Arf-1 (ADP-ribosylation factor-1) is involved in the activation of a mammalian Na⁺-selective current

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Stimulation of mammalian cells often results in an increase in the intracellular Na⁺ concentration, brought about by Na⁺ influx into the cell via Na⁺-permeable ion channels. In some cell types, particularly renal epithelia and mast cells, non-hydrolysable analogues of GTP, such as GTP[S] (guanosine 5'-[γ-thio]triphosphate), activate a non-voltage-activated Na⁺-selective current. We have carried out whole-cell patch–clamp experiments to examine how GTP[S] activates the Na⁺ current in a rat mast cell line. The ability of GTP[S] to activate Na⁺ influx was prevented by including GTP in the pipette solution, indicating the involvement of small G-proteins. Brefeldin A and Arf-1-(2–17), inhibitors of Arf-1 (ADP-ribosylation factor-1) proteins, suppressed the activation of Na⁺ entry by GTP[S]. However, non-active succinylated Arf-1-(2–17) or an N-terminal myristoylated peptide directed towards Arf-5 were ineffective. Arf proteins modulate the cytoskeleton, and disruption of the cytoskeleton with cytochalasin D or its stabilization with phalloidin impaired the development of the Na⁺ current. Disaggregation of microtubules was without effect. Dialysis with cAMP or inhibition of cAMP phosphodiesterase with caffeine both decreased the extent of Na⁺ entry, and this was not prevented by pre-treatment with broad-spectrum protein kinase inhibitors. Collectively, our results suggest that the mechanism of activation of a mammalian non-voltage-activated Na⁺-selective current requires an Arf small G-protein, most probably Arf-1.

Key words: ADP-ribosylation factor (Arf), cAMP, cytoskeleton, guanosine 5'-[γ-thio]triphosphate (GTP[S]), non-voltage-activated Na⁺ current.

INTRODUCTION

Unlike the case with Ca²⁺, an increase in the intracellular Na⁺ concentration is not considered to be a ubiquitous signal transduction mechanism. Nevertheless, changes in the intracellular Na⁺ concentration have been observed [1], and such changes can affect K⁺ channels [2], the affinity of receptors for agonist (e.g. α₁-adrenergic receptors [3]), glutamate uptake and release in the central nervous system [4], and intracellular Ca²⁺ levels through effects on Na⁺/Ca²⁺ exchangers in both the plasma membrane and mitochondria [5,6]. Hence changes in the intracellular Na⁺ concentration do occur, and such changes modulate quite diverse processes. There is little evidence to support the notion that Na⁺ can be released into the cytosol from intracellular stores upon cell stimulation; instead, the main route for elevating intracellular Na⁺ levels is via its entry across the plasma membrane. This is generally accomplished through plasmalemmal Na⁺ channels. The latter either are non-selective cation channels, permeable to both univalent and bivalent cations, or are Na⁺-selective [7].

The best-characterized Na⁺-selective channels are the voltage-activated channels of excitable tissues, where they are central to the initiation and propagation of action potentials [7]. In non-excitable cells, which do not produce action potentials, non-voltage-activated Na⁺-selective channels have been found, and these represent a major route for Na⁺ entry. In renal epithelial cells, a family of Na⁺-selective channels [the most prominent member of this family being ENaC (epithelial Na⁺ channel)] have been described and are important in salt re-absorption [8]. Renal epithelial non-voltage-activated Na⁺ channels can be activated directly by heterotrimeric G-proteins [9]. Non-voltage-activated Na⁺ channels have also been observed in macrophages [10], A431 carcinoma cells [11] and a human myeloid leukaemia cell line [12].

We have previously described a non-voltage-activated, inwardly rectifying Na⁺-selective current in mast cells [13,14]. This current was activated by GTP analogues, and differed from previously described Na⁺ currents in terms of ionic selectivity, conductance, pharmacology and voltage-dependence. We called this Na⁺ current I_Na, (GTP-induced Na⁺ current). The development of this novel Na⁺ current has an absolute requirement for Mg²⁺ and ATP, just how the channels are activated remains unknown. An important clue came from the finding that GTP analogues such as GTP[S] (guanosine 5'-[γ-thio]triphosphate) and p[NH]ppG (guanosine 5'-[β,γ-imido]triphosphate) were much more effective in activating I_Na than was AlF₄⁻ [13]. GTP[S] affects both heterotrimeric and small GTP-binding proteins, whereas AlF₄⁻ targets only heterotrimeric ones. Hence we considered the intriguing possibility that I_Na is regulated by a small GTP-binding protein. In the present study, we have explored this possibility further, and find that functional activity of Arf-1 (ADP-ribosylation factor-1) is critical for the activation of I_Na. Our results provide further insight into the gating and regulation of a mammalian Na⁺-selective current, and identify a new downstream target for the small G-protein Arf-1.

EXPERIMENTAL

RBL-1 cells were purchased from A. T. C. C., and were cultured as described previously [15,16]. Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–24 °C) as described previously [15,17]. Fire-polished patch pipettes had DC resistances of 3.5–5.0 MΩ when filled with standard internal solution that contained (in mM): caesium glutamate 145, NaCl 8, MgCl₂ 1, MgATP 2, EGTA 10, Hepes 10, GTP[S] 0.5, pH 7.2 with CsOH. A correction of

Abbreviations used: Arf, ADP-ribosylation factor; ENaC, epithelial Na⁺ channel; GTP[S], guanosine 5'-[γ-thio]triphosphate; I_Na, GTP-induced Na⁺ current; p[NH]ppG, guanosine 5'-[β,γ-imido]triphosphate. ¹ To whom correspondence should be addressed (e-mail anant.parekh@physiol.ox.ac.uk).
+10 mV was applied for the subsequent liquid junction potential that arose from this glutamate-based internal solution. In some experiments, 5 mM GTP was included in the internal solution and then the total concentration of MgCl$_2$ was adjusted to 6 mM. Extracellular solution contained (in mM): NaCl 145, KCl 2.8, CaCl$_2$ 10, MgCl$_2$ 2, CsCl 10, glucose 10, Hepes 10, pH 7.4 with NaOH. CsCl was present to block the activity of the inwardly rectifying potassium channel. All chemicals were purchased from Sigma except for B581 (N-[2(S)-[2(R)]-amino-3-mercaptopropylamino]-3-methylbutyl)-Phe-Met-OH; from Affinity Research Products), and pertussis toxin and Y-27632 [I(r)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] (both from Calbiochem). Arf-1(2-17) and succinylated Arf-2(1-17) were kindly provided by Professor G. J. Barritt (Flinders University, Adelaide, Australia). The Arf-5 peptide (sequence GLTVSA-LFSRFPGKK) was synthesized by Thistle Research (Glasgow, U.K.) and myristoylated at the glycine (2) position. Where necessary, pipette solution was titrated back to pH 7.2 following addition of the peptides.

$I_{\text{GINa}}$ was measured by applying voltage ramps (−100 to +100 mV in 50 ms) at 0.5 Hz from a holding potential of 0 mV. Currents were filtered using an 8-pole Bessel filter at 2.5 kHz and digitized at 100 µs. Currents were normalized by dividing the amplitudes, measured from the voltage ramps at −80 mV, by the cell capacitance. Capacitative currents were compensated before each ramp by using the automatic compensation of the EPC 9-2 amplifier.

Data are presented as means ± S.E.M. Statistical evaluation was carried out using Student’s $t$ test or the Mann–Whitney $U$ test. In the Figures, statistical significance is indicated as follows: *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$.

**RESULTS**

**Basic features of $I_{\text{GINa}}$**

Dialysis with 500 µM GTP[S] results in the development of a large Na$^+$-selective current in RBL-1 cells, referred to as $I_{\text{GINa}}$. $I_{\text{GINa}}$ is activated by a variable latency of approx. 50–300 s and develops slowly, taking between 100 and 600 s to peak. Figure 1(A) details a typical time course of development of $I_{\text{GINa}}$, (control, measured at −80 mV), and the current–voltage $(I–V)$ relationship is shown in Figure 1(B). The current is non-voltage-gated, weakly inwardly rectifying and reverses at approx. +60 mV, close to the predicted equilibrium potential for Na$^+$. The current is selective for Na$^+$, not supporting detectable bivalent cation entry [13].

$I_{\text{GINa}}$ is regulated by a small G-protein

Non-hydrolysable analogues of GTP, such as GTP[S] and p[NH]ppG, routinely activate $I_{\text{GINa}}$ in the whole-cell configuration of the patch-clamp technique, whereas GTP and guanosine 5′-[β-thio]diphosphate are ineffective [13]. GTP[S] and p[NH]ppG target both heterotrimeric and small G-proteins. Our previous finding that AlF$_4^-$, which is believed to activate heterotrimeric but not small G-proteins, was less effective that GTP[S] or p[NH]ppG in evoking $I_{\text{GINa}}$ [13] suggests that a small G-protein may be involved in regulating the Na$^+$ current. GTP antagonizes the effects of GTP[S] on small G-proteins (e.g. [18,19]), an effect thought to reflect direct competition for the guanine nucleotide binding site. We have exploited this property to examine whether a small G-protein is involved in the regulation of $I_{\text{GINa}}$. Figure 1(A) compares the development of $I_{\text{GINa}}$ in a control cell (dialysed with 500 µM GTP[S]) with a recording from another cell in which GTP (5 mM) was included in the pipette solution together with GTP[S]. GTP prevented $I_{\text{GINa}}$ from developing. The $I–V$ relationships, taken at steady state for the two conditions (after approx. 400 s), are shown in Figure 1(B), and aggregate data are summarized in Figure 1(C) ($n = 6$ for control and $n = 7$ for GTP[S]; $P = 0.0012$).

Small G-proteins, such as those of the Ras family, are isoprenylated in the cytoplasm by protein farnesyltransferase, and this is required for the proteins to associate with cellular membranes. To see whether an isoprenylation process was required for the development of $I_{\text{GINa}}$, we inhibited farnesyltransferase by incubating cells with the enzyme inhibitor B581. However, $I_{\text{GINa}}$ was activated normally (Figure 1C; $n = 20$). We also checked for a role for Rho-associated protein kinase by inhibiting the enzyme with the membrane-permeable inhibitor Y-27632, but again $I_{\text{GINa}}$ was unaffected (Figure 1C; $n = 7$).

Consistent with a lack of involvement of heterotrimeric G-proteins were the findings that pertussis toxin, which inhibits
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Figure 2  Effect of pre-incubation with brefeldin A on \(I_{\text{GINa}}\)

(A) Time courses of current development in a control cell (C) and in a cell pre-incubated with 5 \(\mu\)g/ml brefeldin A (●). (B) Aggregate data for the effect of brefeldin A on current amplitude (\(n = 4\) cells each). Significance: *\(P < 0.05\) compared with control.

the regulation by G\(_i\) of inwardly and outwardly rectifying potassium currents in RBL cells [15,20], and \(N\)-ethylmaleimide, a broad inhibitor of heterotrimeric G-proteins [21,22], both failed to interfere with the activation of \(I_{\text{GINa}}\) (Figure 1C; \(n = 12\) cells each). Collectively, these findings support the notion that a small G-protein, and not a heterotrimeric one, is involved in the regulation of \(I_{\text{GINa}}\) in RBL-1 cells.

Arf-1 regulates \(I_{\text{GINa}}\)

Members of the family of Arf proteins are involved in vesicle budding and trafficking events, addition of coat components to Golgi and endosome membranes, and regulation of phospholipase D and phosphoinositide 3-kinase activities. The fungal metabolite brefeldin A inhibits certain vesicle transport and fusion steps by interfering with GTP/GDP exchange on Arf proteins, thereby impairing their function. To examine whether the Na\(^+\) current is regulated by Arf proteins, we pre-incubated cells with brefeldin A for 2 h prior to the onset of whole-cell experiments. The results are summarized in Figure 2. Whereas GTP[S] evoked a large \(I_{\text{GINa}}\) in control cells, the current was largely suppressed following exposure to brefeldin A (\(n = 4\) cells; \(P = 0.0286\)). A similar exposure to brefeldin A (1–2 h) failed to affect the activation of the store-operated Ca\(^{2+}\) current \(I_{\text{CRAC}}\) (Ca\(^{2+}\)-release-activated Ca\(^{2+}\) current) (results not shown), suggesting that the inhibitory effects on \(I_{\text{GINa}}\) were not due to non-selective effects on membrane currents.

The results with brefeldin A imply a central role for a member of the Arf family of small GTP-binding proteins in the regulation of \(I_{\text{GINa}}\). To test this more directly, we took advantage of small peptides that compete selectively with Arf proteins for binding to effectors, thereby inhibiting the functions of Arf proteins. Arf-1-(2–17) is such a peptide that antagonizes the effects of Arf-1 (reviewed in [19]). Figure 3(A) illustrates that dialysis with 50 \(\mu\)g/ml Arf-1-(2–17) largely suppressed the development of \(I_{\text{GINa}}\), and aggregate data are summarized in Figure 3(C). The reduction of \(I_{\text{GINa}}\) by Arf-1-(2–17) was significant (\(P = 0.028\)).

It has been reported that cationic amphipathic peptides such as Arf-1-(2–17) may exert their effects through damaging membranes rather than competing with Arf-1 [23]. However, three lines of evidence suggest that such a mechanism is unlikely to account for our findings. Firstly, the closely related analogue, succinylated Arf-(2–17), often used as a negative control [19], had no inhibitory effect on the development of \(I_{\text{GINa}}\) (Figures 3B and 3C). Secondly, the inhibitory effect of Arf-1-(2–17) was not mimicked by the N-terminal myristoylated peptide Arf-5-(1–15) (Figure 4), which has been used to probe insulin-dependent

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After transient expression of Arf-1-(2–17) failed to affect the development of the current (Figure 6C). Hence the development of the current depends on depletion of the endoplasmic reticulum stores, and thereby passively depleted intracellular Ca²⁺ stores. This protocol activates I_{CRAC} with a delay, so that Arf-1-(2–17) has time to diffuse into the cell before I_{CRAC} activation. Arf-1-(2–17) had no effect on the amplitude of I_{CRAC} (Figure 5). Furthermore, the delay before current development and the time constant τ of current development were not different between control cells and cells dialysed with Arf-1-(2–17) (n = 3; results not shown).

**Actin microfilament dynamics regulate I_{GINa}**

Because Arf proteins can regulate the cytoskeleton [25], we examined the effects of interfering with microfilaments and microtubules on the activation of I_{GINa}. Cytochalasin D disaggregates microtubules in RBL-1 cells, as was seen following changes in the subcellular distribution of rhodamine-labelled phalloidin [26]. Following pre-treatment of RBL-1 cells with cytochalasin D for at least 15 min, I_{GINa} was significantly lowered (Figure 6A; ramp I–V curves are shown in Figure 6B). Aggregate data are summarized in Figure 6(C) (n = 15 for both controls and cytochalasin D pre-incubation). The decrease in the amplitude of the current was significant (P = 0.0162), but the rate of development of the small I_{GINa} in the presence of cytochalasin D was not significantly different from that in the corresponding controls. This decrease in I_{GINa} is not due to a loss of cell viability following microfilament disaggregation, because similar exposure to cytochalasin D had no inhibitory effect on the activation of I_{CRAC} in these cells [26]. Stabilizing the cytoskeleton by including phalloidin in the pipette solution also reduced the amplitude of I_{GINa} (Figure 6C). However, disruption of the microtubules with colchicine did not impair the development of the current (Figure 6C). Hence the development of I_{GINa} seems to be regulated by actin filament dynamics.

**cAMP inhibits I_{GINa}**

Following an increase in the levels of intracellular cAMP, Arf-1 has been found to redistribute from the cytosol to the Golgi membranes. Inhibition of the cAMP-dependent protein kinase suppressed this translocation [27]. To see whether the cAMP pathway regulates I_{GINa}, ostensibly through redistribution of Arf-1,
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(3) cAMP, acting independently of protein phosphorylation, can inhibit the development of \(I_{\text{GINa}}\).

Western blotting studies have revealed that RBL cells express significant amounts of Arf-1 protein, which is important in the regulation of antigen-evoked exocytosis [28]. We found that pre-exposure of cells to brefeldin A or dialysis with the inhibitory peptide Arf-1-(2–17) suppressed the activation of \(I_{\text{GINa}}\). The effects of Arf-1-(2–17) were not mimicked by the succinylated analogue or by a corresponding myristoylated peptide directed towards the closely related Arf-5. Brefeldin A inhibits constitutive exocytosis by impairing GDP/GTP exchange on certain Arf proteins such as Arf-1, but apparently not Arf-6 [29], suggesting that this latter peptide is unlikely to be involved in the regulation of \(I_{\text{GINa}}\). Brefeldin A suppresses vesicular transport from the transitional endoplasmic reticulum to the cis-Golgi cisternae. Retrograde movement from Golgi to the endoplasmic reticulum is unaffected, and so components of the Golgi apparatus are inserted into the endoplasmic reticulum. Disaggregation of the Golgi network results in the loss of both constitutive exocytosis and Golgi-derived vesicles involved in regulated exocytosis. The relatively rapid effects of brefeldin A on \(I_{\text{GINa}}\) (almost complete inhibition after a 2 h exposure) together with the effect of dialysis with Arf-1-(2–17) would seem more consistent with an effect involving Arf proteins directly rather than an effect on constitutive exocytosis. The store-operated Ca\(^{2+}\) current \(I_{\text{CRAC}}\) was unaffected by similar exposure to brefeldin A or following dialysis of RBL-1 cells with Arf-1-(2–17) (Figure 5), arguing against a general loss of cation channels in the plasma membrane.

Arf proteins require their GTP-hydrolysing activity, and therefore GTP[S] inhibits their function [25]. One possibility is that Arf-1 constitutively inhibits either a key component of the activation mechanism or GINa channels themselves, and that this block is removed by GTP[S]. Because GTP[S] is not buffered, metabolized or transported out of the cell to any significant extent, it is possible to calculate the time course of the build-up of intracellular GTP[S] levels in a whole-cell patch-clamp experiment. Under our conditions, equilibration between GTP[S] in the pipette solution and the cytoplasmic space occurred with a time constant of 280 s (broken line in Figure 1). This time course is not incompatible with a role for Arf-1 in gating the Na\(^+\) channels directly, especially if the protein has a relatively low affinity for the nucleotide analogue, such that relatively high concentrations need to be achieved in order for the inhibition of Arf to occur.

Alternatively, GINa channels could be activated following the Arf-1-mediated restructuring of actin filaments. Exposure to cytochalasin D was able to activate 12 pS Na\(^+\) channels in excised patches from human myeloid leukaemia K562 cells, whereas application of intact actin prevented the channels from opening [12]. Furthermore, raising the intracellular Ca\(^{2+}\) concentration with an ionophore was able to activate these same Na\(^+\) channels, and this was again prevented by actin [30]. It was concluded that Ca\(^{2+}\)-dependent modulation of the actin cytoskeleton resulted in the opening of these 12 pS Na\(^+\) channels. However, exposure to cytochalasin D in RBL-1 cells did not facilitate the development of \(I_{\text{GINa}}\), but rather impaired it. Furthermore, elevation of the intracellular Ca\(^{2+}\) concentration did not alter the kinetics or extent of the Na\(^+\) current (results not shown). Hence the mechanism that activates Na\(^+\) channels in RBL-1 cells seems to be markedly different from that seen in K562 cells, but our results do not exclude a role for Arf-1 via alterations in actin filament dynamics. One way of discriminating between these mechanisms of activation of \(I_{\text{GINa}}\) would be to study channel activity in excised patches. However, we have consistently failed to observe any cationic currents following application of GTP[S] to inside-out patches from RBL-1 cells (results not shown). Further work,

**DISCUSSION**

The results of the present study extend our understanding of the regulation of the mammalian Na\(^+\) current \(I_{\text{GINa}}\). Specifically: (1) a member of the Arf family of small GTP-binding proteins, most probably Arf-1, is involved in the mechanism of activation of \(I_{\text{GINa}}\); (2) actin filament dynamics regulate the extent of the current; and
perhaps using giant excised macropatches, is needed to resolve this.

Finally, one potential activation mechanism incorporating Arf-1 and filamentous actin is the possibility that the GINa channels are stored in secretory vesicles and then inserted into the plasma membrane upon stimulation. The small G-protein kir/Gem inhibits voltage-gated Ca\(^{2+}\) channel activity, and this is brought about by its binding to the β subunit, followed by a decrease in the cell-surface expression of the channels [31]. It is therefore conceivable that Arf-1 might alter the cell-surface expression of GINa channels. In non-excitable cells, disruption of actin microfilaments renders secretion events easier, a process attributed to the removal of an inhibitory cytoskeletal clamp that prevents vesicles from docking [32]. However, disruption of actin filaments with cytochalasin D impaired the ability of I\(_{\text{GINa}}\) to develop, opposite to what one might have expected from an inhibitory clamp mechanism involving actin filaments. Furthermore, we did not see any clear increase in membrane capacitance, which would be indicative of vesicle fusion, as I\(_{\text{GINa}}\) was activated. However, this does not necessarily rule out an exocytotic mechanism. The slow development of I\(_{\text{GINa}}\) over several tens of seconds could easily muffle small increases in capacitance as the channels are inserted.

Arf proteins play important roles in vesicle budding and trafficking, coatomer formation at the Golgi apparatus and endosomes, and activation of enzymes such as phospholipase D and phosphoinositide kinases. Although we do not know if Arf-1 gates the Na\(^{+}\) channels directly or whether this effect involves an intermediary signalling pathway, our findings nevertheless identify a new downstream effector of Arf-1, namely a plasmalemmal Na\(^{+}\) channel.

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