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# A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*

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**The tumour-suppressor gene *Neurofibromatosis 1* (*Nf1*) encodes a Ras-specific GTPase activating protein (Ras-GAP)[1–5]. In addition to being involved in tumour formation[6,7], *NF1* has been reported to cause learning defects in humans[8–10] and *Nf1* knockout mice[11]. However, it remains to be determined whether the observed learning defect is secondary to abnormal development. The *Drosophila* NF1 protein is highly conserved, showing 60% identity of its 2,803 amino acids with human NF1 (ref. 12). Previous studies have suggested that *Drosophila* NF1 acts not only as a Ras-GAP but also as a possible regulator of the cAMP pathway that involves the *rutabaga* (*rut*)-encoded adenylyl cyclase[13]. Because *rut* was isolated as a learning and short-term memory mutant[14,15], we have pursued the hypothesis that NF1 may affect learning through its control of the Rut-adenylyl cyclase/cAMP pathway. Here we show that NF1 affects learning and short-term memory independently of its developmental effects. We show that G-protein-activated adenylyl cyclase activity consists of NF1-independent and NF1-dependent components, and that the mechanism of the NF1-dependent activation of the Rut-adenylyl cyclase pathway is essential for mediating *Drosophila* learning and memory.**

We examined olfactory associative learning of adult fruit flies by using a well-defined Pavlovian procedure[16–19]. Significant decrements in olfactory learning performance were shown for two independently isolated *NF1* null alleles[12], $NF1^{P1}$ and $NF1^{P2}$, as compared with K33, the parental line for *NF1* mutants with a P-element inserted nearby the *NF1* locus[12] (Table 1, Fig. 1a). Olfactory avoidance and electric-shock reactivity[20], two sensorimotor activities necessary for performing the learning task, were similar in the mutant and control K33 flies (Table 1). To consider the potential effects of genetic background on behaviour[20], we outcrossed *NF1* mutants and K33 with an isogenic line $w^{1118}$ (*isoCJ1*)[21]. Again, learning scores of *NF1* mutants were significantly reduced (Table 1, Fig. 1a), whereas the parameters of sensorimotor activities were not statistically different from the control with a similar genetic background (Table 1). Even though learning scores and some scores for shock reactivity and odour avoidance are significantly different for K33 in different genetic backgrounds, these behavioural parameters also vary accordingly in *NF1* mutants (Table 1). These results indicate that *NF1* is a learning mutant.

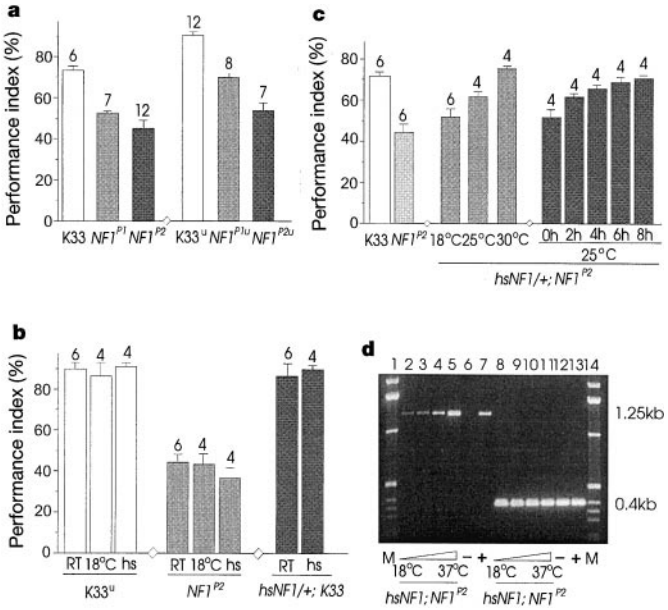


**Figure 1** Rescue of the *NF1* learning defect by inducible expression of the normal *NF1* transgene. **a**, NF1 learning defects observed in both the original and outcrossed isogenic (marked by u in superscript) genetic background. K33 is the parental line of *NF1* mutants. **b**, No effect on learning scores for the heat-shock treatment in controls, including overexpression of the *NF1* transgene in the control background. **c**, Rescue of the learning defect by induced expression of the *NF1* transgene. In the first group, the flies were moved from 18 °C to 25 or 30 °C for 2 h before the learning test ($P < 0.05$, Tukey Kramer Honestly Significant Difference). In the second group, flies were shifted from 18 °C to 25 °C for 0, 2, 4, 6 or 8 h, respectively (significant for 2 h, $P < 0.05$). The number of assays for each group are indicated above each error bar. **d**, Semi-quantitative RT–PCR showing induced expression of the hsNF1 transgene. Lanes 1 and 14, 1-kb DNA ladder (M) (Gibco BRL). Lanes 2–7, RT–PCR using NF1-specific primers with cDNA prepared from *hsNF1; NF1*$^{P2}$ flies grown at 18, 25 and 30 °C or given daily 1 h heat shock at 37 °C, or from $NF1^{P1}$ mutant (–) flies or K33 wild-type (+) flies grown at 18 °C. Lanes 8–13, control RT–PCR from the same cDNA using ribosomal protein rp49-specific primers. Three separate mRNA isolations showed the same pattern of increased expression of the hsNF1 transgene at increased temperature.

**Table 1 Performance indice for olfactor learning, shock reactivity and odour avoidance**

| Genotypes | Learning (*n*) | Shock reactivity | | Odour avoidance | | | |
|---|---|---|---|---|---|---|---|
| | | | | BA dilution | | MCH dilution | |
| | | 60 V | 20 V | 4% | 0.4% | Undiluted | 10% |
| K33 | 73 ± 2 (6) | 72 ± 6 | 25 ± 9 | 78 ± 5 | 28 ± 10 | 73 ± 7 | 40 ± 9 |
| $NF1^{P1}$ | 53 ± 1 (7)* | 77 ± 4 | 30 ± 5 | 80 ± 3 | 25 ± 4 | 66 ± 8 | 36 ± 6 |
| $NF1^{P2}$ | 45 ± 4 (12)* | 76 ± 3 | 26 ± 5 | 71 ± 4 | 19 ± 6 | 67 ± 4 | 29 ± 7 |
| *hsNF1/+*; $NF1^{P2}$ | 75 ± 2 (4) | 65 ± 3 | 23 ± 6 | 79 ± 6 | 32 ± 11 | 83 ± 4 | 34 ± 7 |
| $K33^{u}$ | 90 ± 1 (12) | 89 ± 2 | 61 ± 6 | 92 ± 1 | 41 ± 8 | 77 ± 5 | 63 ± 5 |
| $NF1^{P1u}$ | 70 ± 2 (8) | 80 ± 5 | 56 ± 8 | 85 ± 5 | 36 ± 9 | 84 ± 6 | 59 ± 8 |
| $NF1^{P2u}$ | 54 ± 4 (7)* | 83 ± 3 | 62 ± 7 | 93 ± 2 | 41 ± 7 | 77 ± 4 | 58 ± 5 |

K33, $NF1^{P1}$, $NF1^{P2}$ and *hsNF1/+*; $NF1^{P2}$ have a similar genetic background, whereas $K33^{u}$, $NF1^{P1u}$ and $NF1^{P2u}$ have a different background (see Methods). All scores a
e expressed as PI ± s.e.m. For learning, the number (*n*) of assays are indicated in parentheses. For all shock reactivity and odour avoidance assays, $n = 8$.
* Statistically different from control. No statistical difference at the level of $\alpha = 0.05$ is detected among all the sensorimotor activities. Learning defect is significant at $\alpha \leq 0.001$. Comparison is made between mutants and controls with a similar genetic background using Tukey–Kramer HSD test within the Macintosh software package JMP3.1 (SAS institute, Inc., Cary, North Carolina, USA).

This conclusion is further supported by the observation that the learning defect was rescued by induced expression of the *NF1* transgene (see below) without changing sensorimotor activity significantly (Table 1).

We then examined the effect of heat-shock-induced expression of the *NF1* transgene to determine whether the learning defect is caused by an adult requirement for NF1, or whether it is a secondary consequence of developmental abnormalities, such as the small body size of *NF1* mutants[12]. Heat-shock treatment of *hsNF1* transgenic flies leads to expression of the NF1 protein[12]. Such treatment did not affect learning scores in *NF1* mutants, control flies or *hsNF1*; *K33* (Fig. 1b); however, learning scores were improved when mutant flies carrying the *NF1* transgene were heat shocked.

Heterozygous transgenic *NF1* (*hsNF1/+; NF1$^{P2}$*) flies were raised at room temperature (20–24 °C). These flies showed a learning score PI of 63 ± 3 ($n = 5$), indicating a partial rescue because of leaky expression (see Fig. 1d). To minimize the leaky expression of the heat-shock promoter-controlled *NF1* transgene, flies were shifted to 18 °C overnight before the test. This reduced the learning score significantly to 52 ± 4 (Fig. 1c). Learning scores of transgenic flies (*hsNF1/+; NF1$^{P2}$*) were improved to a better extent when flies were treated at 30 °C as compared with 25 °C for two hours (Fig. 1c), or when the transgenic flies were subjected to 25 °C for successively longer times (Fig. 1c). Presumably, more NF1 was expressed with higher temperatures or for longer times of treatment. This is supported by data from polymerase chain reaction with reverse transcriptase (RT–PCR). Higher temperature treatment led to accumulation of more messenger RNA transcribed from the *hsNF1* transgene (Fig. 1d). Thus, the level of performance improvement may be proportional to the amount of NF1 expressed.

A single heat-shock treatment during larval stages did not change the smaller body size of *NF1* mutants, as quantified by measuring the length of pupal cases. *hsNF1/+; NF1$^{P2}$* pupal cases (2.73 ± 0.14; $n = 66$) were indistinguishable after treatment from *NF1$^{P2}$* (2.69 ± 0.2; $n = 56$), but were smaller than those of control K33 (3.2 ± 0.13; $n = 70$). Repetitive or continuous heat-shock treatments, however, rescue the developmental phenotype[12]. Thus, the learning defect can be rescued by acute expression of the *NF1* transgene during adulthood, but the developmental defect requires repetitive or continuous heat-shock treatment during development. This suggests that NF1 is essential for the learning process.

To test whether the NF1-dependent learning defect involves the cAMP pathway, we compared learning scores of *NF1$^{P2}$* and *rut$^{1}$* single-mutant, and *rut$^{1}$; NF1$^{P2}$* double-mutant flies. The learning scores of all three mutant genotypes were very similar (Fig. 2a). The learning score of another double mutant, *dunce* (*dnc*); *rut$^{1}$*, is reduced when compared with either single mutants[16], which indicates that the two mutations exert additive effects on learning even though both gene products are involved in the cAMP cascade (Rut-adenylyl cyclase (AC) for synthesizing cAMP[14,22] and Dnc-phosphodiesterase for degrading cAMP[23,24]). Therefore, the absence of any further reduction of learning in the double mutant *rut$^{1}$; NF1$^{P2}$* suggests that both gene products function closely in the cAMP pathway.

This idea is supported by studies of *NF1* mutant flies carrying a transgene encoding a mutant catalytic subunit of cAMP-dependent protein kinase (PKA*), which is constitutively active[25]. Sustained expression of this PKA subunit rescues the small body size phenotype of *NF1* mutants[12]. Heat-shock induction of the constitutively active PKA should, in principle, bypass the requirement for the Rut-AC and all other molecules upstream of normal PKA activation. The *hsp70-PKA** transgene completely rescued the learning defect of *NF1$^{P1}$* when the flies were raised at room temperature (Fig. 2b). *NF1$^{P2}$* mutants were partially rescued by the transgene at room temperature, but showed complete rescue with heat shock (37 °C,

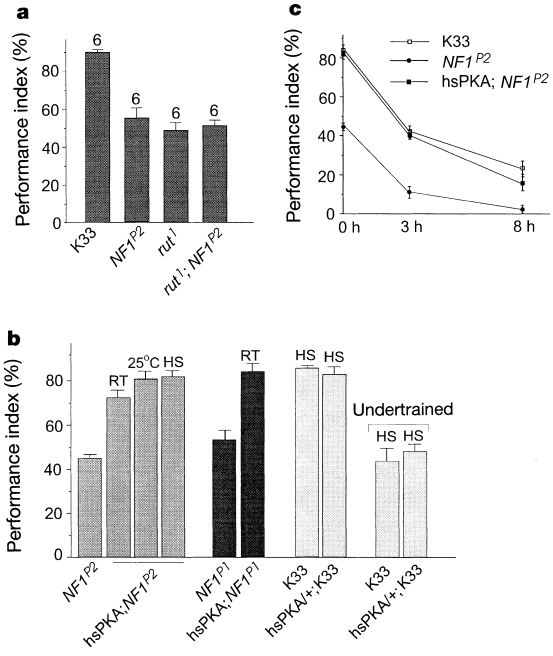


**Figure 2** Effects of the cAMP pathway on NF1-dependent learning and memory. **a**, Effect of the *rut* mutation. Learning defects were similar among *rut*, *NF1* and *rut$^{1}$; NF1$^{P2}$* mutants. **b**, Rescue of *NF1* learning defects by induced expression of a constitutively active catalytic subunit of cAMP-dependent protein kinase (PKA*). Learning scores were rescued or partially rescued in *hsp70-PKA*; NF1$^{P1}$* and *hsp70-PKA*; NF1$^{P2}$* flies, even when raised at room temperature (RT). For undertraining, flies after heatshock (HS) were trained with 3 repeats of electric shock instead of 12 to avoid any ceiling effect on learning scores. The learning scores were reduced in K33 and hsPKA*/+;K33 in parallel. Heat shock was at 37 °C for 30 min and training started after a 3-h rest. Flies were shifted to 25 °C overnight before training (25 °C). From left to right, $n = 6, 6, 6, 5, 4, 6, 6, 6, 4, 4$. **c**, Rescue of short-term memory. Retention at 3 and 8 h after training was also disrupted in *NF1* mutants. This was rescued by induced expression of the PKA* subunit. Flies were raised at room temperature and heat shocked for 30 min at 37 °C and then rested for at least 3 h before training. $n = 6–12$.

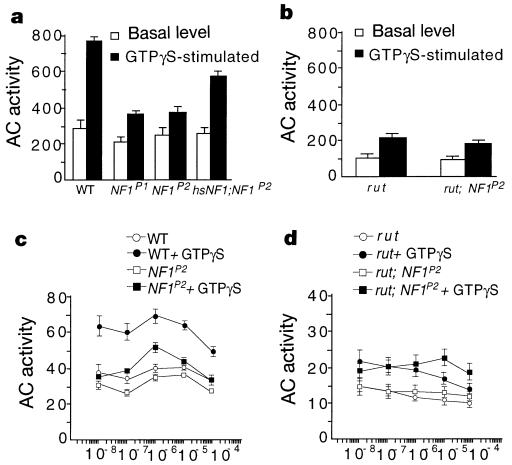


**Figure 3** Biochemical assay of the effects of NF1 on AC activity. **a**, Reduction of GTPγS-stimulated AC activity in brain tissue of *NF1* mutants. Each data point (mean ± s.e.m.) is the average of four independent experiments. To be comparable, all flies were subjected to heat shock (for 2 h at 35 °C and 1 h rest at room temperature). **b**, Diminished *NF1* effect on AC activity in the *rut* mutant background. Flies were also subjected to the same heat shock as in **a** and brain tissues were used. Data points are the average of three independent experiments. **c**, Effects of NF1 on $Ca^{2+}$ dependence of AC activity in abdominal tissues. The average of eight independent experiments is shown. **d**, No significant *NF1* effect on abdominal AC activity in the *rut* mutant background. The average of six independent experiments is shown.

30 min), or with a shift to 25 °C overnight before being tested (Fig. 2b). In addition, *NF1* mutations also caused a short-term memory defect (3- and 8-h retention, Fig. 2c; $NF1^{P1}$ not shown), which was also fully rescued by heat-shock induction of PKA*. To determine whether expression of *hsp70-PKA** induces a nonspecific enhancement of learning, we showed that leaky or induced expression of *hsp-PKA** in the wild-type background did not increase the learning score even if flies were undertrained (Fig. 2b). For undertraining, flies were subjected to 3 repeats of electric shock in a training trail instead of 12 (see Methods, and ref. 16). We conclude that the PKA* effect is not nonspecific and that the learning defect observed in *NF1* mutants can be rescued by induction of PKA activity. Therefore, the biochemical deficiency in the *NF1* mutants must reside upstream of PKA induction in the cAMP pathway.

These behavioural analyses corroborate previously reported electrophysiological data[13] that indicated that NF1 might exert its effect through regulation of the activation of Rut-AC. Biochemical assays provide direct evidence to support the idea. Previous experiments have shown that Rut-AC expressed in a cell line can be stimulated not only by $Ca^{2+}$/calmodulin, but also by reagents that stimulate G-proteins, including GTPγS and $AlF_4^-$ (see ref. 22). We first examined AC activity in membrane fractions of adult brain tissues. The basal level of AC activity was very similar in the control (K33) and *NF1* mutant membranes, but the GTPγS-stimulated AC activity was markedly reduced in $NF1^{P1}$ and $NF1^{P2}$ mutant membranes (Fig. 3a). However, significant GTPγS-stimulated activity occurred above the basal level in the mutants. Overexpression of NF1 in control flies did not increase AC activity (data not shown), whereas the reduction in stimulated AC activity seen in *NF1* mutants was mostly rescued by acutely induced expression of the *NF1* transgene (Fig. 3a), indicating that NF1 is indeed able to regulate cAMP synthesis. Thus, GTPγS-stimulated AC activity consists of two components: one that is NF1 dependent and one that is NF1 independent. To determine whether the NF1-dependent AC activity is due to Rutabaga, we assayed $rut^1$ and $rut^1; NF1^{P2}$ mutant flies. The basal and GTPγS-stimulated level of AC activity were very similar in the single mutant, $rut^1$, and in the double mutant, $rut^1; NF1^{P2}$ (Fig. 3b). Thus, the *NF1* mutation has no impact on AC activity in the absence of Rut-AC. In other words, the NF1-dependent cAMP activity is mediated through Rut-AC.

We also measured the $Ca^{2+}$ dependence of the NF1 effect to determine how Rut-AC is involved. Membrane fractions extracted from abdominal tissues were used because the $Ca^{2+}$-dependent Rut-AC activity is easier to detect (Fig. 3c, d). Our data are consistent with a previous report[14] that the $Ca^{2+}$-dependent peak of AC activity is missing in *rut* mutants. Again, the *NF1* mutation had no effects on AC activity across $Ca^{2+}$ concentrations without Rut-AC (Fig. 3d). Moreover, the $Ca^{2+}$-dependent peak of Rut-AC activity was dependent on G-protein stimulation but not on the presence of NF1 (Fig. 3c).

Together, our results reveal a new mechanism for how G-proteins activate the cAMP pathway for normal learning and memory. The G-protein-activated AC activity is both NF1 dependent and NF1 independent. The NF1-dependent component involves Rut-AC. One possibility for how NF1 regulates AC activity is that NF1 acts as a GAP not only for the small G-protein Ras but also for heterotrimeric G-proteins. Thus, NF1 is required for a functional interaction between AC and heterotrimeric G-proteins, similar to the involvement of IRA, a Ras-GAP that is distantly related to NF1, in the Ras-activated cAMP pathway in yeast[26].

Another possibility is that NF1 regulates AC activity independently of its role as a Ras-GAP. Therefore, NF1 may be important for coordinating activities of multiple signal-transduction pathways. Nevertheless, the expression of the tumour-suppressor gene *NF1* and its regulation of the Rut-AC signal-transduction pathway are critical to the biochemical processes underlying olfactory learning in *Drosophila*. Similar mechanisms are to be expected in vertebrates. □

## Methods

### Fly stocks

$NF1^{P1}$, $NF1^{P2}$ and K33 flies have a similar genetic background[12] and were outcrossed with $w^{1118}$(*isoCJ1*), an isogenic line[21], for five generations. Thus, $NF1^{P1u}$, $NF1^{P2u}$ and $K33^{u}$ have a genetic background that is similar to $w^{1118}$(*isoCJ1*). The transgenic *hsNF1* gene is inserted in the second chromosome[12]. In all experiments related to *hsNF1*, only heterozygous *hsNF1/+* was used, which avoided any recessive effects of the insertion on behaviour. *hsp70-PKA** flies have a murine PKA transgene, with His87Gln and Trp196Arg substitutions that prevent interaction with the PKA regulatory subunit[25].

### Pavlovian learning

Flies were trained by exposure to electroshock (12 pulses at 60 V, duration 1.5 s, interval 5 s) paired with one odour (benzaldehyde (BA, 4%) or methycyclohexanol (MCH, undiluted) for 60 s) and subsequent exposure to the second odour without electroshock. The odour concentrations were adjusted to assume no preference for flies exposed simultaneously to the two odours before the training. Immediately after training, learning was measured by allowing flies to choose between the two odours used during training. No preference between odours results in zero (no learning) PI. Avoidance of the odour previously paired with electroshock produces a $0 < PI \leq 1.00$ (see ref. 16).

### Olfactory acuity

Absolute odour avoidance responses were quantified by exposing naive flies to each odour (BA or MCH) or air in the T-maze. After 120 s, the numbers of flies in each arm of the T-maze were counted, and the performance index was calculated for each odour individually as reported[20].

### Shock reactivity

The ability to sense and escape from electric shock was quantified by inserting electrifiable grids into both arms of the T-maze, and delivering shock pulses to one arm. Flies were transported to the choice point of the T-maze, where they could choose between the two arms. After 60 s, the centre compartment was closed, trapping flies in their respective arms. Individual PI was calculated as defined[20].

### Adenylyl cyclase activity assay

The described AC activity assay[27] was modified as follows. The membrane fraction was extracted from either male heads, with the cuticle removed to leave essentially brain tissue, or from 10 male abdomens. Twenty dissected brains in 850 μl lysis buffer were homogenized, and the membrane fraction was extracted by centrifugation at 178,000*g* for 10 min. GTPγS (final concentration, 20 μM) was added to stimulate G-protein-activated AC activity immediately after homogenization but before centrifugation. When assaying the basal level of AC activity, GTPγS was not added. These modifications seemed to amplify the NF1 effect. Calcium concentrations were calculated according to MaxChelator v1.31 (see ref. 28).

### RT–PCR of induced NF1 expression

Total RNA was prepared from 100 mg of flies using the RNeasy Mini Kit (Qiagen). We isolated mRNA from total RNA (20–100 μg) by using either the Dynabeads mRNA DIRECT Micro Kit or the Dynabeads mRNA Purification Kit (Dynal) and first-strand complementary DNA was synthesized directly from this mRNA using the SuperScript Preamplification System (Gibco BRL). PCR amplification with *Taq* DNA polymerase (Gibco BRL) was carried out in the manufacturer's buffer using 1.5 mM $MgCl_2$ with 30 cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 1 min, followed by extension at 72 °C for 10 min. NF1-specific primers, 5′-tcaccaaagctcagagcacga-3′ and 5′-gccttgttgcaggatttgagt-3′, were designed to amplify a 1,251-base-pair (bp) region of cDNA between bases 2,730 and 3,981 (Genbank L26501)[12]. These primers span a region of genomic DNA containing a 54-bp intron that allows distinction between cDNA products and potential genomic contaminants. Lack of genomic DNA contamination was confirmed by the absence of any PCR products when RT-PCR was carried out in control reactions without reverse transcriptase. Ribosomal protein rp49-specific primers, 5′-atgaccatccgcccagcatac-3′ and 5′-gagaacgcaggcgaccgttgg-3′, were designed to amplify a 391-bp fragment between bases 1 and 391 of the rp49 coding region (Genbank Y13939)[29]. The control rp49 mRNA should be expressed at equal levels in all cells at all stages[29].

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# Three distinct and sequential steps in the release of sodium ions by the $Na^+/K^+$-ATPase


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**The $Na^+/K^+$ pump, a P-type ion-motive ATPase, exports three sodium ions and then imports two potassium ions in each transport cycle. Ions on one side of the membrane bind to sites within the protein and become temporarily occluded (trapped within the protein) before being released to the other side[1,2], but details of these occlusion and de-occlusion transitions remain obscure for all P-type ATPases. If it is deprived of potassium ions, the $Na^+/K^+$ pump is restricted to sodium translocation steps[3], at least one involving charge movement through the membrane's electric field[4,5]. Changes in membrane potential alter the rate of such electrogenic reactions and so shift the distribution of enzyme conformations. Here we use high-speed voltage jumps to initiate this redistribution and show that the resulting pre-steady-state charge movements relax in three identifiable phases, apparently reflecting de-occlusion and release of the three sodium ions. Reciprocal relationships among the sizes of these three charge components show that the three sodium ions are de-occluded and released to the extracellular solution one at a time, in a strict order.**


The main electrical signals generated during $Na^+/K^+$ pumping result from $Na^+$ traversing part of the membrane's electric field in an access channel that connects $Na^+$-binding sites to the extracellular medium[6–9]. To investigate de-occlusion and release of the three transported $Na^+$ ions, we measured pump-mediated charge translocation in voltage-clamped, internally dialysed squid giant axons, using solutions designed to limit $Na^+/K^+$ pumps to phosphorylated conformations with $Na^+$-binding sites either occupied and buried or open to the external solution: $(Na_3)E_1\text{-}P \leftrightarrow P\text{-}E_2\cdot Na_3 \leftrightarrow P\text{-}E_2$ (Fig. 1, dotted box). Pump-mediated charge was assayed as the component of membrane current that was sensitive to dihydrodigitoxigenin ($H_2DTG$), a specific $Na^+/K^+$-pump inhibitor[10] (Fig. 2a (2–3)). With 100 mM external $Na^+$ ($[Na]_o$), the change in pump current induced by a voltage jump (20-ms step from 0 mV to −90 mV; current displayed at 50 μs per point in Fig. 2a) comprised fast (the first ~10 points) and slow ($\tau \approx 4$ ms) components, and relaxed to near zero. As negative internal potentials electrostatically favour the approach of external $Na^+$ to their binding sites within the pump, we interpret the fast component (see ref. 8) as reflecting rapid electrogenic binding of $Na_o^+$ to vacant sites ($P\text{-}E_2$) to satisfy the new $P\text{-}E_2 \leftrightarrow P\text{-}E_2\cdot Na_3$ distribution demanded by the new membrane potential, −90 mV. The resulting increased abundance of pumps in the $P\text{-}E_2\cdot Na_3$ state drives the slower (comparatively electroneutral; see below) occlusion reaction ($P\text{-}E_2\cdot Na_3 \leftrightarrow (Na_3)E_1\text{-}P$), which is tracked by further electrogenic binding of $Na_o^+$ to $P\text{-}E_2$, now rate limited by the slow conformational change and hence appearing as slow charge movement (see, for example, refs 7, 11). We found no recruitment of additional slow charge ($Q_s$; Fig. 2b, left) at extreme potentials, in accord with the expectation that, in the steady state, pumps in the $P\text{-}E_2$ conformation should be

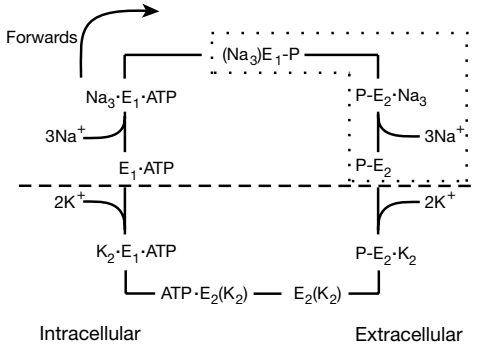


**Figure 1** Simplified Post–Albers transport cycle emphasizing two principal $Na^+/K^+$ pump conformations: $E_1$ with ion-binding sites facing the cytoplasm, and $E_2$ with ion-binding sites open to the extracellular solution. Phosphorylation of $E_1$ by ATP occludes three $Na^+$, which are released to the external medium after the conformational change to $E_2$, whereupon two $K^+$ bind, eliciting dephosphorylation and $K^+$ occlusion. ATP binding favours transition back to $E_1$, prompting $K^+$ release to the cytoplasm and binding of three $Na^+$, completing the cycle. The dashed (horizontal) line separates $Na^+$- and $K^+$-translocation pathways; the dotted box encloses states isolated experimentally to yield the charge movements examined.


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