The study reported here is the first demonstration at the functional level, of changes analogous to those described classically during epigenesis of visual cortex[5,6,11]. It is known that unilateral eye-lid closure following normal visual experience[12] or monocular vision in previously deprived kittens[25] results in a global shift of ocular dominance in favour of the eye remaining open. These rearing procedures could correspond to an S^+ situation, where active or passive visuomotor interaction primes neuronal excitability in response to visual stimulation through the open eye[25,26]. Furthermore, it has been shown recently that after monocular vision associated with blockade of postsynaptic activity (induced by intracortical injection of an agonist of GABA) most cells would respond only to the closed eye[27]. This result is predicted by the covariance hypothesis, and is analogous to the effects we found after S^- pairing alone or interleaved with presentation of a neutral stimulus S^0. Similar reasoning applies to the orientational protocol: our data demonstrate the capacity for certain cells to capture the orientation seen during a restricted visual exposure[13,14]. However, it appears that cells can adapt their preference in favour of the imposed orientation, only if this latter initially evokes some response.

Finally, our study suggests that a hitherto unsuspectedly high level of plasticity is retained in the adult cortex. This favours the hypothesis that hebbian-like mechanisms operate both during development, and in adult learning[4,8]. We suggest that, in the intact animal, extraretinal gating signals[4,16,28]—instead of our pairing artifice—produce covariance changes through normal visuomotor experience during a postnatal critical period in kittens[16], or during selective periods of learning in the adult[28,29]. These would allow cortical neurons to undergo transition from a passive relay mode of transmission to an adaptive state reached only below or above certain levels of membrane potential. It still remains to be determined why there is an age-dependency in the expression of this experience-sensitivity in the intact, behaving animal[8]. In the adult animal, predominance of inhibitory intracortical networks and a lesser efficiency of the mechanisms responsible for the detection and transduction of these covariance changes[24,30], might reduce the probability of cortical synapses reaching the threshold for functional plasticity.

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Removal of phosphorylation sites from the β_2-adrenergic receptor delays onset of agonist-promoted desensitization

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Eukaryotic cells have evolved a variety of mechanisms for dampening their responsiveness to hormonal stimulation in the face of sustained activation. The mechanisms for such processes, collectively referred to as desensitization, often involve alterations in the properties and number of cell-surface hormone receptors[1-3]. It has been speculated that phosphorylation–dephosphorylation reactions, which are known to regulate the catalytic activities of enzymes, also regulate the function of receptors[4]. Highly specific receptor kinases, such as rhodopsin kinase[5] and β-adrenergic receptor kinase[6], which show stimulus-dependent phosphorylation of receptors have been described. Direct evidence for a causal relationship between receptor phosphorylation and desensitization has been lacking however. Here we report that prevention of agonist-stimulated β_2-adrenergic receptor (β_2AR) phosphorylation by truncation of its serine and threonine-rich phosphate acceptor segment delays the onset of desensitization. We also show that selective replacement of these serine and theronine residues by alanine and glycine delays desensitization even further. These data provide the first direct evidence that one molecular mechanism of desensitization of G-protein-coupled receptors involves their agonist-induced phosphorylation.

Figure 1*a* depicts how G-protein coupled receptors, in this case the human β_2AR, may be organized within the plasma membrane. Such proteins are thought to cross the membrane seven times[7-9]. Light-dependent phosphorylation of a homologous G-protein coupled receptor, the visual pigment rhodopsin, by an enzyme termed rhodopsin kinase, occurs on multiple serine and threonine residues at its carboxyl terminus[10-12]. A similar concentration of serine and theronine residues at the β_2AR carboxyl terminus has been proposed as a target for agonist-promoted phosphorylation[8].

To evaluate the role of phosphorylation in desensitization we constructed a mutant human β_2AR complementary DNA encoding a protein truncated after amino-acid residue 365 (T-365). As shown in Fig. 1*a*, this mutant lacks most of the serine and threonine-rich carboxyl segment of the receptor. When expressed in Chinese hamster fibroblast CHW cells, both wild-type and mutant (T-365) β_2AR bound the specific βAR ligand ^{125}I-cyanopindolol (^{125}I-CYP) with high affinity ($K_D \approx 40$ pM) and appropriate β_2AR specificity (data not shown). Moreover, photoaffinity labelling of both receptors in the CHW cells confirmed their expected mobility[13] on SDS-PAGE (Fig. 1*b*).

To determine whether T-365 undergoes agonist-promoted phosphorylation, we equilibrated CHW cells expressing wild-type or T-365 β_2AR with $^{32}P_i$, exposed them to the β-agonist isoprenaline (2 μM) for 15 min and then purified the βARs after solubilization by affinity chromatography on alprenolol-Sepharose. The results are shown in Fig. 2. The levels of basal phosphorylation (apparent stoichiometries, see Fig. 2 legend) of the wild-type and mutant receptor were essentially identical. As previously shown in a variety of cell systems[4], phosphorylation of the wild-type β_2AR increased 2-3 fold when cells were exposed to the agonist isoprenaline (Fig. 2*a*). In contrast, no agonist-promoted increase in phosphorylation of the truncated receptor was observed at any time point investigated (15 min,

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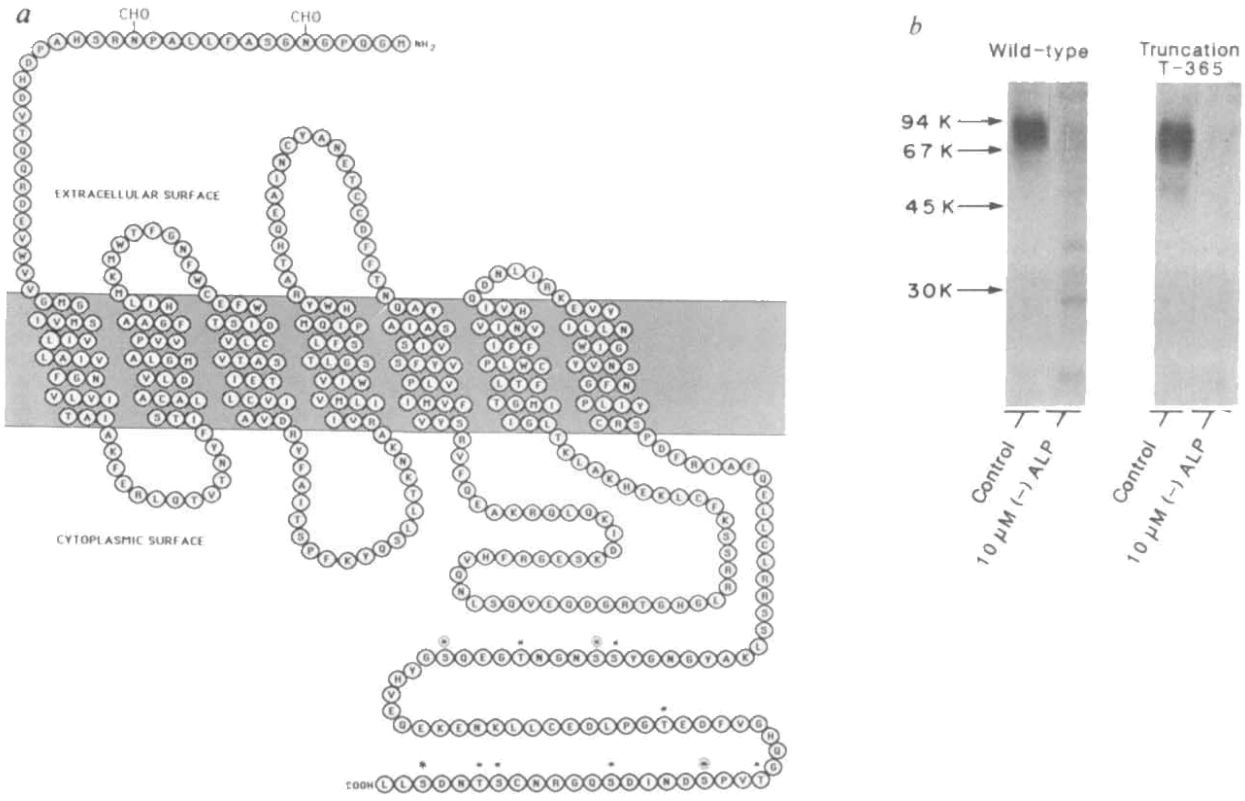

Fig. 1 *a*, Membrane topography of the wild-type and mutant human β_2-adrenergic receptors predicted from hydropathicity analysis of the deduced amino-acid sequence[19]. The arrow indicates the site of truncation of the mutant T-365, and asterisks indicate substituted amino acids in S-351 (*, alanine, ⊛, glycine). *b*, Photoaffinity labelling of β_2AR in membranes from CHW cells expressing wild-type (left panel) or T-365 (right panel) receptors.

Methods. To make the truncation mutant, wild-type β_2AR cDNA was digested with *Eco*RV and religated to a blunt end adapter containing a termination codon and a *Hin*dIII site. The mutation was confirmed by loss of the *Eco*RV and appearance of the *Hin*dIII site. To create S-351, we used an oligonucleotide-directed mutagenesis system (Amersham). The human β_2AR cDNA was cloned into the *Eco*RI-*Hin*dIII sites of the plasmid PTZ (Pharmacia) to enable preparation of single-stranded DNA template; mutagenesis was confirmed by sequencing. The wild-type and mutated human β_2AR cDNAs, containing respectively 190 and 45 bp of 5′-untranslated sequence, were cloned into the eukaryotic expression vectors pKSV10 (Pharmacia) or pBC 12MI (ref. 20). The resulting plasmids were used with pSVNeo to cotransfect CHW cells by calcium phosphate precipitation[21]. Following selection, the clones expressing β_2ARs were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum containing 150 mg ml^{-1} geneticin. Several cell lines expressing the wild-type T-365 and S-351 β_2ARs were generated and those expressing a similar number of receptors, (~1.5 pmol mg^{-1} membrane protein) were selected. Essentially identical EC_{50} concentrations (126 ± 13 nM, 134 ± 14 nM and 121 ± 16 nM respectively) for isoprenaline stimulation of adenylyl cyclase were observed for wild-type, T-365 and S-351 receptors. Characterization of the parental CHW cells and CHW cells expressing wild-type human β_2AR has been published elsewhere[13]. For photoaffinity labelling, membranes were incubated in the dark with [^{125}I]iodocyanopindolol diazarine (25 pM) in phosphate-buffered saline (PBS) containing 5 mM EDTA, in the presence or absence of 10 μM alprenolol to define specific labelling, for 3 h at 25 °C. Following three washes with PBS-EDTA the membranes were resuspended in 1 ml of PBS-EDTA and UV-irradiated with a Hanovia 450 W medium pressure mercury lamp for 5 min. Autoradiograms of samples run on 12% SDS-PAGE (ref. 22) are shown. Results are representative of two separate experiments.

Fig. 2*b*; 5 min, 2 h, 5 h, data not shown).

These results indicated that truncation of the receptor prevents its agonist-induced phosphorylation; we therefore sought to determine the effect on agonist-promoted desensitization. We exposed wild-type and mutant receptor-containing CHW cells to 2 μM isoprenaline for 2–180 min and examined the effect on subsequent isoprenaline-stimulated adenylyl cyclase activity in a cell membrane fraction and the extent to which receptors were internalized. When the wild-type receptors were exposed to agonist, desensitization was apparent even at the earliest time point studied (2 min). This continued to evolve at 10, 60 and 180 min and involved both a decrease in the maximum isoprenaline-stimulated activity, and a rightward shift in the agonist concentration response curve (Fig. 3*a*). No reduction in basal, NaF- and prostaglandin E_1-stimulated adenylyl cyclase activities was observed at 10 min (data not shown), indicating that the early desensitization is agonist-specific (homologous). Similar early desensitization was observed at 2, 5 and 10 min using the human adenocarcinoma cell line A431 which naturally expresses β_2AR (data not shown).

In contrast, no such early desensitization (2 and 10 min) was observed in experiments with the truncated receptor (Fig. 3*b*). By 60 min, however, cells containing the mutant receptor had become desensitized to the same extent as the cells containing the wild-type receptor (Fig. 3*b*). This delay in the onset of desensitization was observed in each of four different clonal lines of T-365.

Agonist-induced sequestration (internalization) of the β_2AR, however, as assessed by the binding of the hydrophilic ligand [^{3}H]CGP12177 to surface receptors in whole cells, appeared to be amplified in the cells expressing the truncated β_2AR (Fig. 4). The absence of desensitization at 10 min, despite sequestration of ~30% of the receptors in the T-365 cells, suggests that the full contingent of β_2ARs is not required to maximally stimulate adenylyl cyclase in cells containing such a large number of receptors.

Truncation of the serine and threonine-rich carboxyl terminal segment removes ~12% of the β_2AR mass, and might lead to conformational changes in the receptor protein unrelated to the absence of phosphorylation at the carboxyl terminus. Thus, we

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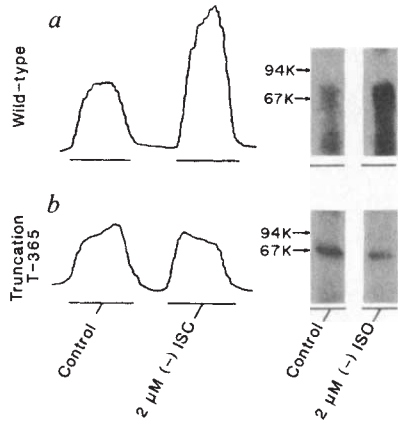

Fig. 2 Whole-cell phosphorylation of the β_2AR in CHW cells expressing wild-type or T-365 β_2AR. Right: autoradiograms of affinity-purified β_2AR solubilized from CHW cells expressing wild-type (*a*) or T-365 (*b*) β_2AR that were prelabelled with carrier-free $^{32}P_i$ and incubated in the presence or absence of isoprenaline (2 μM) for 15 min. The amount of ^{32}P in the excised bands was quantified by liquid scintillation spectrometry (wild-type 3,522 c.p.m. pmol^{-1} in control and 8,645 c.p.m. pmol^{-1} in isoprenaline exposed cells; T365, 3,285 c.p.m. pmol^{-1} in control and 2,934 c.p.m. pmol^{-1} in isoprenaline exposed cells). Left: densitometric scans of the autoradiograms corrected for the amount of β_2AR (as determined by ^{125}I-CYP binding) loaded in each lane (*a*, lanes 1 and 2, 25 fmol each; *b*, lanes 1 and 2, 40 fmol each). This figure is representative of four experiments.

Methods. Nearly confluent cells expressing either wild-type or T-365 β_2AR were detached from flasks by treatment with collagenase (1 mg ml^{-1}) containing soybean trypsin inhibitor (0.05 mg ml^{-1}) for 60 min at 37°. Following a thorough washing, the cells were resuspended in phosphate-free DMEM and incubated with carrier-free $^{32}P_i$ (0.25 mCi ml^{-1} of cell suspension) at 37° for 60 min to allow labelling of the cellular ATP pool. The cells were then exposed to isoprenaline (2 μM) for 15 min and the incubation was terminated by centrifugation at 200*g*. After washing with ice-cold phosphate-free DMEM, the cells were disrupted and plasma membranes prepared as described[13]. The β_2AR was then solubilized with digitonin (2%) and purified by alprenolol-Sepharose affinity chromatography as described[23].

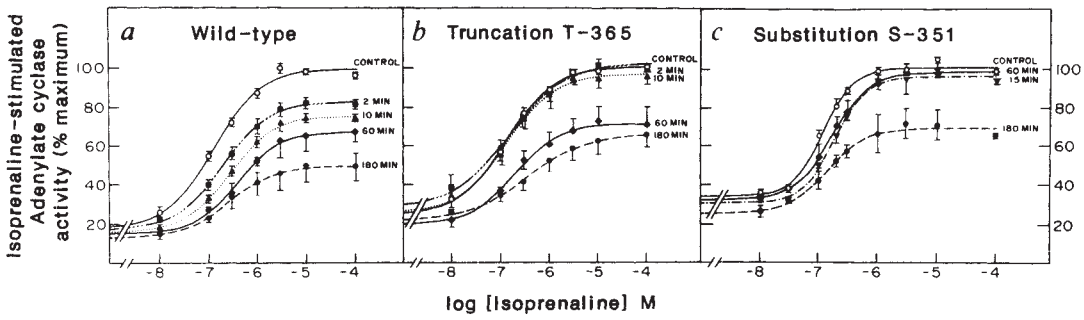

Fig. 3 Effects of isoprenaline pretreatment of β_2AR-containing CHW cells on isoprenaline-stimulated adenylyl cyclase activity. CHW cells expressing the wild type (*a*) the T-365 (*b*) or the S-351 β_2AR (c) were incubated in the absence (control) or presence of isoprenaline (2 μM) for 2–180 min, and the ability of increasing concentrations of isoprenaline to stimulate adenylyl cyclase in membranes derived from these cells was subsequently tested. The data shown are the mean of the following number of experiments: (control: wild-type $n = 13$; T-365 $n = 10$; S-351 $n = 5$). (2 min: wild-type $n = 8$; T-365 $n = 5$. 10 min: wild-type $n = 10$; T-365 $n = 8$. 15 min: S-351 $n = 6$. 60 min: wild-type $n = 5$; T-365 $n = 3$; S-351 $n = 5$. 180 min: wild-type $n = 4$; T-365 $n = 2$; S-351 $n = 3$.) The absolute (pmol min^{-1} mg^{-1}) basal and maximal isoprenaline-stimulated adenylyl cyclase activity were: (wild-type: basal activity: control, 9.3 ± 1.1; 2 min, 10.2 ± 1.2; 10 min, 9.2 ± 1.2; 60 min, 6.6 ± 1.2; 180 min, 6.1 ± 1.8. Maximum isoprenaline stimulation: 54.0 ± 6.8) (T-365: basal activity: control, 8.7 ± 1.5; 2 min, 12.1 ± 1.5; 10 min, 9.2 ± 1.2; 60 min, 4.8 ± 1.4; 180 min, 7.9 ± 1.2. Maximum isoprenaline stimulation, 29.0 ± 4.3) (S-351: basal activity: control, 23 ± 2.3; 15 min, 22.3 ± 3.6; 60 min, 21.5 ± 1.6; 180 min, 12.8 ± 1.8. Maximum isoprenaline stimulation: 68.2 ± 8.9). Values are expressed as the mean ± s.e.m..

Methods. Isoprenaline (2 μM) was added to the cells with fresh DMEM containing 10% fetal calf serum and the cells were incubated at 37 °C for the indicated period of time. The cells were then detached by rapid scraping and plasma membranes were prepared as described[13]. Adenylyl cyclase activity was determined by the method of Salomon *et al.*[24] and expressed as % ± s.e.m. of the maximum stimulation in the control.

examined the pattern of agonist-induced desensitization of a β_2AR mutant with a full length carboxyl terminus, but in which the potential phosphate acceptor serines and threonines (a total of 11) had been mutated to alanine or glycine (S-351, Fig. 1*a*). As shown in Fig. 3*c*, in cells containing such a mutant receptor no desensitization was observed following exposure to isoprenaline for 15 min and 60 min. By 180 min, however, these cells did show significant desensitization. Agonist-induced sequestration of S-351 was identical to that observed in the cells expressing the wild-type receptor (data not shown).

The data described here represent the most direct evidence yet developed for a causal relationship between agonist-promoted phosphorylation of the β_2AR and its desensitization. In addition, these results indicate that the serine and threonine-rich carboxyl terminus of β_2AR is a major site of agonist-promoted phosphorylation in whole cells. This segment of the receptor has been proposed as the target of the β-adrenergic receptor kinase *in vitro*[8] and thus this enzyme is strongly implicated as being responsible for the early agonist-promoted phosphorylation of the receptor in whole cells.

Our data further show, in agreement with several other lines of evidence[14,15], that the early phase of homologous desensitization can be distinguished from the sequestration of the receptor. Indeed, in cells expressing T-365 or S-351 in which the onset of desensitization is significantly delayed, the sequestration was either greater than, or identical to, that observed in cells expressing the wild-type receptor. Agonist-induced phosphorylation of the receptor at the carboxyl terminus, therefore, does not appear to be essential for sequestration. The biological significance of the amplified sequestration in T-365, however, which is in contrast with a previous report[16] that a very similar mutant sequestered normally when expressed in mouse L cells, remains to be determined.

Desensitization is delayed rather than abolished in the mutants lacking the carboxy-terminal phosphorylation sites. Thus, additional mechanisms such as receptor sequestration, heterologous desensitization or binding of a putative arrestin-like molecule to the receptor[17] may contribute to the more slowly evolving adaptation. Alternatively, phosphorylation may modulate the rate rather than the extent of desensitization. Such an effect has been proposed for the nicotinic cholinergic receptor where phosphorylation of the receptor-channel by the cAMP-dependent protein kinase *in vitro* enhances the rate at which the channel desensitizes[18].

Parallels can also be drawn between our data and the recently suggested involvement of the serine and threonine-rich carboxyl terminus in the adaptation processes of yet another putative member of the G-protein-coupled receptor family. Mutants of the yeast α-mating factor receptor, lacking the carboxyl terminus, display a supersensitive phenotype with regard to the cell division arrest normally induced by the α-factor (Remeke, J. and Thorner, J., manuscript in preparation). Such truncation

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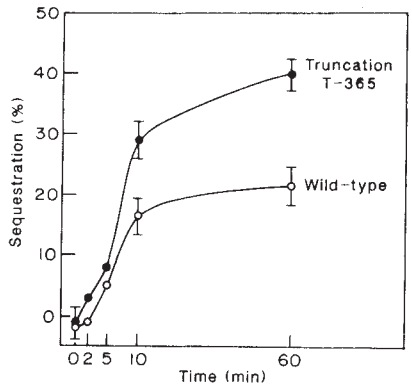

Fig. 4 Isoprenaline-induced sequestration of β2AR in CHW cells expressing wild-type or T-365 β_2AR. Cells were incubated with isoprenaline (2 μM) at 37° for the indicated times, extensively washed and detached by rapid scraping with cold PBS as described[13]. A trypan-blue exclusion test showed that >95% of the cells were intact following scraping. Two different aliquots from the same flask were used for measuring total and surface receptors. Total receptors were determined by [^{125}I]iodopindolol (~150 pM) binding at 37° for 60 min. Surface receptors were determined by [^{3}H]-CGP 12177 (~3 nM) binding at 4 °C for 18 h[25]. Nonspecific binding was defined in the presence of 1 μM (−) propranolol. The % of sequestration was calculated as: [1 − (surface receptors ÷ total receptors)] × 100. The total number of cell receptors did not change over the time course shown. Following 3 h isoprenaline treatment (not shown) a decrease in total cell receptors of ~25% was observed in both wild-type and T-365 containing cells. The data with error bars represent the mean ± s.e.m. of four separate experiments. The data without error bars represent the mean of two separate experiments.

mutants are thus resistant to the 'desensitization' ordinarily induced by the mating factor. Such analogies suggest that receptor phosphorylation on carboxy-terminal cytoplasmic domains, probably mediated by receptor-specific kinases, may be of very broad regulatory significance.

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Conformational changes associated with ion permeation in L-type calcium channels

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The mechanism by which ions deliver their message to effector proteins involves a change in the protein conformation which is induced by the specific interaction of the ion with its binding site on the protein. In the case of an ion-channel protein, conformational changes induced by permeant ions and the consequences for channel function have received little attention. Here we report that binding of permeant cations to an intra-channel binding site of the dihydropyridine (DHP)-sensitive (L-type) Ca^{2+} channel leads to a conformational change which destabilizes the protonated state of a group on the external channel surface, and can shift its apparent *p*K value by more than 2 *p*H units. The lifetime of the protonated state correlates with the occupancy of an intra-channel binding site by the permeant cation. The demonstration of such conformational changes in a channel protein induced by the permeant ion has important implications for realistic models of the mechanism of ion permeation.

We have recently demonstrated that protonation of a site located at the external surface of the L-type Ca channel greatly reduces the channel conductance when Na^+ ions carry the current in the absence of divalent ions[1]. When 110 mM Ba^{2+} is the charge carrier, however, the conductance of L-type Ca channels is little affected between values of external *p*H (pH_0) of 6-9, suggesting a correlation between the protonation reaction and the species of permeant ion. Figure 1 shows that this is indeed the case, and that the protonation kinetics are vastly different even when three monovalent charge carriers are compared. The top traces in Fig. 1 show the typical elementary currents of L-type Ca channels in the presence of the DHP Ca channel agonist (+)-(S)-202-791 (refs 2, 3) when Cs^+ is the charge carrier. The opening of the channel is followed by transitions between two different conducting levels. Our interpretation[1] that these transitions reflect binding and unbinding of individual protons at a single protonation site is supported by the finding that the mean time the channel spends at the high conductance level decreases linearly with increasing H^+ concentration (ref. 1 and unpublished data). A similar pattern of single channel currents is seen when K^+ is the charge carrier (Fig. 1, middle traces), but the lifetime of the low conductance state is shorter than with Cs^+. With Na^+ as the charge carrier (Fig. 1, bottom traces), transitions between the two levels are so fast that they can no longer be resolved at the bandwidth of our recording system (5 kHz) and become apparent as large open channel noise[1]. The pH_0-values for the traces in Fig. 1 were chosen such that with each permeant ion the open channel spends approximately equal time in the protonated and unprotonated state, as seen most directly from the similar peaks at the two levels in the amplitude histogram for each ion (Fig. 1). Therefore the apparent *p*K of the site is close to the pH_0 in each ionic condition shown, and it is clear that the *p*K shifts by more than 1 *p*H unit as Na^+ replaces Cs^+ as the permeant ion.

A plot of the protonation and deprotonation rate constants with each of the three ions (Fig. 2*a*) shows that the shift of the *p*K value results mainly from differences in the deprotonation rate constant ($k_{off,H}$). The average time the channels spend at the low conductance level decreases from 480 μsec in the presence of Cs^+ to 60 μsec when Na^+ is the charge carrier.

Because the L-type Ca channel distinguishes between permeant ions primarily on the basis of the ion's affinity to intra-

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