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Alamethicin channel permeation by Ca^{2+}, Mn^{2+} and Ni^{2+} in bovine chromaffin cells

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Alamethicin causes a concentration-dependent increase of $[Ca^{2+}]_i$ in suspensions of bovine adrenal chromaffin cells loaded with fura-2. The basal levels of Ca_i^{2+} (234 ± 37 nM; $n = 4$) increased to a maximum of 2347 ± 791 nM ($n = 3$) with 100 μg/ml alamethicin. In the presence of 1 mM Ca_e^{2+} the increase reached a plateau within about 2–5 s. This increase was due to Ca^{2+} entry into chromaffin cells, since in the absence of Ca_e^{2+} alamethicin did not modify $[Ca^{2+}]_i$. This contrasts with ionomycin (1 μM) which produced a Ca_i^{2+} transient even in the absence of Ca_e^{2+}. Mn^{2+} ions also entered chromaffin cells in the presence of alamethicin, as measured by the quenching of fura-2 fluorescence following excitation at 360 nm. Resting chromaffin cells had a measurable permeability to Mn^{2+} which was drastically increased by cell depolarization by K^+ (50 mM) addition. This suggests that Mn^{2+} is able to permeate voltage-dependent Ca^{2+} channels. Ni^{2+} uptake into either resting or K^+-stimulated chromaffin cells was undetectable, but addition of alamethicin induced rapid uptake of this cation. The alamethicin-induced entry of Ni^{2+} was decreased by 50 mM K^+. Overall, the results are compatible with the formation by alamethicin of ion channels in chromaffin cell plasma membranes.

Alamethicin; Calcium signal; Manganese; Nickel; Fura-2; Chromaffin cell

1. INTRODUCTION

Alamethicin is a 20-amino acid peptide that forms voltage-dependent channels across membranes [1,2] and has been used as a model for voltage-gated channels. Recently, we demonstrated that this ionophore causes the release of catecholamines from perfused cat adrenal glands in a temperature-, concentration- and Ca_e^{2+}-dependent manner [3]. The time-course of secretion evoked by alamethicin (quick activation followed by a decline) considerably differs from the adrenal catecholamine release pattern seen with carrier-type ionophores such as A23187 [4] and ionomycin [5]. Rather, the profile of secretion evoked by alamethicin resembles that of nicotinic or high-K^+ stimulation of cat adrenal medullary chromaffin cells [6]. In the light of these results, we suggested that alamethicin might form Ca^{2+} permeable artificial channels in chromaffin cell plasma membranes [3]. This possibility has been explored now in bovine adrenal chromaffin cell suspensions loaded with the Ca^{2+} fluorescent indicator fura-2. To study the cell permeabilities to Ca^{2+}, Mn^{2+} and Ni^{2+} we have taken advantage of the spectral characteristics of fura-2 to estimate the entry of Mn^{2+} and Ni^{2+} without interference with Ca^{2+} signals when Ca_e^{2+} was present [7,8].

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2. MATERIALS AND METHODS

Bovine adrenal chromaffin cells were isolated as previously described [9]. A cell layer from a percoll gradient enriched in adrenaline cells was taken to perform these experiments. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM; 10^6 cells/2 ml), supplemented with 10% heat-inactivated fetal calf serum containing 50 iu/ml penicillin and 50 μg/ml streptomycin. Cells were kept in suspension under smooth continuous agitation, in bottles with an MCS-140 microcarrier stirrer, in an incubator (Hearaeus) under an atmosphere of 5% CO_2 in air at 37°C, for 2–3 days before the experiments.

Cells were loaded with fura-2/AM (4 μM) for 45 min at room temperature with continuous shaking in a standard medium (pH 7.4) containing (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; Sodium-HEPES, 10; glucose, 10. The loading incubation was terminated by dilution with 3 volumes of fresh standard medium followed by centrifugation at 80 × *g* for 10 min. The cells were then suspended in standard medium at 1.5×10^6 cells/ml. Fluorescence measurements were performed at 37°C with magnetic stirring in a fluorescence spectrophotometer which allowed rapid alternation (30–300 Hz) of up to 6 different excitation wavelengths (Cairn Research, Kent, UK). Emitted fluorescence was measured at 530 nm. Fluorescent signals were integrated at 1 second periods. $[Ca^{2+}]_i$ was estimated from the ratio of the fluorescence values excited at 340 nm and 380 nm [10]. Uptake of Mn^{2+} and Ni^{2+} was estimated from the quenching of fluorescence excited at 360 nm [7,8]. Alamethicin was obtained from Sigma, dissolved in ethanol and diluted in saline solution.

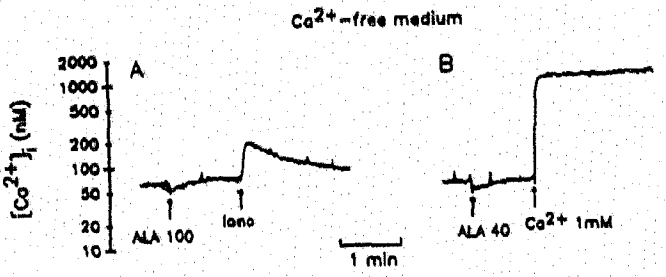

Fig. 1. Effects of alamethicin and ionomycin on intracellular levels of cytosolic Ca^{2+}. Cells (2.5×10^6/assay) loaded with Fura-2 were suspended in a Ca^{2+}-free medium containing 1 mM EGTA. (A) After the fluorescence signal reflecting stabilized basal levels of Ca_i^{2+}, alamethicin (ALA, 100 μg/ml) was added. One min later, ionomycin (1 μM) was added to the same aliquot of cells. (B) A separate aliquot of cells suspended in Ca^{2+}-free solution (1 mM EGTA) was exposed to alamethicin (ALA, 40 μg/ml). 1 min later, Ca^{2+} (1 mM) was added to the bathing solution. The recordings are copies of the originals obtained in a typical experiment.

3. RESULTS AND DISCUSSION

3.1. *Effects of alamethicin on intracellular calcium levels in the absence and in the presence of external calcium*

The basal levels of cytosolic Ca^{2+}, in the presence of 2.5 mM Ca_e^{2+}, were around 234 ± 37 nM ($n = 4$). In the absence of extracellular Ca^{2+} (with 1 mM EGTA present), alamethicin (100 μg/ml) did not affect such levels. However, addition of ionomycin (1 μM) still in the presence of alamethicin, increased $[Ca^{2+}]_i$ from 60 to 200 nM (Fig. 1A). The Ca^{2+} signal decayed slowly with time.

In a separate aliquot of cells, bathed in Ca^{2+}-free medium, alamethicin (40 μg/ml) did not alter basal $[Ca^{2+}]_i$; however, addition of 1 mM Ca^{2+} to the cell suspension produced a sharp, quick rise of $[Ca^{2+}]_i$ to about 1000 nM (Fig. 1B). The new $[Ca^{2+}]_i$ plateau was stable and did not suffer inactivation for at least a 5 min period. This was a signal specifically associated to alamethicin, since in the absence of the ionophore, Ca_e^{2+} addition did not increase the basal levels of $[Ca^{2+}]_i$.

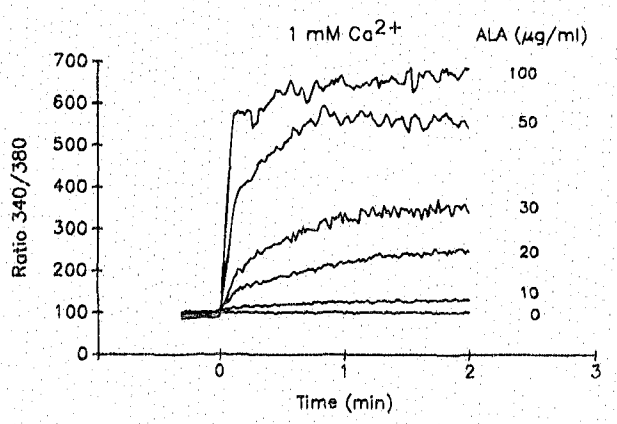

Fig. 2. Effects of alamethicin on Ca_i^{2+} levels. Each ionophore concentration was tested in separate aliquots of cells. Cells were first incubated in Ca^{2+}-free solution (30 s), then exposed to 1 mM Ca_e^{2+} (40 s) and finally treated with each concentration of alamethicin during the 2 min recording period shown in the abscissa. $[Ca^{2+}]_i$ is expressed as the fluorescence ratio between excitation wavelengths of 340 and 380 nm.

Fig. 2 shows the effects of increasing concentrations of alamethicin on $[Ca^{2+}]_i$ levels. Cells were first incubated in Ca^{2+}-free medium for 30 s. Then 1 mM Ca^{2+} was added and, after 40 s, alamethicin (10–100 μg/ml) was added (each individual concentration was tested in separate aliquots of cells). The Ca^{2+} signal was recorded for an additional 2 min period. The threshold concentration producing a clear, measurable response was 20 μg/ml; from then onwards, a gradual concentration-dependent increase in $[Ca^{2+}]_i$ was observed. Note that the higher the ionophore concentration, the faster the rise of the $[Ca^{2+}]_i$ signal. Also note that the signal reached a plateau (especially at 50 and 100 μg/ml alamethicin) and stabilized for the 120 s period of recording.

Ionomycin is known to mobilize Ca^{2+} from intracellular stores, and more specifically from smooth endoplasmic reticulum, therefore, the Ca^{2+} signal seen with this ionophore in the absence of Ca_e^{2+} was likely to be due to Ca^{2+} mobilization from some internal store. The fact that alamethicin was unable to modify the $[Ca^{2+}]_i$ in the absence of external Ca^{2+}, and that Ca_e^{2+} addition suddenly increased the levels of Ca_i^{2+}, suggests that the chromaffin cell plasma membrane, and not the endomembrane, is the target for alamethicin action. In this respect, alamethicin behaves as nicotinic agonists or high-K^+—two stimuli which produce Ca_i^{2+} transients in bovine chromaffin cells loaded with fura-2 only in the presence of external Ca^{2+} [11]. This analogy, and the fast rise of $[Ca^{2+}]_i$ are compatible with an ion channel formed by alamethicin in the chromaffin cell plasma membrane.

3.2. *Permeation of alamethicin-treated cells by Mn^{2+} and Ni^{2+}*

In order to examine more directly, and on a more adequate time scale, the kinetics of cell ion permeabilities activated by alamethicin, we tried the 'Mn^{2+} quenching' technique, which has been successfully used to demonstrate agonist-activated divalent cation entry into several cell types [7,8,12–16]. This technique detects Mn^{2+} entry into cells by monitoring the Mn^{2+}-induced quenching of intracellular fura-2 fluorescence excited at 360 nm (the isosbestic point for Ca^{2+}). Under these conditions, the fluorescence signal is insensitive to changes of $[Ca^{2+}]_i$.

In the absence of Ca_e^{2+}, addition of 0.5 mM Mn^{2+} produced a quenching of fluorescence which developped slowly (Fig. 3A). This quenching presumably reflects gradual entry of Mn^{2+} into cells, suggesting that

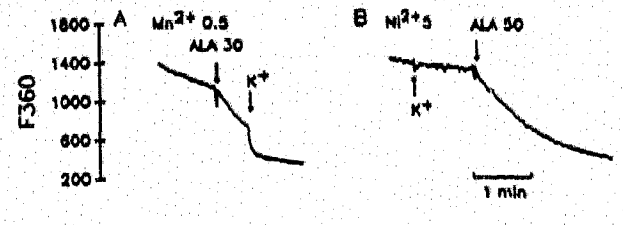

Fig. 3. Quenching of the fluorescence signal by Mn^{2+} and Ni^{2+} in Fura-2 loaded chromaffin cells. (A) Mn^{2+} (0.5 mM) was added and then, sequentially, at the points indicated by arrows, alamethicin (30 μg/ml) and K^+ (60 mM). (B) In a different cell aliquot, Ni^{2+} (5 mM), K^+ (50 mM) and alamethicin (50 μg/ml) were added as indicated. The cell bathing solution had no Ca^{2+} (1 mM EGTA). Arbitrary units of fluorescence at 360 nm are expressed in the ordinate.

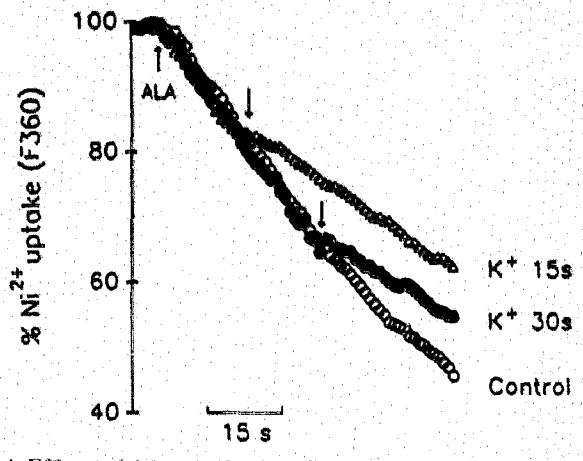

Fig. 4. Effects of delayed addition of K^+ on the rate of dissipation by Ni^{2+} of fluorescence of fura-2 loaded chromaffin cells treated with alamethicin. Cells were excited at 360 nm and their fluorescence estimated (arbitrary units in the ordinate). Alamethicin (100 μg/ml) was added at time 0 and the rate of fluorescence decay followed for the next 60 s (abscissa). In 2 different aliquots of cells, K^+ (50 mM) was added 15 and 30 s after alamethicin (arrows).

unstimulated chromaffin cells are quite permeable to Mn^{2+}. The rate of decline of fluorescence was further accelerated by alamethicin (30 μg/ml), suggesting that Mn^{2+} was also entering the cells through alamethicin pores. Finally, K^+ addition (50 mM) caused an additional drop in fluorescence suggesting that voltage-sensitive Ca^{2+} channels are highly permeable to Mn^{2+}.

In the light of these results, it seemed that Mn^{2+} would not be an adequate Ca^{2+} surrogate to study the kinetics of alamethicin channels in chromaffin cells. Therefore, Ni^{2+} was tried as an alternative divalent cation. Voltage-gated Ca^{2+} channels are not permeable to Ni^{2+} which, in addition, is a potent blocker of such channels as well as of K^+-evoked catecholamine release [17]. Ni^{2+} did not affect the basal fura-2 fluorescence in unstimulated cells. Neither did K^+ addition alter this fluorescence (Fig. 3B) confirming that Ni^{2+} was not permeating voltage-dependent Ca^{2+} channels. Alamethicin (50 μg/ml) caused a gradual fluorescence quenching, suggesting that Ni^{2+} was permeating chromaffin cells only in the presence of the ionophore. This double component (blockade of Ca^{2+} channels and permeation of alamethicin channels) makes Ni^{2+} an ideal cation to study the kinetics of the latter channels, as demonstrated by the following experiments.

Ni^{2+} permeation of alamethicin channels was a slow process, as deduced from the experiment shown in Fig. 4. Upon addition of alamethicin (100 μg/ml) to cells incubated with 3 mM Ni^{2+}, the dissipation of the fluorescence declined gradually with an approximate $t_{1/2}$ of 40 s. This contrasts with the quick rise of Ca_i^{2+} levels evoked by this same concentration of alamethicin (Fig. 2), which exhibited a $t_{1/2}$ of about 1 s. The Ni^{2+} uptake process did not apparently exhibit inactivation, since fluorescence decayed almost linearly.

Alamethicin-evoked Ni^{2+} entry was insensitive to the 1,4-dihydropyridine derivative nisoldipine (1 μM), an L-type Ca^{2+} channel blocker [8]. Thus, dissipation of the fluorescence signal at 360 nm followed the same rate either in the presence or the absence of nisoldipine (not shown). In a different experiment, 1 μM nitrendipine (another dihydropyridine) did not affect Ni^{2+} entry into alamethicin-treated cells (not shown).

Thus, Ca^{2+}, Mn^{2+} and Ni^{2+} permeated alamethicin channels. The Ca^{2+} permeability was very fast, followed by the intermediate Mn^{2+} permeability and the slowest permeability to Ni^{2+}. These permeabilities differ only slightly from those for Ca^{2+} channels. For instance, in high-K^+ solutions, which recruit voltage-dependent Ca^{2+} channels in chromaffin cells [19], fura-2 loaded cells were highly permeable to Ca^{2+}, less so to Mn^{2+} and not at all permeable to Ni^{2+}. We took advantage of the differential permeabilities of alamethicin channels and Ca^{2+} channels to Ni^{2+} in order to study, in isolation, the effects of the membrane potential on the kinetics of ionophore channels.

3.3. *Modulation (by voltage) of the kinetics of alamethicin channels*

Fig. 4 shows the fluorescence decay evoked by alamethicin (100 μg/ml) in the presence of Ni^{2+} (3 mM). When K^+ (50 mM) was added 15 or 30 s after alamethicin, the rate of decay was almost immediately slowed down. This indicates that cell depolarization may be closing the alamethicin channels thereby decreasing Ni^{2+} uptake through them. These results are consistent with previous reports in lipid bilayers, where depolarization decreased the conductance of alamethicin channels [2]. This behaviour is consistent with the model inferred from the crystal structure of this ionophore where gating of the channels is facilitated by burial of the channel in the membrane driven by a transmembrane potential difference [20].

3.4. *Functional correlates*

The concentration-dependence of $[Ca^{2+}]_i$ increase

(this paper) and of adrenal catecholamine release [3] evoked by alamethicin are closely correlated. Thus at 10 μg/ml, where $[Ca^{2+}]_i$ was only slightly modified, alamethicin caused only a mild increase in secretion. But 20–100 μg/ml caused parallel increases in both parameters, indicating that Ca^{2+} entry through alamethicin channels exhibited a functional role in activating the secretory machinery underneath the plasma membrane.

The time-courses of the increase in $[Ca^{2+}]_i$ and secretion cannot be compared appropriately. In our previous experiments [3] secretory rates were measured at 2 min intervals but here, Ca_i transients caused by alamethicin are very rapid, specially at 50–100 μg/ml (Fig. 2). Nevertheless, we can infer that the secretory effects also develop quickly since peak secretion is always reached during the first 2 min collection period. The rapidity of the effects of alamethicin contrasts with those of ionomycin and A23187 which, although causing a rapid rise of $[Ca^{2+}]_i$ in bovine chromaffin cells [21,22], nevertheless initiate a slowly-developing secretory response [4,5,23].

In conclusion, alamethicin seems to form ionic channels in chromaffin cells which are permeable to Ca^{2+}, Mn^{2+} and Ni^{2+}. As previously shown in artificial membranes [2], these channels exhibit voltage-dependence: they undergo inactivation in depolarized cells and reactivate under polarizing conditions. Because alamethicin mimicks the functional and kinetic aspects of the natural voltage-dependent Ca^{2+} channels, it may become a useful tool to investigate the access and role of Ca^{2+} in activating secretory processes. Conversely, chromaffin cells are a good model alongside the widely used artificial membranes or membrane vesicles, to study the kinetics of ion permeation of alamethicin channels. They offer the advantage of a functional correlation between these ion permeabilities and a fast cellular event such as the exocytotic release of catecholamines.

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