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H89, an inhibitor of protein kinase A (PKA), stimulates Na^+ transport by translocating an epithelial Na^+ channel (ENaC) in fetal rat alveolar type II epithelium

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Abstract

The present study was performed to clarify the effect of H89, an inhibitor of cAMP-activated protein kinase (protein kinase A; PKA), on Na^+ absorption in fetal rat alveolar type II epithelium. H89 stimulated the Na^+ absorption by increasing the open probability (Po) and number of a nonselective cation (NSC) channel composed of four α subunits of epithelial Na^+ channel (ENaC). Brefeldin A (BFA), an inhibitor of intracellular protein translocation, blocked the stimulatory action of H89 on the Na^+ absorption by interrupting the action of H89 on the Po and number of the NSC channel. H85, an inactive form of H89, showed an effect similar to H89, suggesting that H89 does not show its effect by inhibiting PKA, but acts on the channel depending the structure. These observations indicate that: (1) the H89 induced increase in number of the channel at the apical membrane is due to translocation of α subunit of ENaC to the apical membrane, (2) the elevation of Po of the channel is mediated through translocation of a protein activating α subunit of ENaC, and (3) the effect of H89 is dependent on its structure without any relation to PKA.
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Keywords: ENaC; Single channel current; PKA; Terbutaline; cAMP; Amiloride

1. Introduction

The fetal lung fluid stimulates development, differentiation and growth of the fetal lung by generating positive pressure from the luminal space to the interstitium, and this lung fluid is secreted into the lung's lumen throughout gestation by the fetal lung epithelium [1]. Although this fluid plays an important role in the lung development, it must be cleared from the alveolar space immediately after birth to allow normal gas exchange. Stimulation of the alveolar epithelium by a β-agonist enhances clearance of the lung fluid at birth [2] by activating the benzamil (or amiloride)-sensitive Na^+ absorption [3–5]. Further, the benzamil (or amiloride)-sensitive Na^+ absorption keeps the amount of lung fluid constant for maintenance of normal lung function [6–8].

A β-agonist acts on the epithelium by increasing the cytosolic cAMP concentration, suggesting that the stimulatory action of β-agonist is mediated through a cAMP-dependent pathway such as cAMP-dependent PKA. Namely, a β-agonist would possibly stimulate the Na^+ absorption by activating PKA. Therefore, to clarify the possibility we applied an inhibitor of PKA, H89, which was expected to block the stimulatory action of β-agonist on the Na^+ absorption. Against our expectation, H89 did not block the Na^+ absorption, but rather stimulated the transport. In the present study, we report the action of H89 on the Na^+ transport and an ENaC, a NSC channel, which

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Abbreviations: β-agonist, β adrenoceptor agonist; BFA, brefeldin A; db-cAMP, dibutyryl cAMP; ENaC, epithelial Na^+ channel; I_{sc}, short circuit current; No, number; N.S., no significant difference; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate; NSC, nonselective cation; PKA, cAMP-activated protein kinase (protein kinase A); Po, open probability; PTK, protein tyrosine kinase.

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is responsible for the Na^+ absorption in the epithelium [9,10].

2. Materials and methods

2.1. Solutions and chemicals

The solution used in the present study contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). The pH of the solution was adjusted to 7.4 with NaOH. All chemicals were purchased from the Sigma Chemical co.

2.2. Cell culture

Alveolar type II epithelia were isolated from fetal Wistar rats of 20-day gestational age (term, 22 days) [11,12], and were subsequently cultured on permeable support dishes [11–13] for 2 or 3 days after seeding under a confluent condition for experiments. To get the statistical results, we measured each parameter shown in the present study using individual dishes obtained from individual rats; i.e. when "N = 9" is represented, 9 individual pregnant rats were used for the experiment.

2.3. Measurement of short circuit current (I_{sc})

The measurement of I_{sc} was performed using the method previously reported [14]. Since benzamil is a specific blocker of ENaC [15–17], we measured the benzamil (10 μM)-sensitive I_{sc} as an indicator of the transcellular macroscopic Na^+ absorption.

2.4. Single channel recordings and data analysis

Single channel recordings were performed using the method previously reported [10,18,19]. The patch pipette had a resistance of about 5 Ω, and was applied to the apical membrane of the epithelium. Single channel currents were recorded from cell-attached patches, digitized at a sampling rate of 5000 Hz, and analyzed with a 2000-Hz low-pass Gaussian filter [13].

2.5. Open probability (Po) and number (No) of a single channel

Channel activity is expressed as Po as shown below:

$$\mathrm{Po} = \left(\frac{1}{N}\right)\sum_{i=1}^{N}\frac{iT_i}{T_t}$$

where N is the maximum number of the channel being simultaneously open observed in a patch, i is the number of the channel being simultaneously open, T_i is the time of just i channels being simultaneously open and T_t is the total recording time. As previously reported [9], the channel studied in the present report was activated by cytosolic Ca^{2+}. Even though the Po of an unstimulated channel was small, we could estimate the number of the channel per patch by forming an inside-out patch with

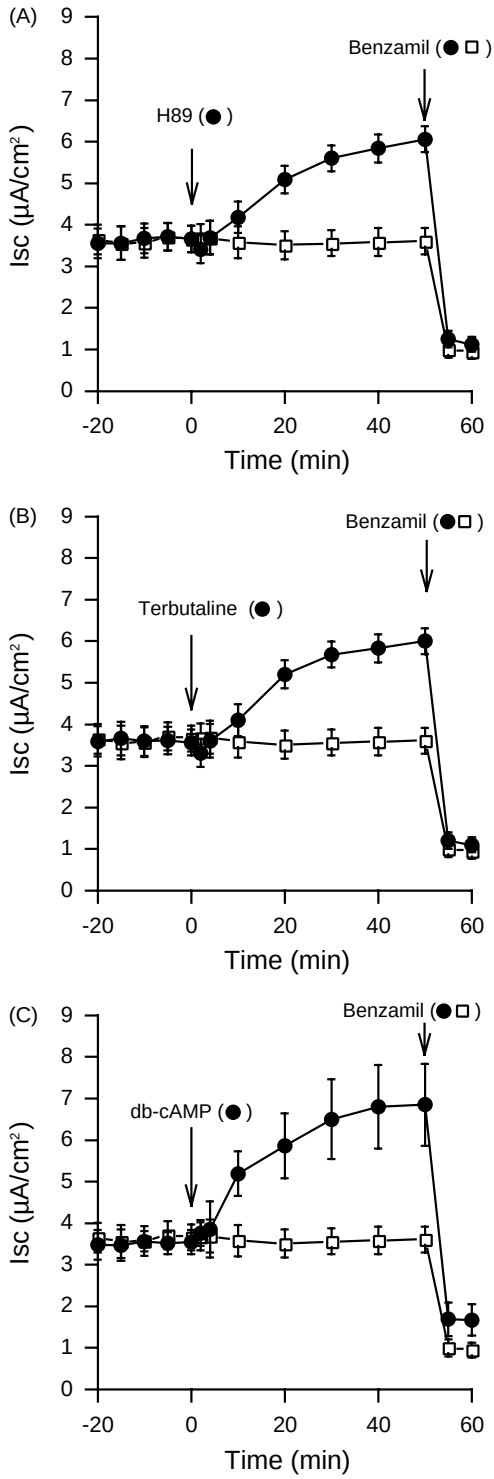

Fig. 1. Effects of H89 (A), terbutaline (B) and db-cAMP (C) on short-circuit current (I_{sc}). H89 (10 μM), terbutaline (10 μM) or db-cAMP (1 mM) was applied at 0 min. Benzamil of 10 μM was applied at 50 min in A, B and C. Values are means ± SD, N = 5.

the cytosolic surface of the channel exposed to a high Ca^{2+} concentration (10 mM) leading to high Po; and then a low Ca^{2+} concentration (1 nM) with a high Cl^- concentration (140 mM) to produce a very low Po [10]. Such treatments allowed us to observe both events with all channels open and all channels closed. Therefore, we could accurately determine the actual number (No) of active channels in a patch. Moreover, our estimate of the number of channels per patch membrane could be statistically substantiated at the 95% confidence level using methods we have previously described [20]. We made only one patch on a permeable support dish under each condition when we obtained the Po and No of the channel.

2.6. Application of H89, terbutaline (a β-agonist) and db-cAMP

We applied 10 μM H89, 10 μM terbutaline or 1 mM db-cAMP from the basolateral side. When we studied the effect of H89, terbutaline or db-cAMP on I_{sc}, and Po and No of the channel, we recorded the parameters 50 min after application of H89, terbutaline or db-cAMP. When we studied the dose dependency of H89, we applied H89 of 0.01–10 μM.

2.7. Application of brefeldin A (BFA)

We applied 1 μg/mL BFA 20 min before addition of H89, terbutaline or db-cAMP. BFA of 1 μg/mL was also present after addition of H89, terbutaline or db-cAMP.

2.8. Temperature

All experiments were performed at 37°.

2.9. Statistical analyses

Results are presented as mean ± SD. Statistical significance was tested with Student's *t*-test or ANOVA as appropriate. A P value of <0.05 was considered significant. If we did not observe any significant difference, it is represented as no significant difference (N.S.).

3. Results and discussion

Fig. 1 shows the time course of I_{sc} with application of H89 (10 μM, Fig. 1A), terbutaline (10 μM, Fig. 1B) or db-cAMP (1 mM, Fig. 1C). H89 increased the I_{sc}, and benzamil of 10 μM diminished the I_{sc} (closed circles in Fig. 1A).

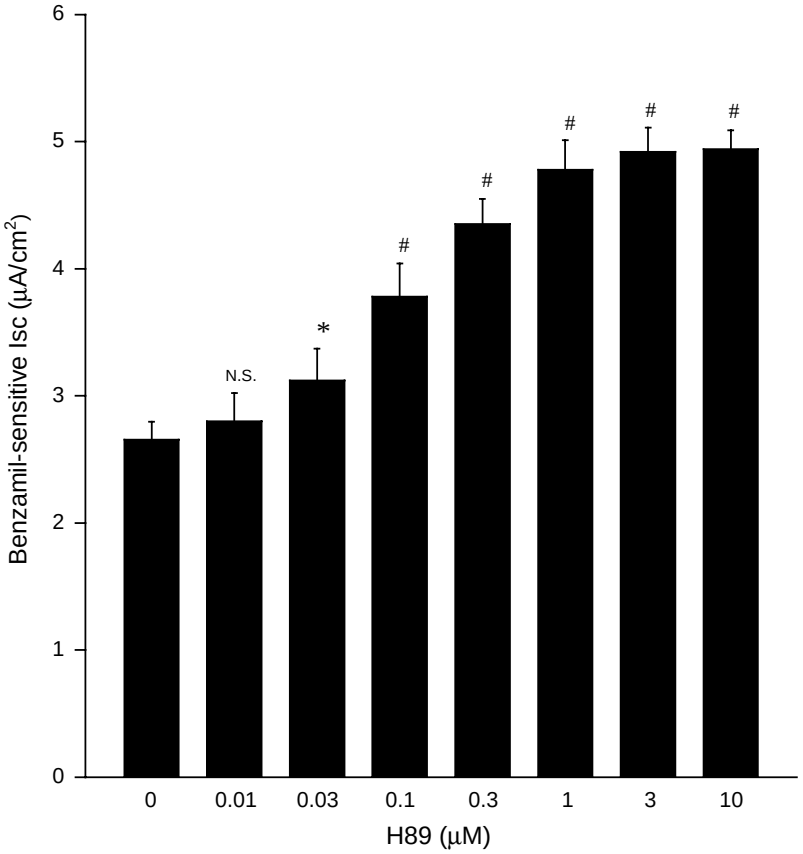

Fig. 2. Effects of H89 of various concentrations on the benzamil-sensitive I_{sc}. Values are means ± SD, N = 5. (∗) $P < 0.01$; (#) $P < 0.0001$. These are compared with those in the absence of H89, respectively.

The amount of the benzamil-sensitive I_{sc} with H89 (closed circles in Fig. 1A) was larger than control (open squares in Fig. 1A). Terbutaline and db-cAMP (Fig. 1B and C) had the action similar to H89. H89 dose-dependently increased the benzamil-sensitive I_{sc} (Fig. 2).

As we previously reported [9], the cell has two types of benzamil-sensitive Na^+-permeable channels, which are responsible for Na^+ absorption as a Na^+ entry pathway across the apical membrane; (1) a NSC channel, and (2) a Na^+ channel. To clarify which channel contributes to the stimulatory action of H89, we studied the effect of H89 on the NSC and Na^+ channels. H89 increased the Po and No of the NSC channel (Fig. 3A). On the other hand, the Po or No of the Na^+ channel was not affected by application of H89 (Fig. 3B). Terbutaline and db-cAMP had the action identical to H89 (Fig. 3).

To study whether the stimulatory action of H89 on the benzamil-sensitive I_{sc} is mediated through a pathway dependent on translocation of proteins, we applied BFA, which is an inhibitor of intracellular protein trafficking [21]. As shown in Fig. 4A, BFA blocked the stimulatory action of H89 on I_{sc}. The statistical results on the benzamil-sensitive I_{sc} are shown in Fig. 4B. H89 increased the benzamil-sensitive I_{sc} 1.90 ± 0.08-fold in control (open columns in Fig. 4B) and 0.90 ± 0.12-fold in the presence of BFA (closed columns in Fig. 4B); i.e. BFA completely blocked the stimulatory action of H89 on the benzamil-sensitive I_{sc} ($P < 0.001$).

As described above, H89 elevated the benzamil-sensitive I_{sc} by increasing the Po and No of the NSC channel. Therefore, we further studied whether BFA treatment blocks the stimulatory action of H89 on the Po and No of the NSC channel. The action of H89 on the Po and No of the NSC channel (open columns in Fig. 5A and B) was abolished by BFA (closed columns in Fig. 5A and B). These observations suggest that: (1) H89 stimulates translocation of the NSC channel to the apical membrane, and (2) H89 activates the NSC channel (i.e. elevation of Po) by translocating a protein activating the NSC channel to the place at which the protein can access to the NSC channel, although further studies are required to clarify what kind of protein contributes to activation of the NSC channel. As mentioned above, BFA abolished the stimulatory action of H89 on Po of the channel (the open and closed columns at H89 (+) in Fig. 5A), but BFA did not affect the Po under the basal condition (open and closed columns at H89 (−) in

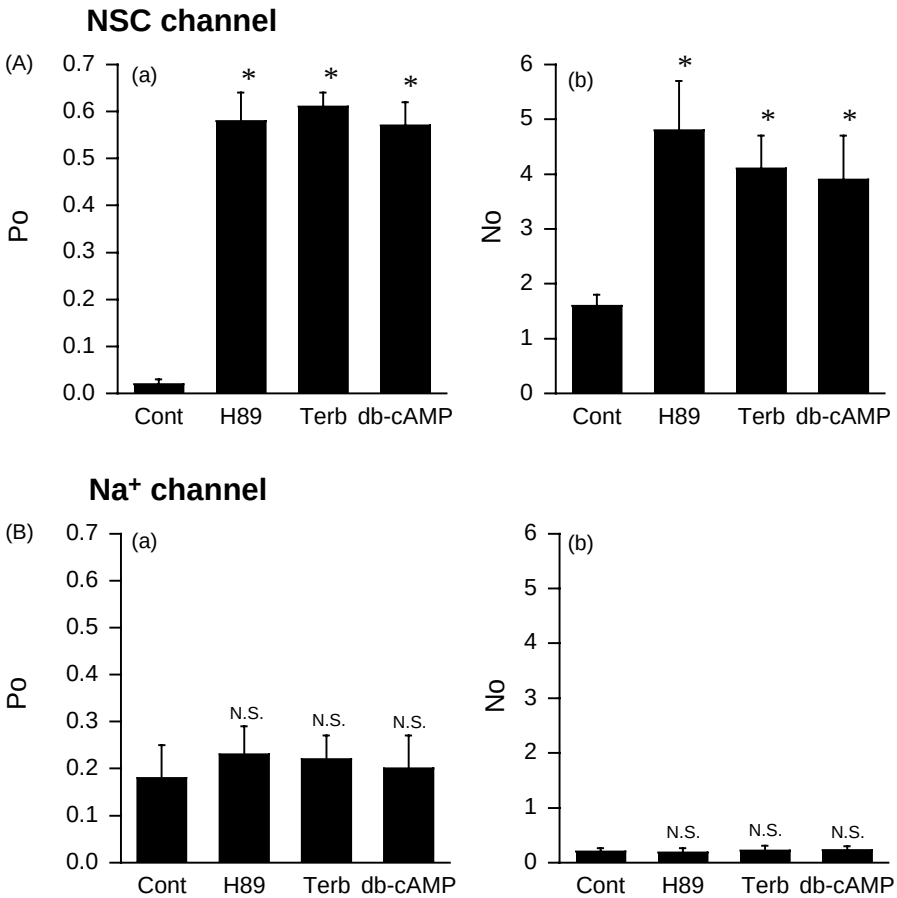

Fig. 3. Effects of H89, terbutaline (Terb) and db-cAMP on the open probability (Po) and number (No) per patch membrane of the NSC (A) and Na^+ channels (B). These values were recorded in cell-attached patches at no applied potential. Values are means ± SD, N = 9. This means that nine patches were obtained from nine individual permeable support dishes under each condition. (*) $P < 0.0001$. These are compared with control (Cont), respectively.

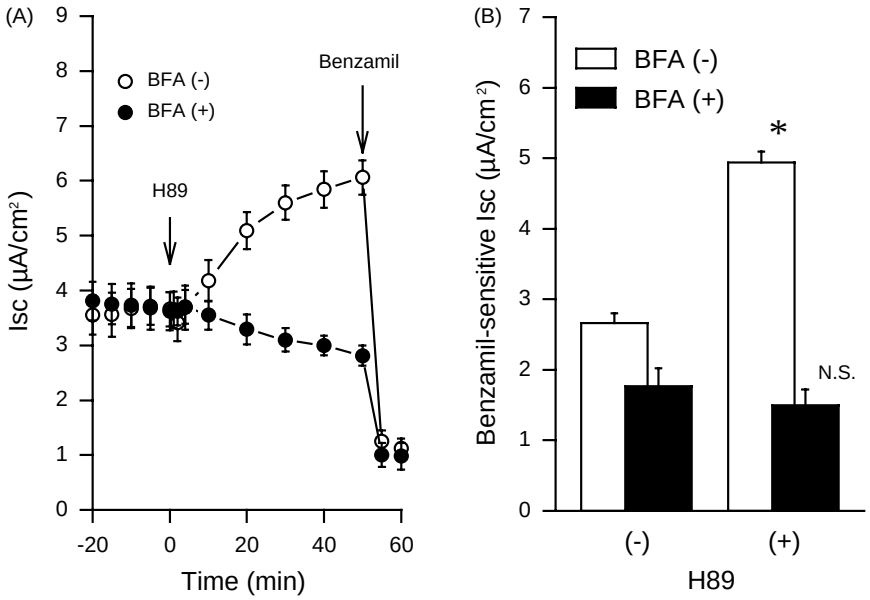

Fig. 4. Effects of BFA on the benzamil-sensitive I_{sc}. Values are means ± SD, N = 5. (∗) $P < 0.0001$. These are compared with those in the absence of H89 in each case of BFA presence or absence.

Fig. 5A). This means that the BFA action would be directly related to the H89 action. On the other hand, BFA diminished the No of the channel under the basal condition (open and closed columns at H89 (−) in Fig. 5B). However, the No of the channel in the presence of BFA was a half of the control (no BFA treatment), but was not zero. These observations indicate that the basal translocation of the channel is partially blocked by BFA, while the H89 action on No of the channel is completely blocked by BFA. Therefore, the inhibitory action of BFA on the channel is a specific one on the H89 effect, but not a nonspecific one on other protein trafficking processes (e.g. the basal ENaC trafficking).

The stimulatory action of H89 on the channel was much larger than that on the benzamil-sensitive I_{sc}. This seems to be discrepancy. However, our previous report [12] indicates that under the basal condition the rate limiting step of the transepithelial Na^+ transport in the epithelium is the Na^+ entry step through the channel. On the other hand, under the stimulated condition the rate limiting step is the Na^+ extrusion step by the Na^+–K^+–ATPase, but is not the Na^+ entry one through the channel [12]. The amount of I_{sc}

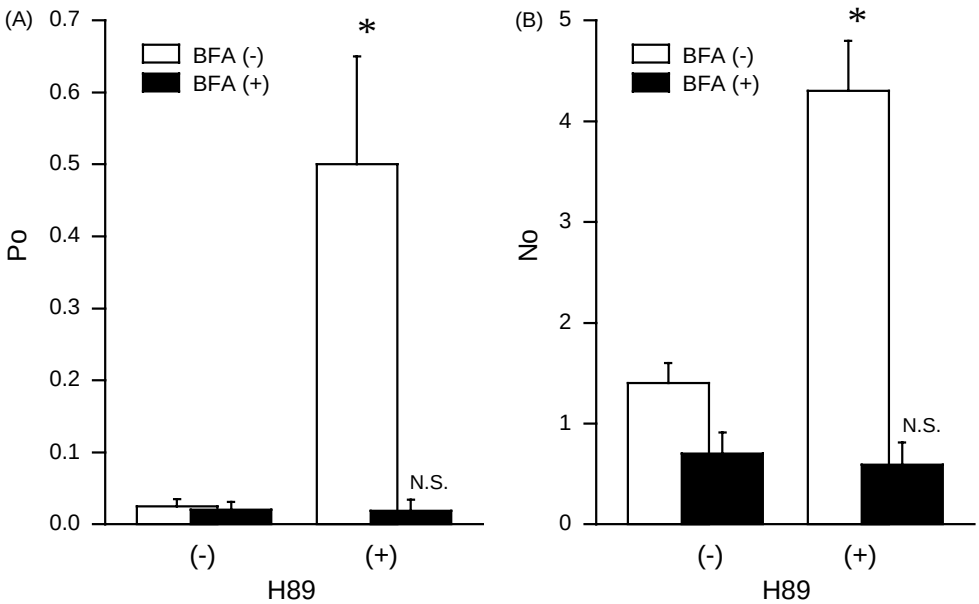

Fig. 5. Effects of BFA on Po and No of the NSC channel under the basal and H89-stimulated conditions. These values were recorded in cell-attached patches at no applied potential. Values are means ± SD, N = 9. This means that nine patches were obtained from nine individual permeable support dishes under each condition. (∗) $P < 0.0001$. These are compared with those in the absence of H89 in each case of BFA presence or absence.

depends upon the rate limiting step. Therefore, under the H89-stimulated condition, the amount of I_{sc} depends upon the capacity of the Na^+–K^+–ATPase activity, but not on the channel activity. This switch in the rate limiting step of the Na^+ transport by H89 causes the large difference in magnitude between the stimulatory action of H89 on the channel and the benzamil-sensitive I_{sc}.

Three types of subunits, α, β, γ, have been cloned [16,17] and are expressed in alveolar type II cells [22]. A highly Na^+-selective channel is composed of two α, one β, and one γ subunits [23], while four α subunits form an NSC channel [24]. Further, it is reported that antisense oligonucleotide against the α-subunit of ENaC diminishes the expression of an NSC channel in alveolar type II epithelia [25]. These reports indicate that the NSC channel reported in the present study is composed of four α subunits of ENaC, and that H89 stimulates translocation of α subunit of ENaC to the apical membrane of the alveolar type II epithelium, and that H89 also increases the Po of the NSC channel composed of α subunit of ENaC by translocating a protein activating α subunit of ENaC.

H89 is well known as an inhibitor of PKA. Therefore, H89 might possibly show its action reported in the present study by inhibiting PKA. Therefore, we applied an H89 analogue, H85. H85 has no inhibitory action on PKA, but the structure of H85 is almost identical to H89. H85 showed an effect similar to H89 (data not shown), suggesting that H89 does not show its effect by inhibiting PKA, but acts on the channel depending the structure. Further, to confirm this point, we studied the effect of another type of PKA inhibitor, KT5720, which has a completely different structure from H89. KT5720 diminished the PKA activity in the fetal alveolar type II epithelium [11], but did not show an H89-like effect on the Po or No of the channel (data not shown). These observations suggest that the stimulatory action of H89 on the I_{sc} and the channel would not be mediated through inhibition of PKA activity. Although more evidence is required to conclude that translocation of the channel is regulated by H8 compounds via their structures, we can suggest that an H8 compound-dependent mechanism contributes to the channel trafficking.

Acknowledgments

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