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PRACTICAL USAGE CONCENTRATIONS OF MONENSIN HAVE NON-SPECIFIC ACTIONS OTHER THAN AS A SODIUM IONOPHORE IN RAT PAROTID ACINAR CELLS

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Abstract—Monensin is used as a sodium ionophore to examine the effect of Na^+ on cellular function in a variety of cell types. In the present study, we investigated the effects of different concentrations of monensin on the signal transduction system in exocrine parotid acinar cells. Monensin increased cytosolic free Na^+ concentration, measured by the Na^+ indicator sodium-binding benzofuran isophthalate in a concentration-dependent manner (0.01 to 100 μM). Likewise, monensin concentration-dependently increased amylase release and intracellular Ca^{2+} concentration in the presence and the absence of extracellular Ca^{2+}. Low concentrations (0.01 to 1 μM) of monensin did not release Ca^{2+} from non-mitochondrial intracellular pools in permeabilized cells with saponin but high concentrations (10 and 100 μM) of monensin which are of practical usage did. Monensin itself did not change the cyclic AMP accumulation, whereas high concentrations (10 and 100 μM) but not low concentrations (0.01 to 1 μM) of monensin inhibited cyclic AMP accumulation elevated by isoproterenol in the presence and absence of extracellular Na^+. These results indicate that high concentrations of monensin, which are practically used, have nonspecific actions in rat parotid acinar cells, and lower concentrations of monensin are recommended for use as a sodium ionophore.

Salivary secretion is regulated by the autonomic nervous system, and salivary acinar cells have their own receptors related to their neurotransmitters. Amylase release from parotid acinar cells induced by cholinergic and α-adrenergic agonists involves Ca^{2+} and protein kinase C; on the other hand, β-adrenergic agonists increase amylase release via cyclic AMP [1–4]. It is known that cholinergic and α-adrenergic agonists increase Na^+ entry from extracellular medium into parotid acinar cells [5–8]. Although Na^+ entry has a role in electrolyte transport, a functional role of intracellular free Na^+ concentration ($[Na^+]_i$)‡ in the signal transduction of exocytotic amylase release is less well known.

Monensin, a Na^+ ionophore, has been used widely to examine the effect of Na^+ on cellular function in a variety of cell types. This ionophore increases amylase release from mouse parotid acini probably due to the mobilization of Ca^{2+} from intracellular Ca^{2+} stores [9, 10] but it inhibits amylase release induced by β-adrenergic agonists from rat parotid acinar cells [11]. We have used different concentrations of monensin to examine the effect of $[Na^+]_i$ on amylase release, Ca^{2+} mobilization and cyclic AMP accumulation in exocytosis, and have found that high concentrations of monensin, even those concentrations that are practically used, have non-specific actions other than as a Na^+ ionophore in rat parotid acinar cells. A preliminary report of these results has been presented [12].

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‡ Abbreviations: $[Na^+]_i$, intracellular free Na^+ concentration; SBFI, sodium-binding benzofuran isophthalate; KRH, Krebs–Ringer–HEPES buffered solution; AM, acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; and IP_3, inositol 1,4,5-trisphosphate.

MATERIALS AND METHODS

Preparation of parotid acinar cells. Male Wistar rats (weighing 150–200 g) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). The parotid acinar cells were prepared from parotid glands by sequential trypsin and collagenase digestion as described previously [13] and resuspended in Krebs–Ringer–HEPES-buffered solution (KRH) under oxygenation. The composition of KRH was as follows: 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1 mM $CaCl_2$, 11.1 mM glucose and 20 mM HEPES (pH 7.4).

Measurement of $[Na^+]_i$. The cell suspension was incubated for 60 min in 10 μM sodium-binding benzofuran isophthalate (SBFI) acetoxymethyl ester (AM) at 37° in the presence of 0.1% pluronic acid and 0.5% bovine serum albumin. The cells were washed, resuspended in fresh KRH, and kept at room temperature under oxygenation. Just before use, aliquots of cells were washed and resuspended with fresh medium containing 0.2% bovine serum albumin at 37° in a quartz cuvette as described previously for fura-2-loaded parotid cells [13]. The fluorescence of SBFI-loaded cells was measured with an Hitachi spectrophotometer (650–10S) with

excitation at 340 nm and emission monitored at 500 nm. Because calibration to calculate $[Na^+]_i$ in the cell suspension is extremely difficult [14], the change of fluorescence of SBFI was regarded as the change in $[Na^+]_i$.

Determination of the amount of amylase released. The release of amylase from parotid acinar cells into the medium during a 30-min incubation was measured as described previously [15]. Amylase activity was assayed by the method of Searcy *et al.* [16]. Amylase released in the presence of monensin was expressed as the percentage of total amylase activity of cells before stimulation. The amylase activity of parotid cells before stimulation was 811.5 ± 9.0 (N = 9) U/mg of cell protein. Units of this enzyme activity were expressed as micromoles of maltose formed per minute at 37°.

Measurement of intracellular free Ca^{2+} *concentration* ($[Ca^{2+}]_i$). The cell suspension was incubated with 2 μM fura-2/AM for 45 min at 37°, washed, resuspended, and then kept at room temperature as described previously [13]. The fluorescence of fura-2-loaded cells was measured as for SBFI-loaded cells. $[Ca^{2+}]_i$ was calculated with maximum and minimum fluorescence determined by Triton X-100 and ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), respectively, as described in Ref. 17.

Determination of Ca^{2+} *release from permeabilized acinar cells.* Parotid acinar cells were permeabilized with 60 μg/mL saponin in a quartz cuvette at 37° in a medium containing ATP-regenerating system and mitochondrial inhibitors as described previously [18]. The composition of the medium was as follows: 20 mM NaCl, 100 mM KCl, 5 mM MgCl, 20 mM HEPES, 3 mM ATP, 10 mM phosphocreatine, 10 U/mL creatine phosphokinase, 10 μM antimycin A, 10 μg/mL oligomycin, and 1 μM fura-2 at pH 7.2.

Assay of cyclic AMP accumulation. Cyclic AMP in cell suspension 3 min after stimulation with drugs was assayed by a commercial assay kit (Yamasa Shoyu Co.) as described previously [15]. For measurements in the absence of extracellular Na^+, Na^+ in KRH was substituted by equimolar amounts of *N*-methyl-D-glucamine. Cellular protein was assayed by the method of Bradford [19].

Chemicals. Monensin, collagenase and *N*-methyl-D-glucamine were obtained from Wako Pure Chemical (Osaka, Japan). Trypsin, trypsin inhibitor, saponin, ATP disodium salt, phosphocreatine, creatine phosphokinase, and antimycin A were from the Sigma Chemical Co. (St. Louis, MO). D-*myo*-Inositol 1,4,5-trisphosphate (IP_3) was obtained from Funakoshi Pure Chemical (Tokyo, Japan), and oligomycin from Calbiochem (La Jolla, CA). Sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI/AM), fura-2 pentapotassium salt, fura-2/AM and pluronic F-17 were purchased from Molecular Probe (Eugene, OR).

Statistical analysis. The significance of differences between values was examined by Student's *t*-test.

RESULTS

Because the effect of monensin on $[Na^+]_i$ has not been determined although monensin increases $^{22}Na^+$ uptake into salivary acinar cells [7, 20], we examined whether monensin increases $[Na^+]_i$ using the Na^+ indicator SBFI. As shown in Fig. 1, monensin increased $[Na^+]_i$, which reaches a plateau within 1 min [12], in a concentration-dependent manner in the range of 0.01 to 100 μM. In addition, $[Na^+]_i$ increased linearly at higher concentrations of monensin (10 and 100 μM).

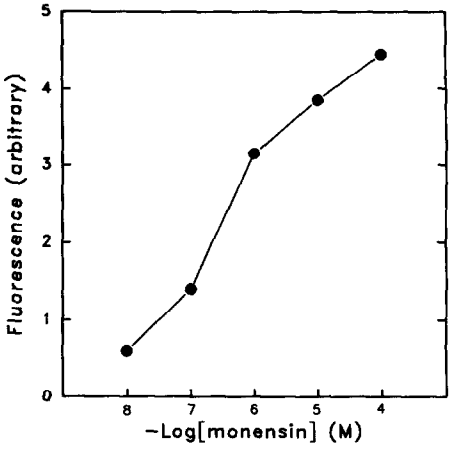

Fig. 1. Representative concentration–response curve of $[Na^+]_i$ for monensin in rat parotid acinar cells. The cell suspension was incubated for 60 min in 10 μM Na^+ indicator SBFI/AM at 37°. The cell suspension was washed and resuspended in fresh KRH, and then the fluorescence of the cells was measured as described in Materials and Methods.

Monensin increased amylase release in a concentration-dependent manner in the range of 0.1 to 100 μM in the presence of extracellular Ca^{2+} (Fig. 2). However, the amount of net amylase release induced by the highest dose of monensin (100 μM) was only about 3% of the total cell content. In the absence of extracellular Ca^{2+}, monensin still increased amylase release in a concentration-dependent manner in the range of 1 to 100 μM although the amount of release induced by monensin in the absence of extracellular Ca^{2+} was smaller than that induced in the presence of extracellular Ca^{2+}.

The addition of monensin gradually increased $[Ca^{2+}]_i$ followed by a plateau of $[Ca^{2+}]_i$ within 5 min in the presence and absence of extracellular Ca^{2+} (Fig. 3). Figure 4 shows that monensin increased $[Ca^{2+}]_i$ in a concentration-dependent manner in the range of 0.01 to 100 μM in the presence of extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, monensin still concentration-dependently increased $[Ca^{2+}]_i$ although the level of $[Ca^{2+}]_i$ was lower than that in the presence of extracellular Ca^{2+}, suggesting that monensin releases Ca^{2+} from intracellular Ca^{2+} pools. Therefore, by using permeabilized acinar cells, we examined whether monensin releases Ca^{2+} from a non-mitochondrial intracellular Ca^{2+} pool. Figure 5 shows that inositol 1,4,5-trisphosphate (IP_3) caused a transient release of Ca^{2+} from non-mitochondrial pools in parotid acinar cells as described previously [18]. On the

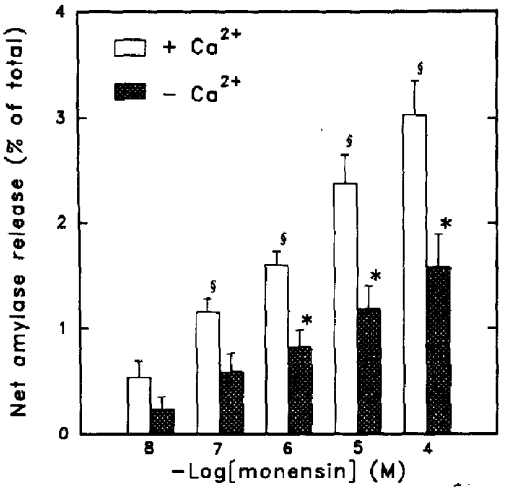

Fig. 2. Concentration–response curves of amylase release for monensin in the presence and absence of extracellular Ca^{2+}. Amylase release was measured during a 30-min stimulation with monensin and is expressed as a percentage of total amylase activity [811.5 ± 9.0 U/mg protein (N = 8)] of cells before stimulation. Net amylase release was obtained by subtracting amylase release in the absence of monensin from amylase release in the presence of monensin. Each value is the mean ± SEM (N = 3–5). Key: *P < 0.05 and §P < 0.05 compared with amylase release in the absence of monensin in Ca^{2+}-free KRH and amylase release in the presence of monensin in Ca^{2+}-free KRH, respectively.

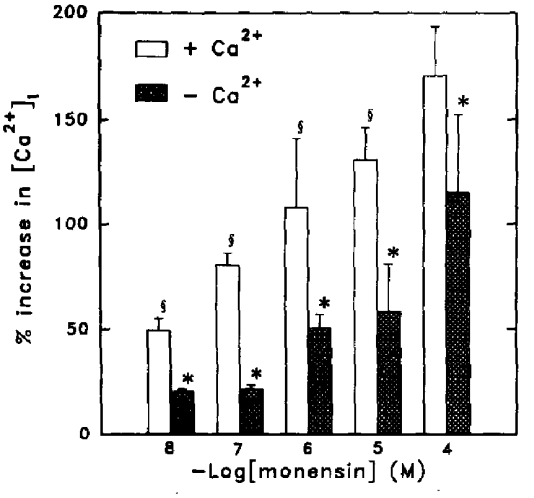

Fig. 4. Concentration–response curves of $[Ca^{2+}]_i$ for monensin in the presence and absence of extracellular Ca^{2+}. $[Ca^{2+}]_i$ was measured in fura-2-loaded cells as described in Materials and Methods. Monensin-induced $[Ca^{2+}]_i$ is expressed as the percent increase from basal $[Ca^{2+}]_i$ in the absence of monensin. The values of basal $[Ca^{2+}]_i$ in the presence and the absence of extracellular Ca^{2+} were 58.1 ± 1.9 (N = 16) and 46.9 ± 2.3 (N = 13) nM, respectively. Each value is the mean ± SEM (N = 3–4). Key: *P < 0.05 and §P < 0.05 compared with basal level and the monensin-treated level in the absence of extracellular Ca^{2+}, respectively.

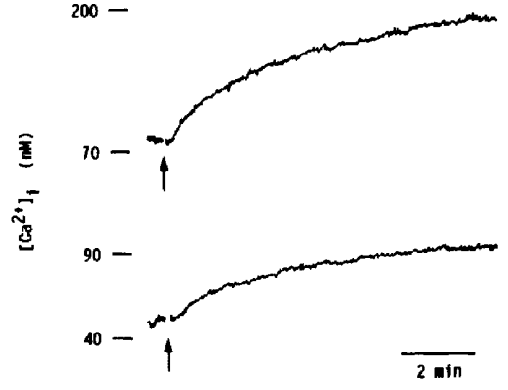

Fig. 3. Time course of $[Ca^{2+}]_i$ in the presence (upper trace) and absence (lower trace) of extracellular Ca^{2+} induced by 10 μM monensin added at the arrows. $[Ca^{2+}]_i$ was measured in fura-2-loaded cells as described in Materials and Methods.

other hand, 1 μM monensin did not release Ca^{2+}. In addition, lower concentrations (0.01 and 0.1 μM) of monensin produced no change in $[Ca^{2+}]$ (data not shown). In contrast, higher concentrations (10 and 100 μM) of monensin released Ca^{2+} from non-mitochondrial pools dependent on the concentrations of monensin added. However, the addition of 30 mM Na^+ into extracellular medium did not change $[Ca^{2+}]$.

Takuma and Ichida [11] have reported that monensin inhibits isoproterenol-induced amylase release even through monensin alone slightly increases this enzyme from rat parotid cells. We examined the effect of monensin on cyclic AMP accumulation in the presence and absence of extracellular Na^+ (Fig. 6). The resting accumulations of cyclic AMP in the presence and absence of extracellular Na^+ were 3.16 ± 0.74 (N = 6) and 2.69 ± 0.21 (N = 5) pmol/mg protein, respectively. Monensin by itself, at any concentration used, did not change cyclic AMP accumulation in the presence or absence of extracellular Na^+ (data not shown). Low monensin concentrations (0.01 to 1 μM) did not affect cyclic AMP accumulation elevated by 1 μM isoproterenol, whereas high concentrations (10 and 100 μM) of monensin significantly reduced isoproterenol-induced cyclic AMP accumulation. Although cyclic AMP accumulation elevated by isoproterenol was not modified in the absence of extracellular Na^+, the inhibitory effects of high concentrations of monensin on cyclic AMP accumulation induced by isoproterenol were more potent in the absence of extracellular Na^+ than in its presence.

DISCUSSION

Monensin, a carboxylic Na^+/H^+ ionophore, has been used widely as a Na^+ ionophore in a variety of cell types. We have demonstrated here that high concentrations (10 and 100 μM) of monensin, which

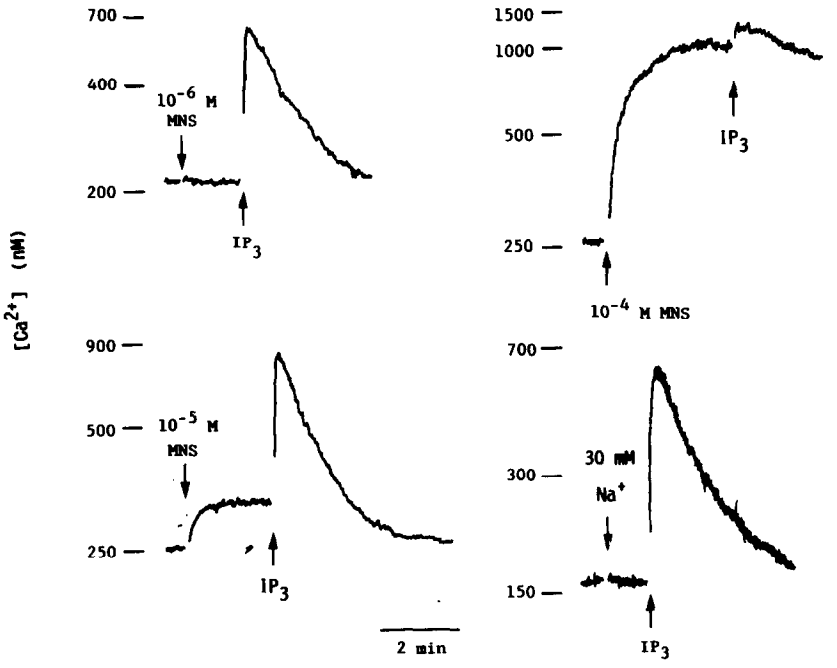

Fig. 5. Ca^{2+} release induced by monensin (MNS) and 1 μM inositol 1,4,5-trisphosphate (IP_3) from a non-mitochondrial Ca^{2+} pool. Parotid acinar cells were permeabilized with 60 μg/mL saponin at 37° in the presence of 1 μM fura-2, ATP, ATP-regenerating system and mitochondrial inhibitors.

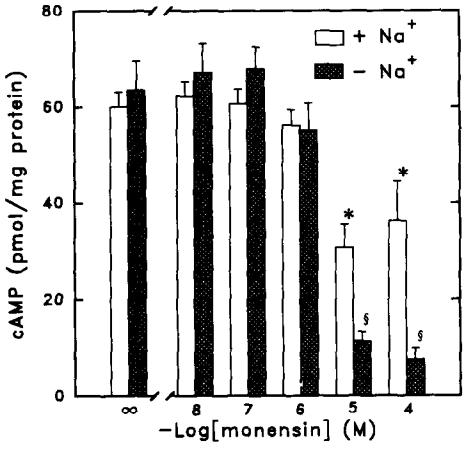

Fig. 6. Effect of monensin on cyclic AMP accumulation induced by 1 μM isoproterenol in the presence and absence of extracellular Na^+. Cyclic AMP was measured 3 min after stimulation with drugs. Each value is expressed as mean ± SEM (N = 5–7). Key: *P < 0.05 and §P < 0.05 compared with isoproterenol alone and isoproterenol-induced cyclic accumulation in the presence of extracellular Na^+, respectively.

are practically used, had non-specific actions and low concentrations of monensin were enough to increase $[Na^+]_i$ in rat parotid acinar cells.

Although the level of $[Ca^{2+}]_i$ elevated by monensin in the absence of extracellular Ca^{2+} was smaller than that in the presence of extracellular Ca^{2+}, the present findings that monensin concentration-dependently increased $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+} suggest that monensin mobilizes Ca^{2+} from extracellular medium as well as from intracellular stores. Ca^{2+} mobilization evoked by low concentrations (0.01 to 1 μM) of monensin seems to result from a rise of $[Na^+]_i$ induced by monensin, because low concentrations of monensin increased $[Ca^{2+}]_i$, in the absence of extracellular Ca^{2+} and did not release Ca^{2+} from non-mitochondrial Ca^{2+} pools in permeabilized cells nor did the addition of 30 mM NaCl. Thus, an elevation of $[Na^+]_i$ in parotid cells could release Ca^{2+} from mitochondria caused by inhibition of Na^+–Ca^{2+} exchange in mitochondrial membrane [21] as well as increase Ca^{2+} entry from extracellular medium into cells due to inhibition of Na^+–Ca^{2+} exchange in plasma membrane [22]. Since a limited amount of amylase release from parotid acinar cells is stimulated by only Ca^{2+} [1], monensin is able to cause a slight increase in amylase release from parotid acinar cells due to mobilization of Ca^{2+} from both extracellular medium and intracellular Ca^{2+} pools. Furthermore, it is suggested that not only the Ca^{2+} released from IP_3-sensitive intracellular pools but also the Ca^{2+} released from mitochondria induced by an elevation of $[Na^+]_i$ have an important role in exocytosis.

Martinez *et al.* [7] have suggested that 10 μM monensin may release Ca^{2+} from an IP_3-sensitive intracellular Ca^{2+} pool in intact rat submandibular cells. Monensin at 10 and 100 μM released Ca^{2+} from a non-mitochondrial intracellular Ca^{2+} pool

(Fig. 3), most likely from an IP_3-sensitive pool, suggesting that high concentrations of monensin directly release Ca^{2+} from the non-mitochondrial pool in intact cells. We have indicated that Ca^{2+} released by IP_3 is resequestered into the intracellular Ca^{2+} pool in permeabilized rat parotid acinar cells [23]. The mechanism of this non-specific action of high concentrations of monensin remains unknown but may be as follows: (1) sustained stimulation of IP_3 receptors or activation of IP_3-gated channels in microsomal membrane and inhibition of resequestration of Ca^{2+} into the intracellular Ca^{2+} pool; or (2) inhibition of the Ca^{2+}-ATPase pump in microsomes by compounds such as the microsomal Ca^{2+}-ATPase inhibitor thapsigargin [24].

In contrast to the present results, Watson *et al.* [25] have reported that Na^+ potentiates isoproterenol- or forskolin-induced elevation of cyclic AMP activity in a mouse parotid plasma membrane preparation. However, they examined the effects of extracellular Na^+ but not $[Na^+]_i$ on cyclic AMP accumulation and amylase release induced by isoproterenol in intact parotid cells [25, 26]. The present results show that isoproterenol-induced cyclic AMP accumulation was not affected at low concentrations of monensin but was inhibited at high concentrations of monensin in both the presence and the absence of extracellular Na^+. This suggests that an elevation of $[Na^+]_i$ does not modify cyclic AMP accumulation induced by activation of β-adrenergic receptors; however, the inhibitory effect of high concentrations of monensin on isoproterenol-induced cyclic AMP accumulation is due to a non-specific effect rather than that of a Na^+ ionophore. High concentrations of monensin did not affect the basal accumulation of cyclic AMP, indicating that monensin may not modify the activity of phospodiesterase. Monensin is known to inhibit surface binding of agonist to its receptors due to internalization and to arrested transport of secretory proteins in the Golgi apparatus in a variety of cell types although long treatment with monensin is needed to produce such inhibitory effects [27–34]. Therefore, it is suggested that the mechanism of the inhibitory effect of monensin on cyclic AMP accumulation may be inhibition of the binding of isoproterenol to β-adrenergic receptors. It has been reported that monensin inhibits isoproterenol-induced amylase release in a concentration-dependent manner [11]. Although this inhibition may be related to the inhibitory effect of high concentrations of monensin on cyclic AMP accumulation, whether an increase in $[Na^+]_i$ results in an inhibition of amylase release induced by isoproterenol remains unknown. Further study is needed to clarify the exact role of $[Na^+]_i$ on amylase release.

In conclusion, caution is needed when using high concentrations of monensin, even those which are practically used, as a Na^+ ionophore, and the use of low concentrations of monensin is recommended for examining the effect of $[Na^+]_i$ on cellular function.

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