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 Ca^{2+}, 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. α_2-adrenergic receptors [3]), glutamate uptake and release in the central nervous system [4], and intracellular Ca^{2+} levels through effects on Na^+/Ca^{2+} 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^+ 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_{GINa} (GTP-induced Na^+ current). Although the development of this novel Na^+ current has an absolute requirement for Mg^{2+} 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_{GINa} than was AlF_4^- [13]. GTP[S] affects both heterotrimeric and small GTP-binding proteins, whereas AlF_4^- targets only heterotrimeric ones. Hence we considered the intriguing possibility that I_{GINa} 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_{GINa}. 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, $MgCl_2$ 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_{CRAC}, Ca^{2+}-release-activated Ca^{2+} current; I_{GINa}, GTP-induced Na^+ current; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate.

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+ 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 [(*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] (both from Calbiochem). Arf-1-(2–17) and succinylated Arf-(2–17) were kindly provided by Professor G. J. Barritt (Flinders University, Adelaide, Australia). The Arf-5 peptide (sequence GLTVSALFSRIFGKK) 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_{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_{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_{GINa}. I_{GINa} is activated after 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_{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_{GINa} is regulated by a small G-protein

Non-hydrolysable analogues of GTP, such as GTP[S] and p[NH]ppG, routinely activate I_{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_{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_{GINa}. Figure 1(A) compares the development of I_{GINa} in a control cell (dialysed with

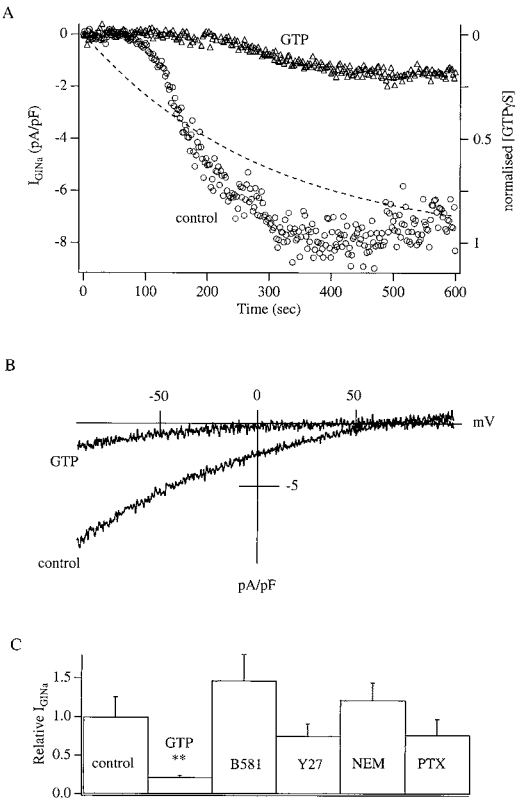

Figure 1 Effects of GTP and agents that affect G-proteins on I_{GINa}

(**A**) Time course of current development under control conditions (dialysis with 500 μM GTP[S]; ○) and for a cell dialysed with a pipette solution containing 5 mM GTP (△). The currents were measured at − 80 mV during voltage ramps (− 100 to + 100 mV in 50 ms). The broken line represents the estimated build-up of intracellular GTP[S] (GTPγS) against time following the onset of whole-cell dialysis. The time constant of the build-up of GTP[S] is proportional to the product of the series resistance (reflecting the diameter of the pipette in the whole-cell configuration) and the cube root of its molecular mass, as described in [33]. The mean series resistance was 10 MΩ, and the mean cell capacitance was 14 pF. (**B**) Corresponding *I–V* relationships (constructed with peak currents). (**C**) Effects of GTP and agents that affect G-proteins on the amplitude of I_{GINa}: 5 mM GTP, 1 mM *N*-ethylmaleimide (NEM; in pipette solution), 200 ng/ml pertussis toxin (PTX; pre-incubation for 16–20 h), 50 μM B581 (farnesyltransferase inhibitor, pre-incubation) and 10 μM Y-27632 (pre-incubation). The current amplitudes were normalized to control recordings. Numbers of cells were: seven (GTP), 12 (NEM), 12 (pertussis toxin), 20 (B581) and seven (Y-27632). Significance: $^{**}P < 0.005$ compared with control.

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_{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_{GINa}, we inhibited farnesyltransferase by incubating cells with the enzyme inhibitor B581. However, I_{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_{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

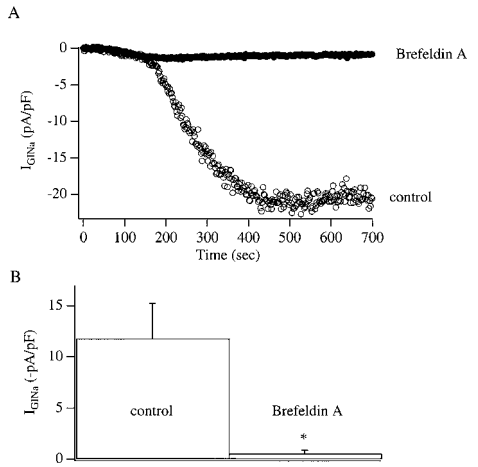

Figure 2 Effect of pre-incubation with brefeldin A on I_{GINa}

(**A**) Time courses of current development in a control cell (○) and in a cell pre-incubated with 5 μg/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_{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_{GINa} in RBL-1 cells.

Arf-1 regulates I_{GINa}

Members of the family of Arf proteins are involved in vesicle budding and trafficking events, addition of coatomers 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_{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_{CRAC} (Ca^{2+}-release-activated Ca^{2+} current) (results not shown), suggesting that the inhibitory effects on I_{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_{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 μg/ml Arf-1-(2–17) largely suppressed the development of I_{GINa}, and aggregate data are summarized in Figure 3(C). The reduction of I_{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

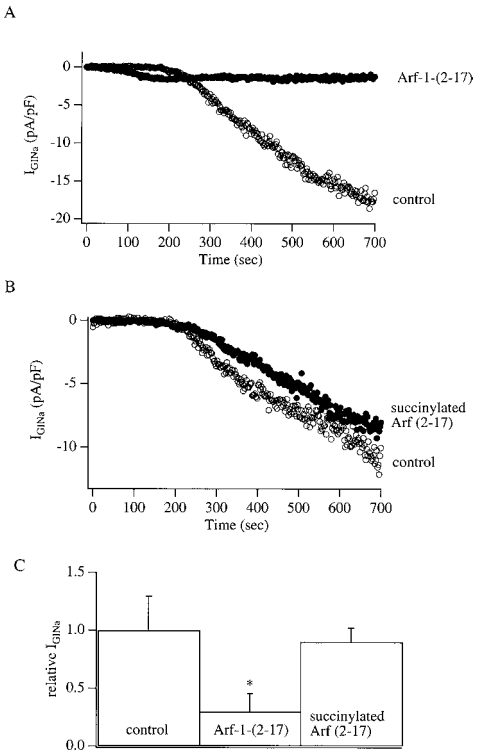

Figure 3 Effect of dialysis with Arf-1-(2–17) on I_{GINa}

(**A**) Time courses of current development in a control cell (○) and in a cell dialysed with 50 μg/ml Arf-1-(2–17) (●). (**B**) Time courses of current development in a control cell (○) and in a cell dialysed with 50 μg/ml succinylated Arf-(2–17) (●). (**C**) Aggregate data for the effects of Arf-1-(2–17) and succinylated Arf-(2–17) on current amplitude ($n = 6$–8 cells). Significance: $^*P < 0.05$ compared with control.

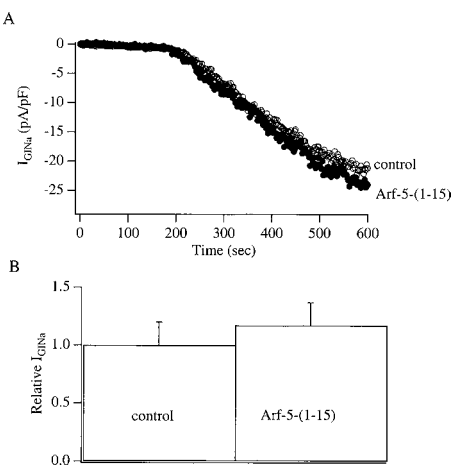

Figure 4 Effect of dilalysis with Arf-5-(1–15) on I_{GINa}

(**A**) Time courses of current development in a control cell (○) and in a cell dilalysed with 50 μg/ml Arf-5-(1–15) (●). (**B**) Aggregate data for the effect of Arf-5-(1–15) on current amplitude ($n = 8$ cells).

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_{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

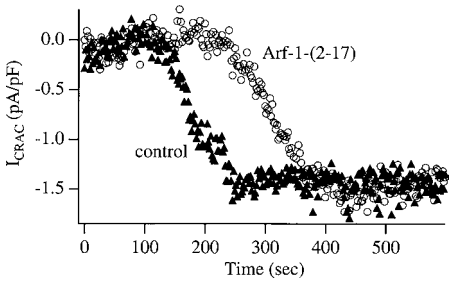

Figure 5 Effect of dialysis with Arf-1-(2–17) on I_{CRAC}

Shown are sample time courses of I_{CRAC} development in a control cell (dialysis with 10 mM EGTA; ▲) and in a cell dialysed with 50 μg/ml Arf-1-(2–17) in addition to 10 mM EGTA (○) ($n = 3$ cells per condition).

stimulation of the glucose transporter [24]. Arf-5-(1–15) impairs the functions of Arf-5, but not Arf-1, and its lack of an effect suggests that Arf-1 is central to the gating of I_{GINa}. Finally, Arf-1-(2–17) failed to affect I_{CRAC}. The activation of this store-operated Ca^{2+} current depends on depletion of the endoplasmic reticulum stores of Ca^{2+}, and should therefore be affected by a non-specific disruption of intracellular membrane systems. We evoked I_{CRAC} by dialysing cells with 10 mM EGTA to strongly buffer cytoplasmic Ca^{2+} and thereby passively depleted intracellular Ca^{2+} 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 microfilaments 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 has 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,

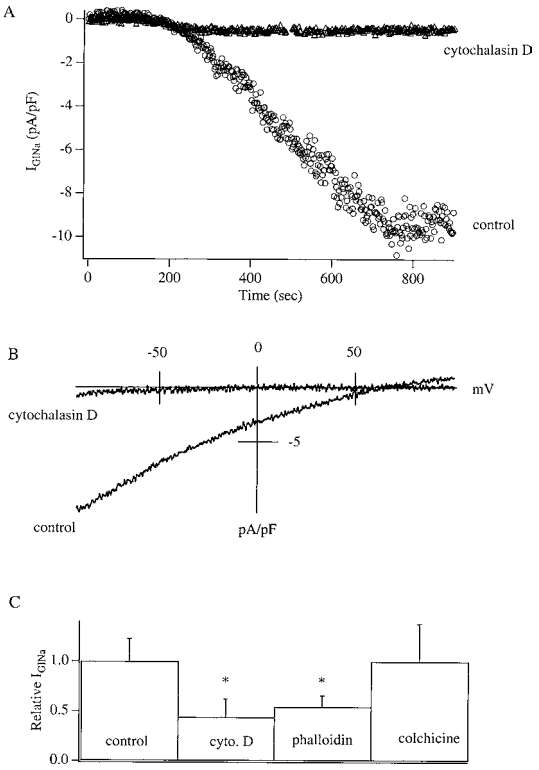

Figure 6 Effects on I_{GINa} of agents that affect the cytoskeleton

(**A**) Time courses of current development under control conditions (○) and for a cell preincubated in 5 μM cytochalasin D (△). (**B**) Corresponding I–V relationships. (**C**) Overview of the effects on current amplitude of the agents used: 5 μM cytochalasin D (pre-incubation), 50 μM phalloidin (pipette solution), and 10 μM colchicine [agent present in bath (pre-incubation) and pipette solution]. The amplitudes were normalized to control recordings that were obtained at the same time. Numbers of cells were: 15 (cytochalasin D), 16 (phalloidin), and 11 (colchicine). Significance: $^*P < 0.05$ compared with control.

we examined the effects of the cyclic nucleotide on the Na^+ current. Inclusion of cAMP in the pipette solution significantly reduced the extent of I_{GINa} (Figure 7A), amounting to an approx. 65 % decrease in amplitude at -80 mV [Figure 7C; $n = 10$ cells (cAMP) and $n = 13$ cells (control)]. This reduction by cAMP was maintained over a range of voltages (-100 to $+100$ mV; Figure 7B), indicating that it was not a rapid voltage-dependent process. Moreover, the shape and reversal potential of the I–V relationship was similar during the experiment, demonstrating that the decrease in the Na^+ current was not caused by induction of an outward current, but rather by a specific decrease in I_{GINa} (Figure 7B).

The inhibitory effects of cAMP were mimicked by pre-incubating cells with the phosphodiesterase inhibitor caffeine (Figure 8), a protocol that increases basal levels of cAMP. It is unlikely that the effects of caffeine involved ryanodine receptors, because application of caffeine to cells loaded with fura-2 failed to generate an intracellular Ca^{2+} signal (results not shown). However, the inhibitory effects of cAMP were not prevented by pre-exposing cells to the broad kinase inhibitors staurosporine or H-89 (*N*-{2-[(*p*-bromocinnamyl)amino]ethyl}-5-isoquinolinesulphonamide) (Figure 8), suggesting that the inhibition probably does not involve cAMP-dependent protein phosphorylation. Pre-exposing cells to either staurosporine or H-89 alone failed to alter the extent of I_{GINa} in control cells (Figure 8), ruling out non-specific effects of the kinase blockers on the development of I_{GINa}.

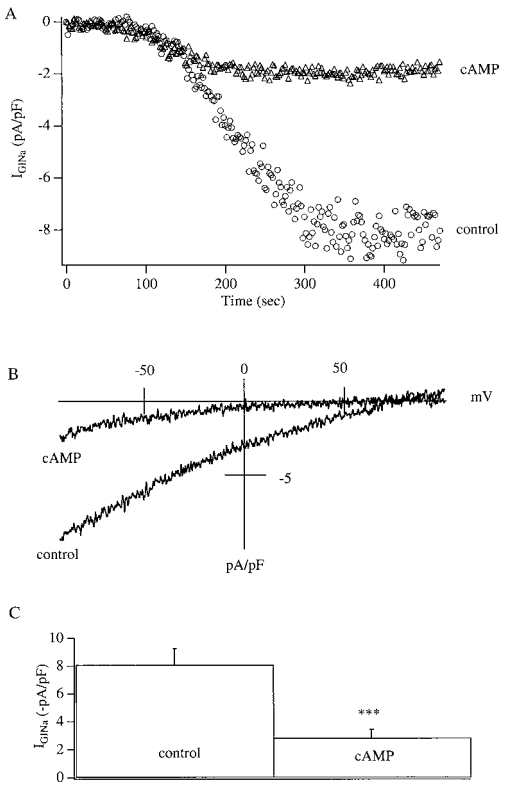

Figure 7 Effect of cAMP on I_{GINa}

(**A**) Time course of I_{GINa} development under control conditions (○) and for a cell dialysed with an internal solution containing 500 μM cAMP (△). (**B**) Corresponding *I–V* relationships. Aggregate data are shown in (**C**); $n = 13$ cells (control) and 10 cells (cAMP). Significance: $^{***}P < 0.001$ compared with control.

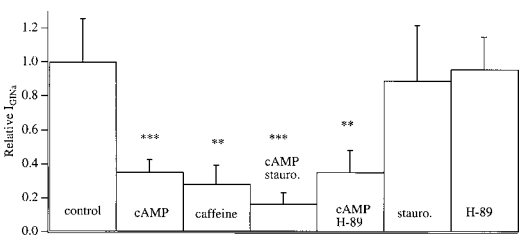

Figure 8 Effects of protein kinase inhibitors on the blockade of I_{GINa} by cAMP

This overview demonstrates the effects of 500 μM cAMP (in pipette solution) and 10 mM caffeine (in bath) to lower the amplitude of I_{GINa}. The effect of cAMP could not be reversed by the protein kinase inhibitors staurosporine (stauro.; 2 μM) or H-89 (1 μM). Both protein kinase inhibitors were present in both bath and pipette solutions. Staurosporine and H-89 by themselves had no effect on I_{GINa}. All current amplitudes are normalized to control recordings that were obtained at the same time. Numbers of cells were: 10 (cAMP), 11 (caffeine), seven (staurosporine and cAMP), seven (H-89 and cAMP), four (staurosporine alone) and eight (H-89 alone). Significance: $^{**}P < 0.005$, $^{**}P < 0.001$ compared with control.

DISCUSSION

The results of the present study extend our understanding of the regulation of the mammalian Na^+ current I_{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_{GINa}; (2) actin filament dynamics regulate the extent of the current; and (3) cAMP, acting independently of protein phosphorylation, can inhibit the development of I_{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_{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_{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_{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_{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_{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_{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,

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 decease 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_{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_{GINa} was activated. However, this does not necessarily rule out an exocytotic mechanism. The slow development of I_{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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