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# Diverse effects of monensin on capacitative $Ca^{2+}$ entry and release of stored $Ca^{2+}$ in vascular smooth muscle cells

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**Abstract**

The effects of monensin, an activator of $Na^+/H^+$ exchanger (NHE), on capacitative $Ca^{2+}$ entry (CCE) were investigated using A7r5 cells. Capacitative $Ca^{2+}$ entry was induced by elevation of extracellular $Ca^{2+}$ concentrations of A7r5 cells in which stored $Ca^{2+}$ had been depleted by previous administration of thapsigargin. Capacitative $Ca^{2+}$ entry was abolished by pretreatment of the cells with SKF-96365 (1-[β-(3-[4-methoxyphenyl]propoxy)-4-methoxyphenethyl]-1*H*-imidazole hydrochloride) but was not affected by pretreatment with verapamil. Monensin significantly increased capacitative $Ca^{2+}$ entry. On the other hand, 5-hydroxytryptamine-induced inositol monophosphate accumulation and subsequent intracellular $Ca^{2+}$ release from its stores were significantly inhibited by monensin, while thapsigargin-induced $Ca^{2+}$ release was not affected by monensin. These results suggest that monensin has diverse actions on capacitative $Ca^{2+}$ entry and agonist-induced release of stored $Ca^{2+}$ in vascular smooth muscle cells.


*Keywords:* $Ca^{2+}$ channel; $Ca^{2+}$ entry, capacitative; Inositol 1,4,5-trisphosphate; Monensin; Smooth muscle, vascular; Vasoconstriction

## 1. Introduction

Phosphoinositide hydrolysis is an initial response following stimulation of plasmalemmal receptors by G protein-coupled receptor agonists and triggers cellular activation in a variety of cells, including vascular smooth muscle cells (Abdel-Latif, 1986). Inositol trisphosphate ($IP_3$), a metabolite of phosphoinositide hydrolysis, induces release of $Ca^{2+}$ from its intracellular stores. The depletion of $Ca^{2+}$ stores then induces voltage-independent plasmalemmal $Ca^{2+}$ entry, known as capacitative $Ca^{2+}$ entry (CCE) (Putney, 1986). Thus, CCE is thought to be involved in a mechanism of receptor-stimulated $Ca^{2+}$ entry into vascular smooth muscle cells (Putney, 1997; McFadzean and Gibson, 2002). Diacylglycerol, another metabolite of phosphoinositide hydrolysis, activates protein kinase C (PKC), which plays crucial roles in cellular functions. In vascular smooth muscle cells, PKC activation results in activation of plasmalemmal voltage-dependent $Ca^{2+}$ channels (Gollasch and Nelson, 1997) as well as increase in $Ca^{2+}$ sensitivity of the contractile apparatus (Rasmussen et al., 1987). However, the mechanism of $Ca^{2+}$ channel opening following PKC activation is still not clear. On the other hand, PKC is known to activate the $Na^+/H^+$ exchanger (NHE), which is a ubiquitously expressed membrane transport protein, through phosphorylation of its serine residues (Sardet et al., 1990). In vascular smooth muscle cells, receptor stimulation by agonists such as angiotensin II results in activation of NHE, which is involved in a mechanism of proliferative action of the agonists (Berk et al., 1987; LaPointe and Batlle, 1994). However, it is not known whether modulation of NHE affects the mechanism of $Ca^{2+}$ entry, including CCE, into vascular smooth muscle cells. In the present study, we therefore investigated the effects of monensin, an activator of NHE, on CCE and its related mechanisms such as phosphoinositide hydrolysis and $Ca^{2+}$ release from its stores. CCE was induced by elevation of extracellular $Ca^{2+}$ concentrations in the presence of thapsigargin, a $Ca^{2+}$-ATPase inhibitor (Treiman et al., 1998), and phosphoinositide hydrolysis was evaluated by accumulation of inositol monophosphate in the presence of LiCl (Berridge et al., 1982).

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## 2. Materials and methods

### 2.1. Cell culture

A7r5 rat aortic smooth muscle cells were obtained from Dainippon Pharmaceutical (Osaka, Japan) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal calf serum, 4 mM glutamate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B in a humidified atmosphere at 37 °C under 5% $CO_2$–95% air. The cells were spread in 12-well or 100-mm dish and cultured until reaching a confluent condition. Then, confluent cells were used for the assays.

### 2.2. Measurement of intracellular free $Ca^{2+}$ concentration

$[Ca^{2+}]_i$ (intracellular free $Ca^{2+}$ concentration) was measured using a fluorescent $Ca^{2+}$ indicator, fura-2. A7r5 cells were loaded with fura-2/acetoxymethyl ester (AM) (final concentration, 5 μM) at 37 °C for 30 min. After loading, the cells were washed once with physiological salt solution (PSS) buffered by HEPES (mM: NaCl 135, KCl 5, $KH_2PO_4$ 1, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4), and they were resuspended in $Ca^{2+}$-free PSS buffered by HEPES and containing 0.01 mM EGTA (nominally $Ca^{2+}$-free solution).

Fluorescence measurements were carried out with a dual-wavelength spectrofluorometer (F2500 Fluorescence Spectrophotometer, Hitachi, Tokyo, Japan) using a 0.4-ml cuvette maintained at 37 °C. The wavelengths used for excitation were 340 and 380 nm, and the wavelength used for emission was 510 nm. Using a ratio ($R$) of fluorescence intensity ($F$) of $F_{340}/F_{380}$, the fractional changes in $[Ca^{2+}]_i$ were determined. The fluorescence after sequential addition of 0.2% Triton X-100 and EGTA (5 mM) to the cuvette provided the maximum fluorescence ratio ($R_{max}$) and minimum fluorescence ratio ($R_{min}$), respectively. $[Ca^{2+}]_i$ was calculated using the formula described by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R) \times \beta \times K_d,$$

where $\beta$ is the ratio of the emission fluorescence values at 380 nm excitation in the presence of Triton X-100 and EGTA, and $K_d$, the dissociation constant for $Ca^{2+}$, is 224. CCE was expressed as a net increase in $[Ca^{2+}]_i$ evoked by extracellular addition of $CaCl_2$ following thapsigargin stimulation in nominally $Ca^{2+}$-free medium.

### 2.3. Measurement of inositol monophosphate accumulation

The A7r5 cells were stabilized in each well containing 0.5 ml of DMEM, and then *myo*-[2-$^3$H]inositol (0.05 μM) was added to the medium. After 24 h of incubation, the cells were used for measurement of inositol monophosphate accumulation. Inositol monophosphate accumulation was measured according to the method described previously (Berridge et al., 1982). The cells were rinsed three times with fresh warm (37 °C) phosphate-buffered saline. Then, LiCl at a final concentration of 10 mM was added to each well containing 0.5 ml of DMEM, and 5-hydroxytryptamine (5-HT, 100 μM) or the vehicle was added to the well 30 min later and further incubated at 37 °C for 20 min. The reaction was then terminated by addition of 0.9 ml of chloroform–methanol solution (1:2, v/v), followed by addition of 0.3 ml of chloroform and vortexing. Water (0.3 ml) was then added, followed by vigorous vortexing. The medium in each well was collected in a glass tube and then centrifuged at 1000 × $g$ for 5 min, allowing the aqueous and chloroform phases to separate. An aliquot of 0.9 ml of the upper phase was then loaded onto AG 1X8 (formate form) resin packed in a disposable column. The columns were then sequentially washed with 9 ml of water, 9 ml of 60 mM sodium formate/5 mM sodium borate, and 9 ml of 1 M ammonium formate/0.1 M formic acid to selectively elute [$^3$H]inositol monophosphate. Aliquots (3 ml) of the eluant were mixed with scintillant, and its radioactivity was counted in a liquid scintillation spectrophotometer. The level of inositol monophosphate accumulation was expressed as [$^3$H]inositol monophosphate level (dpm) of each aliquot.

### 2.4. Drugs

Fura-2/AM (Dojin, Kumamoto, Japan) and thapsigargin (Sigma, St. Louis, MO, USA) were dissolved in dimethylsulfoxide to make a stock solutions of 5 mM and 1 mM, respectively, and stored at −30 °C. 1-[β-(3-[4-Methoxyphenyl]propoxy)-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SKF-96365, Calbiochem, La Jolla, CA, USA) was dissolved in distilled water to make a stock solution of 10 mM and stored at −30 °C. Verapamil (Wako, Osaka, Japan) and 5-hydroxytryptamine hydrochloride (Sigma) were dissolved in distilled water to make stock solutions of 10 mM, and stored at 4 °C. Monensin (Sigma) was dissolved in ethanol to make a stock solution of 50 mM, which was stored at −30 °C and diluted with distilled water just before use. (1-[6-([(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1*H*-pyrrole-2,5-dione) (U73122, Sigma) was dissolved in dimethylsulfoxide to make a stock solution of 1 mM, which was stored at −30 °C and diluted with distilled water just before use.

### 2.5. Statistical analysis

Statistical analysis was done using Student's $t$ test or Mann–Whitney $U$ test. $P$ values less than 0.05 were regarded as significant.

## 3. Results

### 3.1. Effects of monensin on thapsigargin-induced CCE

Fig. 1A shows a representative recording of CCE induced in the presence of thapsigargin in A7r5 cells. In nominally

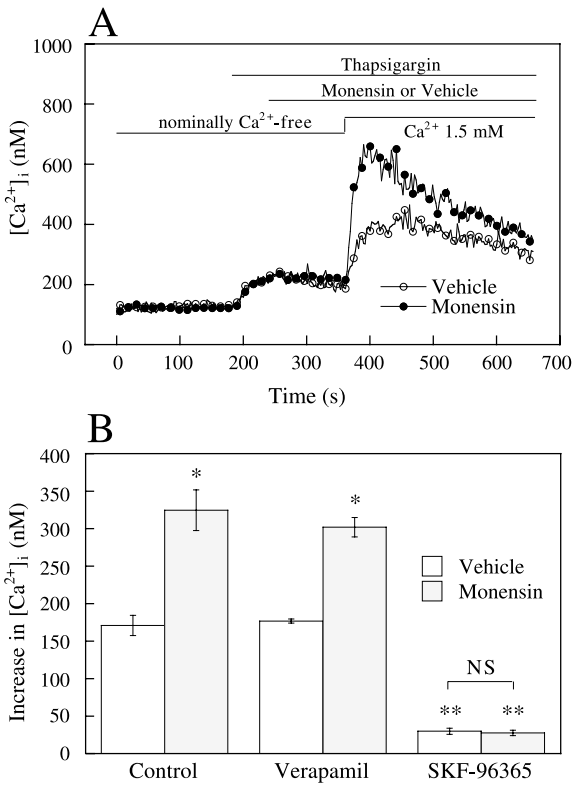


Fig. 1. (A) Representative recording of induction of capacitative $Ca^{2+}$ entry in A7r5 cells. The cells were incubated in nominally $Ca^{2+}$-free solution. After stabilization, the cells were stimulated with thapsigargin (0.1 μM). At 3 min after thapsigargin stimulation, $CaCl_2$ (1.5 mM) was added to the cuvette containing the cells. Monensin (10 μM) or a vehicle was added to the cuvette at 2 min before addition of $CaCl_2$. (B) Effects of monensin on capacitative $Ca^{2+}$ entry in A7r5 cells. In nominally $Ca^{2+}$-free solution, A7r5 cells were incubated with thapsigargin (0.1 μM) for 3 min; then $CaCl_2$ (1.5 mM) was added to the solution. Monensin (10 μM) or a vehicle was added to the solution at 2 min before addition of $Ca^{2+}$. In some experiments, SKF-96365 (100 μM) or verapamil (1 μM) was added to the solution at 1 min before addition of $Ca^{2+}$. $[Ca^{2+}]_i$ at the peak after addition of $Ca^{2+}$ was used for the analysis. Asterisks denote significant differences from the condition without pretreatment with monensin (*) and from the condition without pretreatment with SKF-96365 (**). NS, not significant difference. $n = 4–5$.

$Ca^{2+}$-free solution, the addition of thapsigargin (0.1 μM) resulted in an increase in $[Ca^{2+}]_i$. Then elevation of the $Ca^{2+}$ concentration of the medium from nominal zero to 1.5 mM resulted in a further increase in $[Ca^{2+}]_i$ (CCE). The CCE was strongly inhibited by pretreatment with SKF-96365 but was not affected by pretreatment with verapamil (Fig. 1B).

Pretreatment of the cells with monensin (1 and 10 μM) augmented significantly the CCE (Fig. 1) in a concentration-dependent manner (Fig. 2). SKF-96365 abolished the CCE and the enhancing effect of monensin, but verapamil did not affect them (Fig. 1B).

### *3.2. Effects of monensin on thapsigargin- and 5-HT-induced $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores*

In nominally $Ca^{2+}$-free solution, stimulation of A7r5 cells with thapsigargin (0.1 μM) and 5-HT (100 μM) caused

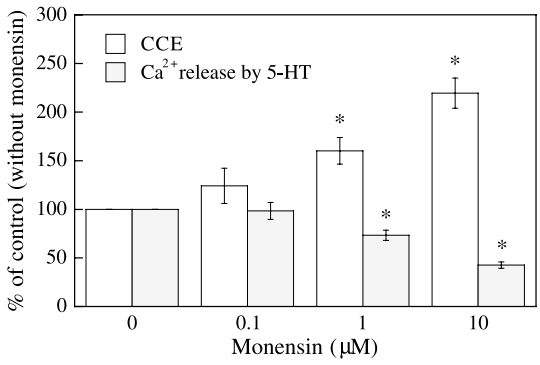


Fig. 2. Effects of monensin on thapsigargin-induced capacitative $Ca^{2+}$ entry (CCE) and 5-hydroxytryptamine (5-HT)-induced increase in $[Ca^{2+}]_i$ ($Ca^{2+}$ release) in A7r5 cells. In the experiments for thapsigargin-induced CCE, A7r5 cells were incubated in nominally $Ca^{2+}$-free solution with thapsigargin (0.1 μM) for 3 min; then $CaCl_2$ (1.5 mM) was added to the solution. Monensin (0.1, 1, 10 μM) or a vehicle was added to the solution at 2 min before addition of $Ca^{2+}$. $[Ca^{2+}]_i$ at the peak after addition of $Ca^{2+}$ was used for the analysis. In the experiments for 5-HT-induced $Ca^{2+}$ release, A7r5 cells were incubated with monensin (0.1, 1, 10 μM) for 4 min. 5-HT (100 μM) was then added to the solution. $[Ca^{2+}]_i$ at the peak after addition of 5-HT was used for the analysis. The data are expressed as the percentage of CCE or 5-HT-induced $Ca^{2+}$ release in the cells treated with a vehicle instead of monensin. Asterisks denote significant differences from the condition without pretreatment with monensin. $n = 5$.

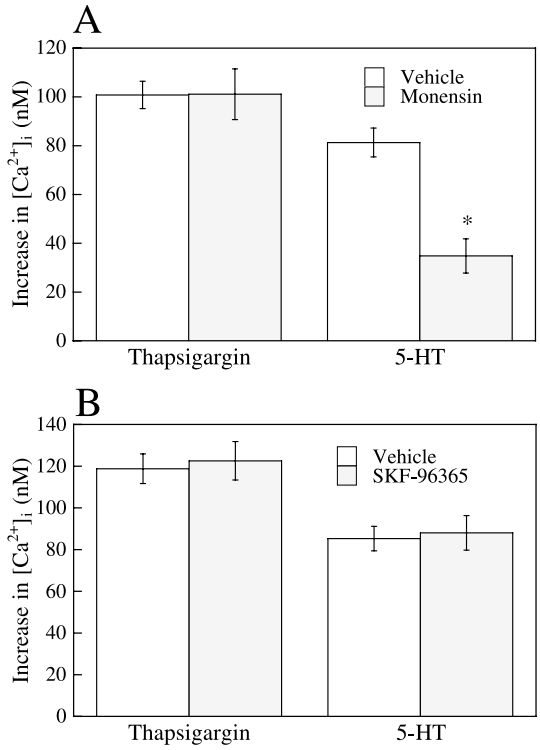


Fig. 3. Effects of monensin (A) and SKF-96365 (B) on thapsigargin-or 5-hydroxytryptamine (5-HT)-induced increases in $[Ca^{2+}]_i$ of A7r5 cells in $Ca^{2+}$-free solution. In nominally $Ca^{2+}$-free solution, A7r5 cells were incubated with monensin (10 μM) or SKF-96365 (100 μM) for 4 min. Thapsigargin (0.1 μM) or 5-HT (100 μM) was then added to the solution. $[Ca^{2+}]_i$ at the peak after addition of thapsigargin or 5-HT was used for the analysis. An asterisk denotes a significant difference compared with the control pretreated with a vehicle. $n = 3–8$.

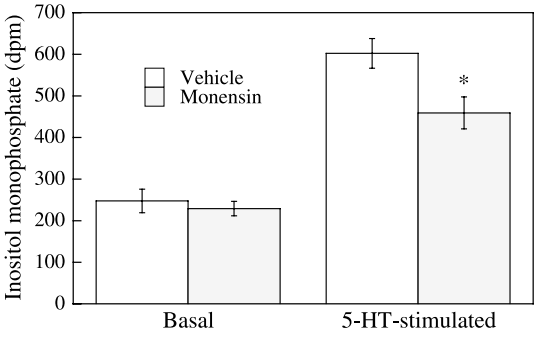


Fig. 4. Effects of monensin on 5-hydroxytryptamine (5-HT)-induced inositol monophosphate accumulation in A7r5 cells. A7r5 cells were pretreated with monensin (10 μM) for 1 min and then stimulated with 5-HT (100 μM) for 20 min. An asterisk denotes a significant difference compared with the control pretreated with a vehicle. $n = 5$.

a transient increase in $[Ca^{2+}]_i$. Monensin (1, 10 μM) inhibited the 5-HT-induced increase in $[Ca^{2+}]_i$ in a concentration-dependent manner but did not affect the thapsigargin-induced increase in $[Ca^{2+}]_i$ (Figs. 2 and 3A). U73122 (4 μM) abolished 5-HT-induced increase in $[Ca^{2+}]_i$ [82.0 ± 7.6 nM (control) vs. 7.4 ± 2.0 nM (U73122-treated) ($P < 0.01$)]. Neither thapsigargin-induced nor 5-HT-induced $Ca^{2+}$ release was affected by SKF-96365 (Fig. 3B).

### 3.3. Effects of monensin on 5-HT-induced phosphoinositide hydrolysis

Inositol monophosphate accumulation was increased by 5-HT (100 μM) stimulation to a level about 2.5 times higher than the basal level. 5-HT-induced inositol monophosphate accumulation was significantly inhibited by monensin (10 μM), while monensin did not affect the basal level of inositol monophosphate accumulation (Fig. 4).

## 4. Discussion

The relationship between NHE activation and CCE, both of which are triggered by accelerated phosphoinositide turnover following agonist stimulation, has not been clarified. The present study has shown that activation of NHE by monensin increases thapsigargin-induced voltage-independent $Ca^{2+}$ entry. Since CCE and NHE are activated by $IP_3$ (through $Ca^{2+}$ store depletion) and diacylglycerol (through PKC activation), respectively, following agonist-stimulated phosphoinositide turnover, it is possible that these two signal pathways work in harmony to increase $Ca^{2+}$ entry due to $Ca^{2+}$ store depletion. In the present study, monensin was added to the medium after $Ca^{2+}$ stores had been depleted by thapsigargin. Thus, the pathway of CCE after $Ca^{2+}$ store depletion may be facilitated by activation of NHE.

Monensin, which activates NHE, enhanced CCE in A7r5 cells, implying that activation of NHE by monensin leads to intracellular alkalinization by extrusion of intracellular protons via NHE. In support of this postulate, we recently found that monensin induced a gradual increase in intracellular pH by about 0.1 pH when elevation of $[Ca^{2+}]_i$ due to CCE reached a peak level in the same condition (Wakabayashi et al., 2002, unpublished observation). SKF-96365, an inhibitor of receptor-operated $Ca^{2+}$ channels (Merritt et al., 1990), abolished the CCE and the enhancing effect of monensin, but verapamil, an inhibitor of voltage-dependent $Ca^{2+}$ channels, did not affect them. We also found that phenylephrine-induced verapamil-insensitive $Ca^{2+}$ entry and contraction of vascular smooth muscle were enhanced by $NH_4Cl$-induced increase in intracellular pH (Wakabayashi et al., 2001). Further study is required to elucidate the causal relationship between intracellular pH and CCE in vascular smooth muscle. Alternatively, it is possible that the enhancing effect of monensin on CCE is due to an increase in $Na^+$ influx by NHE activation and a subsequent increase in $Ca^{2+}$ influx through the $Na^+/Ca^{2+}$ exchanger (Valant et al., 1992). This hypothesis should also be examined in a future study.

Increased NHE activity has been reported in platelets, leukocytes and erythrocytes from primary hypertensive patients (Ng et al., 1989; Semplicini et al., 1989; Rosskopf et al., 1992). The phosphorylation level of NHE protein in cells from Wistar–Kyoto (WHY) rats was found to be only half of that in cells from spontaneously hypertensive (SHR) rats (Siczkowski and Ng, 1996). Moreover, transgenic mice in which NHE was constitutively overexpressed developed hypertension, which was not essential but salt-sensitive (Kuro-o et al., 1995). These findings suggest that NHE plays a significant role, especially in the pathogenesis of salt-sensitive hypertension. Therefore, facilitation of CCE due to increased NHE activity may be, in part, involved in increased contractility of VSMC in primary hypertension.

On the other hand, monensin attenuated 5-HT-stimulated inositol monophosphate accumulation and subsequent release of $Ca^{2+}$ from the stores, while thapsigargin-induced $Ca^{2+}$ release was not affected by monensin. 5-HT-induced $Ca^{2+}$ release was dependent on phosphoinositide hydrolysis, since U73122, a phospholipase C inhibitor, abolished 5-HT-induced increase in $[Ca^{2+}]_i$ of A7r5 cells. These findings suggest that activation of NHE inhibits agonist-induced phosphoinositide hydrolysis, resulting in a decrease in $IP_3$-induced $Ca^{2+}$ release from the stores. The concentration dependence of both of the diverse effects of monensin on CCE and 5-HT-induced $Ca^{2+}$ release supports the speculation that the mechanisms underlying these effects of monensin are the same. In the present study, monensin was used as a stimulant of $Na^+/H^+$ exchanger, which is activated by PKC following diacylglycerol formation. Monensin inhibited 5-HT-induced acceleration of phosphoinositide hydrolysis and thereby the 5-HT-induced $Ca^{2+}$ release. Thus, activation of the $Na^+/H^+$ exchanger following agonist stimulation may play a negative feedback role in agonist-induced phosphoinositide hydrolysis. There have been sev-

eral studies on the effects of monensin on phosphoinositide hydrolysis, and the results of the previous studies, in contrast to the results of the present study, have shown that monensin stimulates phosphoinositide hydrolysis by increasing intracellular $Na^+$ in brain synaptoneurosomes (Gusovsky et al., 1987) and microvessels (Catalan et al., 1996). Thus, the effects of monensin on phosphoinositide hydrolysis differ depending on the cell type. A7r5 cell is a cell line originating from fetal rat aortic smooth muscle and is often used for studies on function of vascular smooth muscle cells. Capacitative $Ca^{2+}$ channels have been demonstrated in A7r5 cells that are activated by agonists such as endothelin-1 (Miwa et al., 1999). However, further studies using native vascular smooth muscle cells are needed to clarify the mechanism of the inhibitory action of monensin on phosphoinositide hydrolysis.

In conclusion, monensin, an activator of NHE, facilitates CCE and inhibits agonist-induced phosphoinositide hydrolysis and subsequent $Ca^{2+}$ release from the stores, suggesting that NHE plays crucial roles in agonist-stimulated $Ca^{2+}$ entry and its related regulation of signal transduction.

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