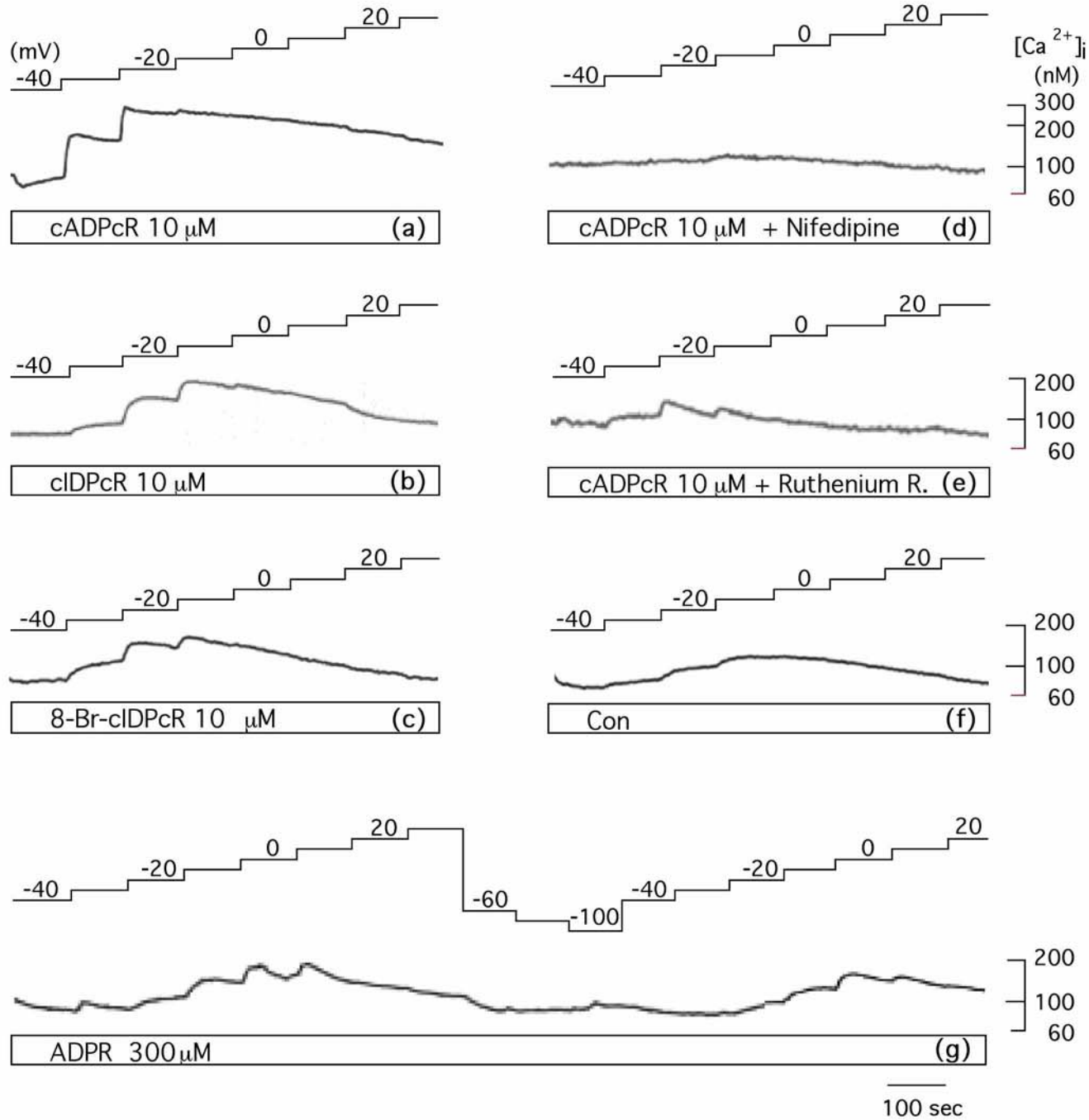

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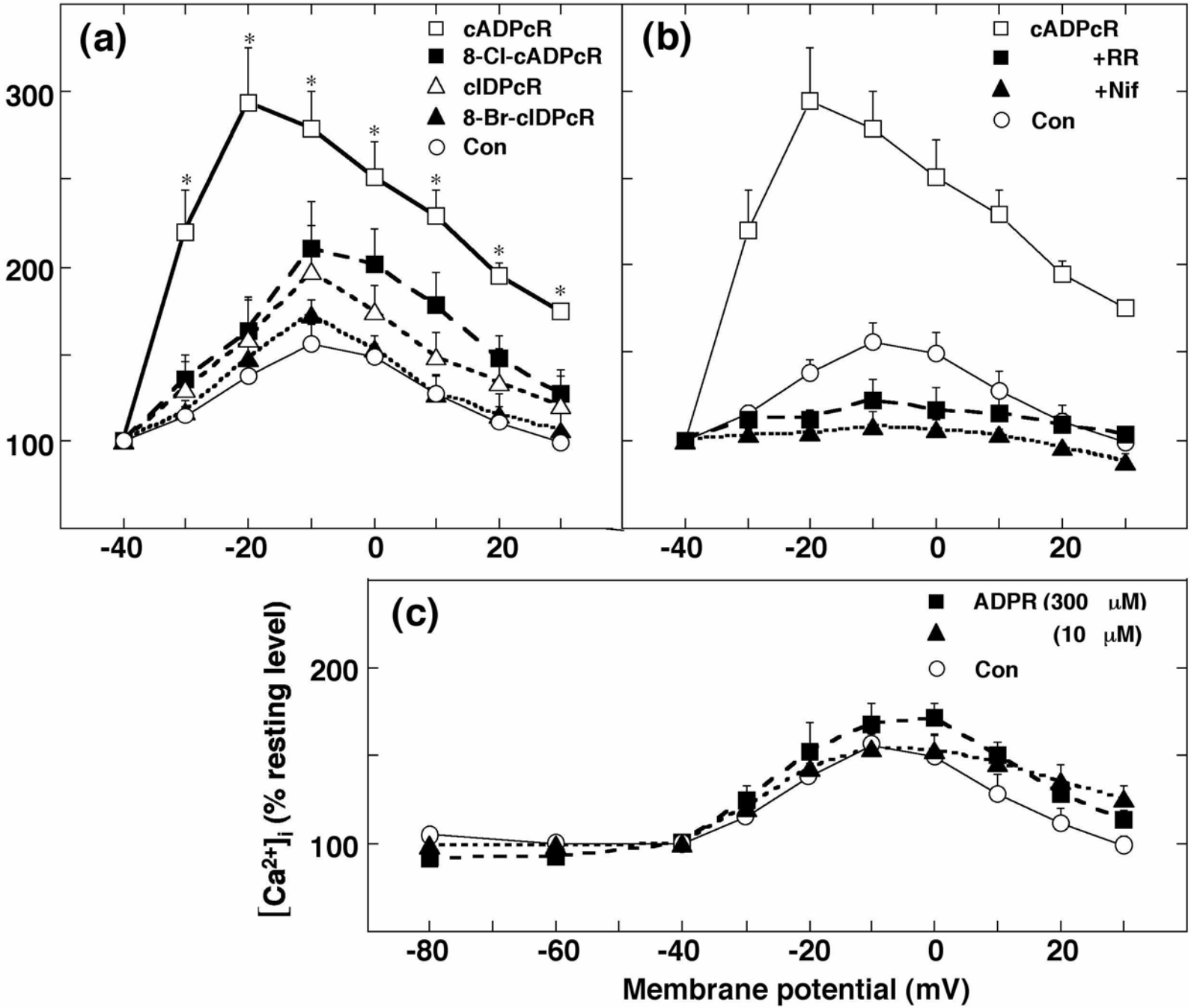
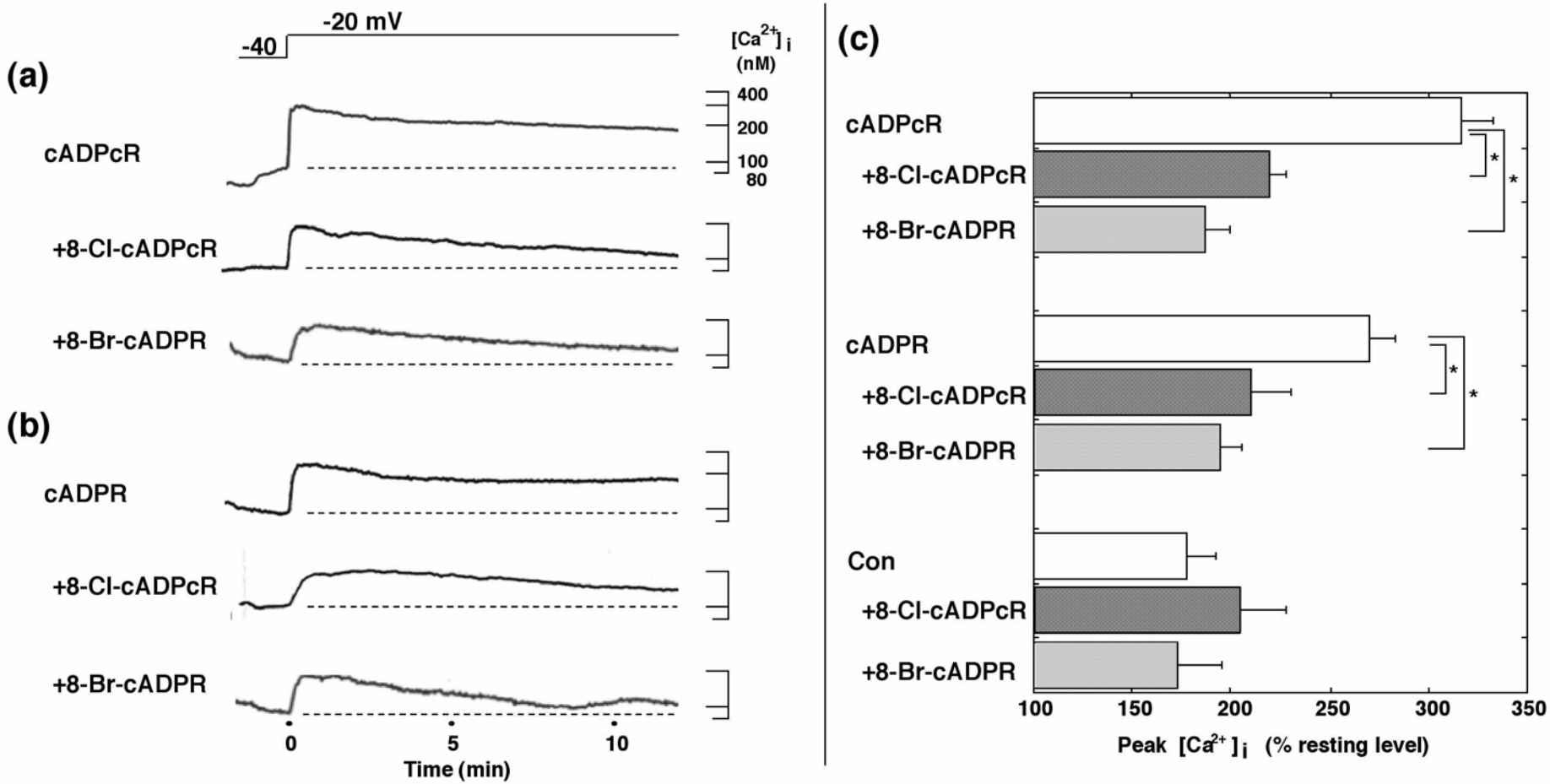
Fig. 5 Hashii et al.