# Chapter 4. Unnatural amino acids with caged side chains

# 4.1 Introduction

# 4.1.1 Caged compounds

The use of masks is practically universal, from the Halloween celebrations of Europe, to Malian *chiwara* dancers, the  $n\bar{o}$  theater of Japan, and the False Face societies of the Iroquois. In the Americas, some of the most dramatic masks were carved by the people of the Northwest Coast. A particular specialty of the Kwakiutl is the intricately worked transformation mask, which is worn over the head of a dancer. At the climactic moment of a dance, the mask is flung open along concealed hinges, effecting a startling transformation of Raven into Sisuitl, a spirit represented by the face of a warrior flanked by coiling serpents. (Figure 4.1)



**Figure 4.1** Kwakiutl transformation mask, which transforms Raven (left) into Sisuitl (right), compared to the photochemical transformation of an unnatural aromatic amino acid side chain (left) into serine (right).<sup>1</sup>

The instantaneous transformation of one material into another is likewise the most dramatic demonstration of chemical change. No popular chemistry demonstration concludes without a bang, a color change, a puff of smoke, a flash of light, a sudden phase transition. Instigating such a transformation with light for more serious purposes has a rather long history in chemical biology. Biologically active compounds may be protected with photo-removable protecting groups, altering important functionality in the molecule so as to block its biological efficacy. The protected compound is often thus referred to as being 'caged' or 'masked.' Irradiation of the system ('de-caging') removes the protecting groups and restores the intrinsic efficacy of the molecule.

This nomenclature disturbs some chemists, as the term is reserved in chemistry to describe solvation effects on reactivity, leading to the proposal that these compounds be referred to as 'phototriggered' rather than 'caged.'<sup>2</sup> However, the term 'caged' is superior grammatically and has found favor among biologists. The term will be used throughout this work, along with 'masked' and occasionally 'phototriggered.'

Classically, caged compounds have been used in biology for rapidly initiating processes which depend on small molecules.<sup>3-6</sup> High-resolution kinetic information may sometimes be obtained this way. Caged compounds can also be used because of their ability to bring small molecule-mediated processes under experimental control. A system dependent on a small molecule is primed by the diffusion of caged compounds into the proximity of the active site. Since the caged compounds lack the efficacy of the natural substrate, the process under consideration is held up until experimentally initiated. It is mostly in this latter sense that the compounds described below will be utilized.

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### 4.1.2 Caged amino acids, particularly tyrosine

Caging drugs and bioactive small molecules has led naturally to the idea of caging peptides, many of which have important regulatory roles. Additionally, peptides offer a number of possibilities for caging. Both amines, such as the N-terminus of a peptide, and carboxylates (C-terminus) may be masked by photoactive protecting groups. Amino acid side chains also offer a number of easily caged functional groups. Caged serine, threeonine, and tyrosine have all been reported.<sup>7-9</sup> Cysteine, methionine, aspartate, glutamate, and lysine have also been caged.<sup>10-19</sup> Given the wide range of possibilities for introducing a photo-labile protecting group and the large number of systems where peptide binding has an important role, it is not surprising that large numbers of caged peptides and amino acids have been reported.

Caged tyrosine, in particular, has been employed in a number of studies. Its earliest reported synthesis was in 1996, where the suitability of nitrobenzyl-protected tyrosine for solid-phase peptide synthesis was demonstrated. In this work, caged neuropeptide Y and caged angiotensin II peptides were synthesized. In the case of neuropeptide Y, the presence of a single caged tyrosine residue severely compromised binding to cells expressing the NK2 receptor, whereas no effect was seen for caged angiotensin II relative to the wild-type peptide.<sup>9</sup> More recent work by the laboratory of Jeffery Walker has used caged tyrosine in solid-phase synthesis to generate caged peptides which interact with calmodulin and the myosin light-chain kinase (MLCK). Photolysis of the caged inhibitory peptides in eosinophils caused contraction of these cells, implying an important role for calmodulin and MLCK in the motility of these immune cells.<sup>20</sup>

# 4.1.3 Caged proteins

The advent of techniques to alter the amino acids of proteins has permitted the consideration of 'caged proteins,' in which particular residues in a protein are provided with photo-removable protecting groups. A number of recent reviews have presented a survey of this literature.<sup>21-23</sup> Interesting experiments have been done with such caged proteins, including a number of caged kinases whose activity is dependent on decaging.<sup>23,24</sup> In addition, protein-protein interactions essential to actin polymerization have been disrupted by caging a lysine on F-actin.<sup>15</sup> The laboratory of Hagan Bayley has a long-standing interest in caged proteins and has recently introduced a caged hemolysin which only conducts ions subsequent to photolysis.<sup>25</sup> A caged antibody which only binds upon irradiation has been developed.<sup>26</sup> Both enzymes, such as galactosidase, and proteins involved in signal transduction, such as p21<sup>Ras</sup>, have been caged.<sup>27,28</sup>

The necessity in these experiments for forming the caged protein *in vitro* has meant that investigation of real cellular processes has been difficult. Additionally, of course, techniques relying on modification of reactive side chains in the protein suffer from limited specificity of caging, as differential reactivity of multiple nucleophilic residues can be difficult to guarantee. To solve the problem of specificity, proteins containing caged amino acids may be synthesized through either solid-phase methods, or by semi-synthesis followed by ligation of the unnatural amino acid-containing portion to the remainder of the protein. Still, experiments with these proteins may be performed *in situ* only insofar as modified proteins can be introduced into the cell. Although such techniques are being continually improved, this limitation provides clear incentive for this work to be done using unnatural amino acid mutagenesis, where the protein is

synthesized inside the cell itself.<sup>29</sup> In addition, caging integral membrane proteins remains a particularly challenging case for conventional methods, whereas unnatural amino acid mutagenesis is increasingly well-established for membrane proteins.

# 4.2 Using nonsense suppression to introduce caged amino acids into proteins

### 4.2.1 Expression systems

The first requirement for introducing an unnatural amino acid into a receptor is a viable expression system for that receptor. The majority of experiments employing nonsense suppression as a means of incorporating unnatural amino acids into proteins have relied on bacterial extracts to produce the proteins of interest. More recent efforts have employed bacterial extracts modified to increase suppression efficiency or extracts from eukaryotic systems.<sup>30,31</sup> *In vitro* systems such as wheat germ and rabbit reticulocyte extracts have been utilized for nonsense suppression of functional soluble proteins.<sup>32,34</sup> In our lab, wheat germ extracts are routinely used to generate full-length membrane proteins for PAGE analysis.<sup>35,36</sup> In principle, receptors containing unnatural amino acids could be reconstituted into bilayers using such a system supplemented with microsomes.<sup>37</sup> However, there is no precedent for unnatural amino acid mutagenesis of an integral membrane protein using a cellular extract. Expression in *Xenopus* oocytes is the only method to date that has been shown to be effective for incorporating unnatural amino acids into functional receptors.<sup>38-42</sup>

# 4.2.2 Caging groups

The choice of caged side chain depends, of course, on the exact nature of the information to be gained from the experiment. The literature contains precedents for

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caged side chain hydroxyls (Tyr<sup>43-45</sup> and Ser<sup>46</sup>) thiols (Cys<sup>44</sup>), acids (Asp<sup>47</sup>), amines (βaminoalanine<sup>48</sup>) and amides (Npg<sup>49</sup> in which backbone cleavage can be seen as photolytic "decaging" of the peptide bond). As mentioned above, the suppression efficiency of an unnatural amino acid is related to the nature of its side chain, but in a complex fashion. Most investigators have employed relatively small caging groups, because the charged tRNA must pass through the ribosome in order for the amino acid to be incorporated into the receptor. As will be discussed further, it is difficult to predict whether one will encounter problems in incorporating a particular amino acid. Workers in the field have generally reported that steric and especially charge conservation of the native side chain help promote efficient expression. In practice, there is no substitute for simply attempting the suppression at the desired position with the desired unnatural amino acid.

The only caging groups precedented for unnatural amino acid suppression thus far are nitrobenzyl (Nb)<sup>43-50</sup> and nitroveratryl.<sup>50</sup> Other photoactive protecting groups have very attractive photochemical properties, but it remains to be seen whether or not these groups can be incorporated by nonsense suppression. There are several considerations in choosing a caging group: 1) synthetic characteristics: the degree to which it is synthetically accessible and compatible with coupling to pdCpA; 2) photochemical characteristics: its action spectrum, quantum yield for photolysis, and speed of the dark reactions that complete photolysis; and 3) reactivity in the system of interest: its stability in water, and the possible reactivity of its photoproducts.

### 4.2.3 Side chain uncaging

Various types of photolysis apparatus have been employed for side chain decaging. Experiments involving photoreactive unnatural amino acids such as diazirine, aryl azides, and benzophenone<sup>32,51-55</sup> are also relevant here, as are experiments with caged small molecules in *Xenopus* oocytes.<sup>56,57</sup> In general, methods of photolysis can be divided into those performed on single oocytes and those performed on batches of oocytes.

An apparatus for real-time decaging in single oocytes (Figure 4.2) was designed for the particular needs of these unusually large cells (~ 1 mm diameter).<sup>43-45,58</sup> In most cases, inexpensive arc lamps (either continuous irradiation or pulsed) are preferred over the much more costly lasers if one wishes to illuminate the entire oocyte.<sup>57</sup>



**Figure 4.2** Protein decaging with real-time electrophysiological monitoring. Apparatus for simultaneous irradiation and electrophysiological recording from *Xenopus* oocytes. The output of the Oriel Hg arc lamp is passed through a 300 - 350 nm bandpass filter and focused onto a fiber optic liquid light guide. The light guide directs the beam onto the oocyte, which is clamped in a standard two-electrode configuration. A concave mirror can be moved into position above the bath, reflecting some of the beam that has passed around the oocyte back to the shadowed upper surface. The mirror increases the overall flash intensity by ~50%. The inset depicts the photolytic de-caging of a caged amino acid incorporated into a channel with attendant response. This example indicates incorporation of Tyr(ONb) into an extracellular domain of the protein, where removal of the caging group results in an increased number of active channels.

Methods for decaging proteins expressed in many oocytes at once are rather simpler.<sup>49</sup> In the particular case of Npg decaging, oocytes were irradiated for 4 hours at 4 °C in Pyrex vials with a 288 W Hg lamp equipped with a 360 nm band pass filter at a distance of 15-30 cm.

### 4.2.4 Choice of receptor

Suppression has been demonstrated in the major classes of neuroreceptors and ion channels. Unnatural amino acids have been incorporated into a number of ligand-gated channels, such as the nAChR (numerous subunits) and 5-HT<sub>3</sub> receptors, a G-protein-coupled receptor (NK1), GIRKs, and several potassium channels including Kir2.1 and Shaker.<sup>38,39,42,43,45,49,59-61</sup> In unpublished work from our lab, we have incorporated unnatural amino acids into CFTR, a P2X receptor, and a neurotransmitter transporter. Among these are monomeric and multimeric receptors, both homomeric and heteromeric versions of the latter. We emphasize that heterologous expression of any novel protein is by no means guaranteed, since it is uncertain whether the protein will be folded, assembled, or transported properly. However, our experience suggests that any protein that can be expressed effectively in *Xenopus* oocytes will be amenable to incorporation of unnatural amino acids by nonsense suppression.

# 4.2.5 Assay

Finally, a fundamental methodological requirement for unnatural amino acid mutagenesis is an assay capable of detecting the effects of photolyzing a caged side chain. In fact, one of the reasons that receptors are such an attractive target for studies of this nature is that electrophysiology may be employed for this purpose. Electrophysiology is exquisitely sensitive and therefore capable of detecting the very small amounts of protein generated by nonsense suppression. To date, only two-electrode voltage clamp recordings have been made with receptors containing caged amino acids; but single-channel recordings are in principle possible. In addition, electrophysiology has been successfully coupled with irradiation in a number of experiments.<sup>56,57</sup>

Other methods may be employed to detect downstream effects of side-chain decaging. There is ample precedent for most biochemical methods on receptors expressed in oocytes.<sup>62</sup> However, given that small amounts of protein are produced in nonsense suppression experiments, these methods often require some adaptation for use. The literature is not terribly extensive, but there are examples on non-electrophysiological techniques that have been successful in the analysis of the effects of single side chain decaging, some of which are presented below.

# 4.2.6 Precedents for using nonsense suppression to introduce caged amino acids

Unnatural amino acid mutagenesis is an attractive technique for generating caged proteins, because of its absolute site-specificity. The Schultz group was the first to employ the technique for this purpose, caging a critical active-site serine in T4 lysozyme, bringing the activity of the enzyme under photocontrol.<sup>47</sup> Caged aspartate was also used by this laboratory to control protein-protein interactions important to the signaling protein p21<sup>Ras</sup>.<sup>28</sup> A final example from the Schultz laboratory is the use of caged serine to control the intein splicing of a DNA polymerase.<sup>46</sup> *In vitro* nonsense suppression has been used to cage aspartate residues in an attempt to bring the dimerization of HIV protease under experimental control.<sup>50</sup> Again, these experiments are somewhat limited in scope by the

fact that the caged protein would have to be introduced into a cell to truly examine a biologically relevant interaction.

*In vivo* unnatural amino acid mutagenesis provides a means to solve both the problem of specificity and biological context of the caged protein. Experiments on nitrobenzyl-protected tyrosine residues have been carried out in this laboratory on the nAChR.<sup>43</sup> Particular tyrosine residues shown to be important in agonist binding were introduced into the receptor by unnatural amino acid mutagenesis in *Xenopus* oocytes. The presence of the caging groups was shown to impair the response of the receptor to acetylcholine. Upon removal of the protecting groups by irradiation of the oocyte, wild-type reactivity was restored. Using this system, important kinetic and optical parameters of caged tyrosine residues were incorporated in an extracellular region of the protein. The experiments reported in Chapter 3 with incorporation of caged cysteine and tyrosine into the M2 transmembrane domain of the nAChR are an example of caged amino acids incorporated into membrane-resident protein domains.

The effectiveness of caged tyrosine in both of these contexts led to a consideration of the use of caged tyrosine to control the accessibility of this particular residue in a functioning ion channel.

# 4.2.7 Application of caged tyrosine to ion channels

Ion channels represent the first-line class of signal-transducing molecules. They are resident in the plasma membrane and are thus in intimate contact with the extracellular environment. In addition, of course, functioning ion channels are an imperative of earthly life, as any non-viral organism must maintain an ion gradient to power its metabolic processes. As a result of these critical functions, ion channels are tightly regulated. All of a cell's regulatory mechanisms are probably brought to bear upon ion channels, including transcriptional control; translational control; RNA editing; post-translational modification of all varieties; dynamic insertion into and removal from the membrane; functional modulation by ligand binding, pressure, light, heat, interaction with other proteins, changes in membrane voltage, proteolysis, etc.

Phosphorylation is a modification that is presumed to be central to ion channel regulation. Surprisingly, however, there is remarkably little evidence that a specific residue in an ion channel under a particular circumstance at a given time has been phosphorylated. The technical barriers inherent in obtaining this information are steep. Any assay to determine whether or not a single residue has been phosphorylated must be extremely sensitive. Since the phosphate linkage is biologically labile (due to enzyme activity, if not metal-promoted hydrolysis), the analyte itself is delicate. Ion channels are integral membrane proteins, and isolating them for analysis is difficult, particularly under the required conditions of sensitivity and gentleness. Finally, the temporal sensitivity to determine whether a functional effect is caused by phosphorylation of a single residue is simply unattainable, although the following chapter will present a technique for perhaps achieving it.

Despite all of this, there are numerous reports of the direct detection of ion channel phosphorylation.<sup>63-68</sup> There are a few which show serine or threonine phosphorylation, but the bulk involve tyrosine phosphorylation. Partly, this could be because tyrosine phosphorylation may have unique importance in mediating protein-protein interactions. But it also reflects the existence of antibodies against phosphotyrosine which do not exist

for phosphoserine and phosphothreonine. In the work presented in this chapter, tyrosine phosphorylation will be the sole focus.

### 4.3 Incorporation of caged tyrosine into the potassium channel Kir2.1

### 4.3.1 Introduction

There is ample evidence to suggest that the activity of ion channels is modulated by the same regulatory mechanisms which operate on other signal transduction pathways. Generally speaking, modulation may be accomplished by modification of the channel itself or through the interaction of other proteins with the channel. Phosphorylation has been mentioned, where a charged phosphoryl group is transferred to a serine, threonine, or tyrosine side chain. An example of the latter mechanism is endocytosis. Through endocytosis, channel activity may be regulated by removal of channels from the cell surface, a process mediated through non-covalent interactions with proteins of the endocytotic pathway. These two fundamental kinds of mechanisms are by no means exclusive.<sup>69-71</sup> Covalent modification by phosphorylation induces conformational changes, which may directly affect the conducting pathway. Alternatively, phosphorylation may regulate the binding of modulatory proteins to ion channels, as it does with G-protein coupled receptors.<sup>72,73</sup>

As an example of the complications which arise from interaction among regulatory pathways, endocytosis is an interesting case. The rules whereby membrane proteins are targeted for endocytosis are not yet completely understood. One targeting motif for which there is a significant body of biochemical evidence is the tyrosine-based endocytosis motif, YXX $\Phi$ , where X may be any amino acid and  $\Phi$  is a hydrophobic amino acid, typically L, F, or M.<sup>74</sup> Tyrosine, however, is a residue which may be phosphorylated by tyrosine kinases. It is likely the case that the phosphorylation state of a channel governs whether or not it is able to be targeted for endocytosis. Furthermore, both phosphorylation and endocytosis are precedented in the literature as mechanisms of ion channel modulation. It would appear, then, that the pathways regulating ion channel modulation are likely to be tightly coupled. In order to determine the molecular-level details of ion channel modulation, it is essential to be able to distinguish the contributions of the various regulatory pathways. We introduce here an experimental technique which allows for making these distinctions. A caged form of tyrosine, a residue which plays a special role in recognition events leading to both phosphorylation and endocytosis, was incorporated into a potassium channel known to be sensitive to tyrosine phosphorylation. (Figure 4.3)



**Figure 4.3** General schematic depicting the caging of an intracellular tyrosine residue in the potassium channel Kir2.1. The tyrosine residue of interest is indicated in red, and the caging group in blue.

Ion channels which have been found to respond to modification by tyrosine kinases include the potassium channel Kir2.1, a member of the family of inwardly rectifying K<sup>+</sup> channels with the important biological function of maintaining membrane potential.<sup>75,76</sup> As the name implies, these channels pass potassium only in the inward direction. They are, in fact, always open and have no apparent gating mechanism, other than the voltage-dependence of their rectification. Their physiological role is often described by analogy

to the latch on a drawer. These channels do permit some outward K<sup>+</sup> flow within several mV of  $E_{\rm K}$ , which allow the cell's membrane potential to approach  $E_{\rm K}$ . However, once V<sub>m</sub> becomes more negative than  $E_{\rm K}$ , these channels no longer pass K<sup>+</sup>. The latch having been released, the cell's V<sub>m</sub> is free to change, with inward rectifiers' ability to allow K<sup>+</sup> into the cell under hyperpolarizing conditions contributing to the return to resting V<sub>m</sub>. Kir2.1 is a particular isoform of inward rectifier found in a variety of tissues in the periphery and CNS.<sup>75</sup> Kir2.1 currents have been found to be strongly inhibited by tyrosine phosphorylation. Mutation of a single tyrosine residue to phenylalanine abolishes this inhibition.<sup>76</sup> Interestingly, this intracellular C-terminal tyrosine (Y242) is also part of a YXX $\Phi$  tyrosine-based endocytosis motif. Such a motif has been demonstrated to regulate the expression of the ENaC sodium channel in oocytes.<sup>77</sup>



**Figure 4.4** Schematic showing the chemistry whereby wild-type tyrosine is revealed by photolysis of Tyr(ONb).

By the nonsense codon suppression method, we have introduced the caged tyrosine analog *O*-nitrobenzyl tyrosine, Tyr(ONb), into Kir2.1 at the critical Y242 position. As introduced, the protected tyrosyl oxygen is incapable of acting as a tyrosine kinase substrate. Nor, based on crystallographic evidence, would it appear that tyrosine protected in this way would permit binding by components of the clathrin-mediated endocytotic pathway.<sup>78</sup> However, UV irradiation of the protein releases the nitrobenzyl protecting group, revealing the wild-type tyrosine residue. (Figure 4.4) Restoration of the native side chain, of course, permits both phosphorylation and/or recognition by the

endocytotic machinery. This technique thus brings the initiation of phosphorylation and endocytosis under some degree of experimental control. The experiments reported herein distinguish the contribution of phosphorylation from that of endocytosis, revealing important details about an interaction which may be fundamental to the regulation of ion channels *in vivo*.

### 4.3.2 Results

### 4.3.2.1 Kir2.1 currents are decreased by tyrosine kinase

Initially, we verified that the inwardly rectifying potassium channel Kir2.1 from *Mus musculus* is modulated by tyrosine phosphorylation under the present experimental conditions.<sup>76</sup> *Xenopus* oocytes expressing Kir2.1 were voltage clamped at 0 mV in a recording solution containing 96 mM KCl. Potassium currents elicited by a voltage step to a test potential of -80 mV were recorded. Table 4.1 shows that oocytes expressing Kir2.1 gave robust  $13.3 \pm 1.2 \,\mu$ A whole-cell potassium currents after 24 hours. Oocytes treated with the tyrosine phosphatase inhibitor phenylarsine oxide (PAO)<sup>79</sup> showed no reduction of current, giving whole-cell potassium currents of  $13.1 \pm 1.4 \,\mu$ A. However, oocytes co-injected with v-Src gave an average current of  $7.8 \pm 0.3 \,\mu$ A, a 57% current reduction relative to those injected with Kir2.1 alone. This effect was enhanced by treatment with PAO; oocytes co-injected with v-Src and treated with PAO gave average currents of only  $5.7 \pm 0.6 \,\mu$ A. Indeed, co-injection with v-Src alone did not always yield significant current reduction. This is consistent with results from other laboratories.<sup>80</sup> Although there appeared to be no strict requirement for the presence of phosphatase

Treatment	Mean current ( $\mu$ A±SEM)	n
-	13.3±1.2	5
PAO	13.1±1.4	5
v-Src	7.8±0.3	5
v-Src + PAO	5.7±0.6	5
PYK-2	12.8±0.5	5
PYK-2 + PAO	6.9±0.8	5
T.L. 41 A		

inhibitors, the phosphatase inhibitor PAO was always added for consistency between experiments.

**Table 4.1** Averaged whole-cell currents for Kir2.1.

The proline-rich tyrosine kinase PyK2 gave results similar to v-Src. 48% current reduction was measured upon PAO treatment of oocytes co-injected with PyK2 (Table 4.1). In this case, co-injection of the kinase without phosphatase inhibitor was not sufficient to cause current reduction: oocytes co-expressing the channel with PyK2 gave  $12.8 \pm 0.5 \mu A$ .

Table 4.2 confirms that Y242 is critical for the effect of tyrosine kinases. The tyrosine residue at position 242 was changed to phenylalanine by site-directed mutagenesis. Oocytes expressing the Kir2.1-Y242F construct were unaffected by treatment with the tyrosine phosphatase inhibitor PAO and/or tyrosine kinases. For instance, untreated oocytes expressing the Y242F construct gave average whole-cell K<sup>+</sup> currents of  $31.4 \pm 2.7 \mu$ A.; oocytes co-expressing v-Src and treated with PAO gave currents of  $31.2 \pm 0.9 \mu$ A. The overall expression for the experiment depicted in Table 4.2 was higher than that for the WT Kir2.1, which represents imprecision in mRNA quantitation or batch-dependent expression differences among oocytes. There were no consistent differences in expression level between the WT (WT) and Y242F constructs. In other experiments on Y242F at expression levels comparable to those of Table 4.1, we

confirmed that no combination of v-Src expression and PAO gave significant decreases for this mutant.

Treatment	Mean current ( $\mu$ A±SEM)	n
-	31.4±2.7	6
PAO	34.0±2.0	5
v-Src	30.3±1.8	5
v-Src + PAO	31.2±0.9	4
PYK-2	34.3±2.1	4
PYK-2 + PAO	32.9±3.1	4
Table 4.2 Assume and sub-st	a call automatic for Kin2 1 V242E	

**Table 4.2** Averaged whole-cell currents for Kir2.1 Y242F.

For both constructs, similar but less extensive results on the inhibition of K<sup>+</sup> currents were obtained with the tyrosine phosphatase inhibitor pervanadate substituting for PAO. Similar data were also obtained when the v-Src cRNA injection was omitted but oocytes were injected with TrkB, a receptor tyrosine kinase, and exposed to BDNF. These data suggest that several different activators of tyrosine phosphorylation can inhibit the function of Kir2.1.

# 4.3.2.2 Photolytic decaging of tyrosine-242 leads to a decrease in current

In order to gain temporal control over the state of the tyrosine side chain at position 242, the unnatural amino acid Tyr(ONb) was incorporated into Kir2.1 at position 242. The nonsense codon TAG was introduced using site-directed mutagenesis. mRNA encoding the Kir2.1-Y242TAG construct was generated by *in vitro* transcription. This message was co-injected into *Xenopus* oocytes along with the nonsense suppressor tRNA which had been chemically charged with the synthetic amino acid Tyr(ONb). In order to verify that functional protein was produced by this procedure and to assess the effect of decaging the caged tyrosine residue, oocytes were studied as in the experiments of Table 4.3. As in most unnatural amino-acid experiments to date, current measurements show that expression levels are rather lower—in this case by a factor of 5 to 10—than the

levels for WT channel or for conventional site-directed mutagenesis (compare the expression levels in Table 4.1 and Table 4.2 above). UV light was delivered to oocytes for 3 s.<sup>43</sup> Figure 4.5 presents normalized current values averaged over several oocytes.

v-Src	hν	Percent decrease±SEM
-	+	5.0±1.5
+	-	1.5±0.9
+	+	32±0.9

**Table 4.3** Normalized average whole-cell current decrease of oocytes expressing Kir2.1 Y242TAG suppressed with Tyr(ONb) thirty minutes after initiation of experiment (n=5).

Control oocytes were injected with Kir2.1-Y242TAG mRNA and tRNA-Tyr(ONb) but not with v-Src mRNA. Current from these oocytes decreased by only  $5.0 \pm 1.5\%$  over the 30 min interval, showing that irradiation itself had only a small effect on Kir2.1 currents. In a second control, whole-cell currents were measured in non-irradiated oocytes co-expressing Kir2.1 containing Tyr(ONb) and v-Src. After 30 min, the average current decrease was only  $1.5 \pm 0.9\%$ . This is consistent with the supposition that while the nitrobenzyl protecting group remains, the residue at 242 is unavailable as a substrate for interaction with other proteins—either tyrosine kinases or adaptor proteins. Importantly, oocytes co-expressing Kir2.1 and v-Src, treated with PAO, and irradiated showed an average current decrease of  $32.0 \pm 0.9\%$  30 min after irradiation. This shows that the decaging of Kir2.1-Y242 under conditions favoring tyrosine phosphorylation leads to a large decrease in current.



**Figure 4.5** Inhibition of current in oocytes expressing Kir2.1-Y242TAG suppressed with Tyr(ONb). Averaged normalized data from batches of oocytes recorded at t = 30 min Average values (and ranges) for 100% current were 8.7 (5.5 to 13), 6.5 (4.5 to 10.4), and 6.6 (1.6 to 15.5)  $\mu$ A for the three groups (left to right). Error bars indicate SEM, n = 4 to 6 oocytes.

Although these data qualitatively agree with the data for the WT and Y242F construct, present limitations of the nonsense suppression technique prevent direct quantitative comparisons. First, suppression is incomplete, leading to a substantial proportion of Kir2.1 subunits truncated at the 242 position. To control for this effect, we expressed the Y242TAG construct alone; there were no currents. When we co-expressed Y242TAG and WT channels, we saw currents whose modulation properties were like those depicted in the experiment of Table 4.1. Therefore, dominant negative effects due to truncated subunits probably play no role in our experiments. Second, read-through may occur, so that natural residues would be incorporated at position 242. It is therefore possible that some channels would contain one or more Kir2.1 subunits with natural residues. Although we know that a channel with four Y242F subunits does not respond to v-Src and PAO (Table 4.2), we do not know how modulation would proceed for channels with varying numbers of subunits containing one or more of the other 18 natural

amino acid residues. In the unlikely event that read-through produces a channel with tyrosine at position 242 in one or more subunits, the modification has presumably occurred during the PAO incubation period (> 30 min). We assume that the general effect of read-through would be to decrease the modulatory effect of irradiation.

### 4.3.2.3 Decaying tyrosine-242 also causes a capacitance decrease

Importantly, Table 4.4 shows that the current decrease observed in oocytes expressing v-Src and Kir2.1-Y242-Tyr(ONb) was accompanied by a significant capacitance decrease. The change in capacitance is a direct measure of net endocytotic activity of a cell (i.e. exocytosis minus endocytosis). In both control cases where irradiation of oocytes caused no current inhibition, the capacitance remained essentially constant over the length of the experiment. Thus, the correlation between current and capacitance decrease is clear. (Figure 4.6)



**Figure 4.6** Decrease of capacitance in oocytes expressing Kir2.1-Y242TAG suppressed with Tyr(ONb). Average values (and ranges) for 100% capacitance were 190 (159 to 227), 187 (167 to 202), and 194 (132 to 234) nF for the three groups (left to right). Error bars indicate SEM, n = 4 to 6 oocytes.

Control oocytes that were irradiated without v-Src co-expression showed an overall capacitance decrease of only  $2.8 \pm 0.8\%$  30 min after irradiation. The second control, in which oocytes co-expressing Kir2.1 with caged tyrosine and v-Src were studied without irradiation, also showed little capacitance decrease  $(3.0 \pm 0.4\%)$  over the time course of recording. In contrast, oocytes expressing v-Src and Kir2.1 in which the Tyr(ONb) residue at 242 is uncaged by irradiation, showed an overall decrease in membrane capacitance of  $14.1 \pm 1.9\%$  over 30 min. This decrease suggests that the membrane surface area has decreased as a result of increased endocytotic activity.

v-Src	hν	Percent decrease±SEM
-	+	2.8±0.8
+	-	3.0±0.4
+	+	14.1±1.9
Table	4.4 Normalized	average whole-cell capacitance decrease of oocytes expressing
Kir2.1Y	242TAG suppress	ed with Tyr(ONB) thirty minutes after initiation of experiment (n=5).

In additional controls, we found no decrease in capacitance when oocytes were injected with v-Src cRNA, treated with PAO, and then irradiated. Therefore the capacitance change does not arise from possible photosensitive processes involving v-Src. In additional controls, oocytes were injected with tRNA-Tyr(ONb) and v-Src, and then treated with PAO. Again, irradiation produced no capacitance change, ruling out possible suppression of stop codons in endogenous proteins as the source of the photosensitivity. Furthermore, there were no changes in capacitance associated with decaging of tyrosine in our previous study of caged tyrosine at the nicotinic acetylcholine receptor. Therefore tyrosine decaging in general does not lead to membrane endocytosis.

Irradiated oocytes co-expressing wild-type Kir2.1 and v-Src showed no decrease over the time course of the experiment. Presumably, the wild-type channel is capable of phosphorylation by v-Src and would perhaps be expected to show a current decrease upon application of PAO. Under certain conditions, current reduction with wild-type Kir2.1 could, in fact, be seen in response to PAO treatment. Oocytes recorded from shortly after addition of the phosphatase inhibitor showed significantly lower maximal currents than those which were untreated. This observation is consistent with the existence of a dynamic equilibrium between kinase and phosphatase activity. Under typical conditions, no special effort was taken to record immediately after phosphatase addition. Consequently, equilibrium was established prior to recording, and oocytes showed an apparent steady-state current. With suppressed proteins, the caged tyrosine provides a controllable switch to initiate a process which occurs on the native protein.

We compared the time course of the current decrease and the capacitance decrease by normalizing to the decrease at 30 min, then interpolating the approximate time to halfcompletion for this decrease. The average values were quite comparable: 11.8 min for current and 11.1 min for capacitance. These data indicate that the capacitance change and the current decrease followed similar time courses. Nonetheless some of our measurements suggest that the capacitance decrease begins after a delay of several minutes. This possible initial delay has not been investigated systematically.

# 4.3.2.4 Dominant negative dynamin eliminates capacitance decrease and partially eliminates current decrease

Capacitance measurements may reflect the contribution of various mechanisms of endocytosis. In order to test specifically whether clathrin-mediated pathways were involved, experiments were performed with a dominant negative dynamin isoform. If Kir2.1 inhibition resulted from clathrin-mediated endocytosis, co-injection of oocytes with dominant negative dynamin (dynamin-K44A)<sup>81,82</sup> at levels capable of blocking

endogenous dynamin activity would eliminate both the current change and the capacitance change. Table 4.5 and Table 4.6 present data that distinguish between these two events.



**Figure 4.7** Data for current and capacitance for oocytes recorded 30 min after irradiation (mean  $\pm$  SEM, n=5). Left-hand y-axis and full bars represent normalized current decrease. Average values (and ranges) for 100% were 4.1 (3.15 to 4.8), 3.1 (2.8 to 3.7), and 4.1 (3.0 to 5.7) µA for the three groups (left to right). Right-hand axis corresponds to hollow bars, representing normalized capacitance decrease. The rightmost column shows capacitance change of uninjected control oocytes. Average values (and ranges) for 100% capacitance were 197 (191 to 205), 225 (191 to 248), 189 (174 to 199), and 197 (190 to 209) nF for the four groups (left to right). Error bars represent SEM.

Figure 4.7 shows averaged current and capacitance data for three to eight oocytes and also the capacitance change of uninjected control oocytes over the course of the experiment. Current change is indicated by filled bars and the left-hand y-axis. When no dynamin mRNA was co-injected, the average current decrease 30 min after irradiation was  $41.1 \pm 0.7\%$ , which is reasonably consistent with the value of 32% obtained in the experiment of Table 4.3 and Figure 4.5. Co-expression with a WT dynamin isoform had an insignificant effect on the current decrease: the average decrease was  $46.5 \pm 5.7\%$ . Dominant negative dynamin affected the current decrease somewhat, but it remained relatively large at  $27.9 \pm 1.5\%$ . The hollow bars and right-hand axis of Figure 4.7

represent the normalized capacitance decrease. Without added dynamin, the average capacitance change associated with decaging tyrosine on Kir2.1 in the presence of v-Src was  $17.7 \pm 5.8\%$ , consistent with the average value of 14.1% reported in Table 4.4. Injection of WT dynamin gave rise to an average capacitance decrease of  $26.0 \pm 6.6\%$ , not significantly larger than that of oocytes containing only endogenous dynamin. The effect of the dominant negative K44A dynamin isoform, however, was dramatic. Oocytes expressing dominant negative dynamin displayed an average capacitance change of  $4.4 \pm 0.7\%$  over 30 min. This degree of change is not significantly different from the baseline capacitance change for uninjected oocytes ( $3.3 \pm 1.4\%$ ).

Channel	Dynamin	% decrease±SEM	n	
Kir2.1	-	41.1±0.7	3	
Kir2.1	WT	46.5±5.7	5	
Kir2.1	K44A	27.9±1.5	8	
Table 4.5 Normalized average whole-cell current decrease of oocytes expressing Kir2.1Y242TAG				
suppressed with Tyr(ONb) treated with the indicated dynamin thirty minutes after initiation of experiment.				

Channel	Dynamin	% decrease±SEM	n	
Kir2.1	-	17.7±5.8	3	
Kir2.1	WT	26.0±6.6	5	
Kir2.1	K44A	4.4±0.7	8	
-	-	3.3+1.4	5	

**Table 4.6** Normalized average whole-cell capacitance decrease of oocytes expressing Kir2.1Y242TAG suppressed with Tyr(ONb) treated with the indicated dynamin thirty minutes after initiation of experiment.

By combining current and capacitance measurements with the expression of dominant negative dynamin, we have shown that significant current decrease occurs in the absence of appreciable capacitance change. The 26% current reduction in the absence of clathrin-mediated endocytosis is smaller than the 41% or 32% reductions seen in cells where the endocytotic machinery has not been perturbed. Thus, clathrin-mediated endocytosis may account for a portion of the whole-cell current reduction. In addition, however, a non-endocytotic mechanism reduces current after the decaging of Tyr242.

### 4.3.2.5 Fluorescent labeling corroborates dynamin-mediated endocytosis

In order to directly observe loss of plasma membrane, and to complement the electrophysiological data on current and capacitance change, we labeled and measured surface proteins on the oocyte. Oocytes expressing Kir2.1 were exposed to the impermeant thiol-reactive fluorophore tetramethylrhodamine-5-maleimide. Since the reagent is not specific for Kir2.1, uninjected oocytes were also labeled as a control. Oocytes treated in this manner displayed fluorescence at the cell surface. Oocytes expressing the channel displayed 65% greater fluorescence intensity than uninjected oocytes, suggesting that one or both of the two putative extracellular cysteines of Kir2.1 are labeled by the maleimide. After 3 s of irradiation, fluorescence was measured at the animal pole at 2- or 2.5 min intervals over a 30-min period.<sup>83</sup> (Figure 4.8)



**Figure 4.8** Normalized, average fluorescence change at t = 30 min for oocytes expressing Kir2.1-Y242TAG suppressed with Tyr(ONb) and treated with PAO, then labeled with tetramethylrhodamine maleimide. The first column represents a control in which oocytes were not co-injected with v-Src. In all other columns, oocytes expressed the Kir2.1-Y242Tyr(ONB) along with v-Src. The second column represents a control in which oocytes were not irradiated. In the third column, no exogenous dynamin was added. The fourth column shows oocytes co-expressing WT dynamin. The fifth column represents oocytes co-expressing dynamin-K44A. Each bar represents 6 or 7 oocytes. The average values (and ranges) for 100% fluorescence were 5.76 (3 to 9.5), 5.74 (3.5 to 7.2), 6.45 (3.7 to 9.5), 5.60 (3.93 to 6.92), and 5.62 (1 to 9.7) V.

Averaged data from six or seven oocytes are collected in Table 4.7. In oocytes expressing Kir2.1 with Tyr(ONb) at Y242 but not v-Src, the fluorescence change at 30 min was similar to that of non-irradiated cells expressing v-Src,  $0.4 \pm 0.4\%$  and  $0.2 \pm 0.2\%$ , respectively. Cells that expressed both channel and kinase without added dynamin showed an average fluorescence change of  $5.7 \pm 0.9\%$  30 min after irradiation. The co-injection of WT dynamin increased endocytosis markedly: there was a  $9.2 \pm 0.7\%$ change over 30 min. As before, dominant negative dynamin suppressed membrane turnover to the level of uninjected oocytes. Oocytes co-injected with dynamin-K44A showed only a  $0.4 \pm 0.2\%$  fluorescence change 30 min after tyrosine decaging. Net membrane internalization is revealed by decreased fluorescence, possibly because the exciting and/or emitted light is partially shielded by intracellular pigment granules and other structures. Corroborating the capacitance measurements, irradiation leads to internalization only in oocytes co-expressing v-Src, caged Kir2.1, and endogenous or added WT dynamin under conditions favoring tyrosine phosphorylation. Thus, both capacitance and fluorescence measurements imply that dynamin is required for decaginginduced endocytotic activity in the cell.

v-Src	hν	WT dynamin	K44A dynamin	% decrease±SEM
-	+	-	-	0.4±0.4
+	-	-	-	0.2±0.2
+	+	-	-	5.7±0.9
+	+	+	-	9.2±0.7
+	+	-	+	0.4±0.2

**Table 4.7** Normalized average surface fluorescence decrease of oocytes expressing Kir2.1Y242TAG suppressed with Tyr(ONb) thirty minutes after initiation of experiment.

# 4.3.2.6 Tyrosine phosphorylation decreases Kir2.1 conductance and decreases total open channels per patch

In single-channel experiments on WT Kir2.1 at -80 mV and -100 mV, we found that co-expression of v-Src and treatment with PAO had two major effects on channel openings. First, conditions favoring tyrosine phosphorylation decrease the conductance of Kir2.1. For instance, Figure 4.9 presents tracings and all-points histograms for oocytes injected either with Kir2.1 alone, untreated with PAO, or with Kir and v-Src and treated with PAO. The single-channel conductances for control and Src and/or PAO oocytes were  $17.5 \pm 0.4 \text{ pS}$  and  $12.3 \pm 0.2 \text{ pS}$  (mean  $\pm \text{ SEM}$ , n = 8 and 5, respectively), amounting to a 30% decrease in single-channel conductance. We found no additional substates, suggesting that the conductance decrease is not graded with the number of affected subunits in a channel. Thus at least part of the phosphorylation-induced decrease in macroscopic currents derives from a decreased single-channel current.

Second, v-Src/PAO patches consistently displayed fewer active channels. In a series of recordings with tightly controlled pipette tip diameter, three of five untreated patches occasionally displayed two simultaneous openings, and the other two displayed only a single opening. Therefore the average peak was 1.6 simultaneous openings. On the other hand, none of five Src/PAO patches displayed more than one simultaneous opening; and two of these patches displayed no openings. Therefore the average number of peak simultaneous openings was 0.6. These data show that v-Src/PAO decreases either the number of active channels per patch or the open probability P<sub>open</sub>. We noted that P<sub>open</sub> was near 0.5 for both untreated and v-Src/PAO patches with only a single channel; therefore

the probable basis of the difference is that v-Src/PAO patches contained fewer active channels.



**Figure 4.9** Conditions favoring tyrosine phosphorylation decrease single-channel conductance of WT Kir2.1. Left panel: Traces showing exemplar single-channel currents from cell-attached patches at -100 mV. The arrows point to the closed states and to the open states for control oocytes (left) and for oocytes co-injected with v-Src and treated with PAO (right). Right panel: Normalized all-points amplitude histograms for exemplar patches. Data from an oocyte injected with Kir2.1 alone are shown as heavy lines, and data from an oocyte injected with Kir2.1 + v-Src and exposed to PAO are shown as light lines. The arrows show open-channel amplitudes from Gaussian fits to the data, 1.69 and 1.15 pA for the control and v-Src/PAO patches, respectively.

### 4.3.2.7 There Is No Direct Evidence for Tyrosine Phosphorylation of Kir2.1

Active tyrosine kinases decrease the activity of WT Kir2.1 (Table 4.1), are required for the decreases in both current and membrane area after tyrosine decaging (Table 4.3 and Table 4.4), and produce decreases in active channel density per patch (Figure 4.9). None of these effects is observed for the Y242F mutant channel. The most direct interpretation of these data is that covalent modification of Y242 causes these effects, as proposed in earlier work on this system. It is also possible that this tyrosine residue is the interaction partner of proteins that must themselves be tyrosine phosphorylated for full activity, as is the case for many of the proteins in the clathrin-mediated endocytotic pathway.

Therefore, we sought evidence that Kir2.1 is phosphorylated at position 242 in the presence of kinases and phosphatase inhibitors. The primary strategy used to detect phosphorylation of Y242 was Western blotting with anti-phosphotyrosine antibodies. For

the purposes of immunoprecipitation and immunostaining of the channel, a Kir2.1 construct with the hemagglutinin (HA) epitope at the carboxyl terminus was generated. We verified in electrophysiological experiments that functional expression and inhibition by v-Src/PAO of Kir2.1-HA were similar to WT Kir2.1. In addition, a Y242F-HA construct was made and shown to behave like the Y242F channel.

Also, a panel of anti-phosphotyrosine antibodies was examined to optimize sensitivity under the present experimental conditions, with the result that 4G10 gave the highest sensitivity, followed closely by PY72. (Figure 4.10)



**Figure 4.10** Comparison of anti-phosphotyrosine antibody affinities in a Western blot of positive control extract from EGF-stimulated cells ( $a = 2 \mu g$ ,  $b = 0.2 \mu g$  total protein). Lane 1: Upstate Biotech 4G10. Lane 2: Covance 2G8-D6. Lane 3: Covance 6G9. Lane 4: Covance 1G2. Lane 5: Covance PY72. Lane 6: Covance PY20.

To establish whether Western blotting with anti-PY antibodies was sufficiently sensitive to detect tyrosine phosphorylation of integral membrane proteins expressed in oocytes, control experiments were performed using the receptor tyrosine kinase TrkB. This kinase undergoes auto-phosphorylation on tyrosine in response to the binding of its ligand, BDNF. Plasma membranes from BDNF-stimulated oocytes expressing TrkB were isolated by physical dissection. Western blotting with an anti-TrkB antibody (Santa Cruz C14) shows readily observable staining at the expected molecular mass (Figure 4.11). As expected, uninjected oocytes show no anti-TrkB staining. Blotting with an anti-phosphotyrosine antibody (4G10) detects numerous phosphoproteins in the oocyte membrane, which is consistent with the effects of treating the oocytes with a tyrosine phosphatase inhibitor. Oocytes expressing TrkB contain a tyrosine-phosphorylated protein that co-localizes with TrkB and that is not present in uninjected oocytes. Densitometry showed that the phosphorylated TrkB band is roughly as intense as the nearby bands corresponding to endogenous phosphorylated proteins.



**Figure 4.11** Detection of phosphorylated TrkB serves as a positive control for the detection of phosphorylated integral membrane protein from dissected oocyte plasma membranes. Western blot of membranes from BDNF-stimulated oocytes expressing TrkB shows positive staining with both anti-TrkB and anti-PY antibodies. The left panel was stained with Santa Cruz C14 anti-TrkB antibody. Lane 1: Uninjected oocytes. Lane 2: TrkB-expressing oocytes. The right panel was stained with Upstate Biotech 4G10 anti-phosphotyrosine antibody. Lane 1: Uninjected oocytes. Lane 2: TrkB-expressing oocytes. Lane 2: TrkB-expressing oocytes.

Comparable experiments were carried out with Kir2.1-HA constructs (Figure 4.12). Kir2.1 constructs are clearly detectable by the anti-HA antibody (HA.11) at the appropriate molecular mass. A protein produced by *in vitro* translation of Kir2.1-HA mRNA from wheat germ extract confirms the assignment of monomeric Kir2.1 subunits. The stained upper molecular mass bands presumably represent oligomers that are not denatured, even under SDS-PAGE conditions. In contrast, staining Western blots with the anti-phosphotyrosine antibody gives no convincing signal corresponding to Kir2.1 monomer or oligomer. Phosphoproteins are clearly present at a variety of other molecular masses, which is consistent with the TrkB results and corroborates that the assay does detect membrane phosphoproteins. That these phosphoproteins are also present in uninjected oocytes treated with PAO confirms that they do not arise from Kir2.1. Any staining in the region corresponding to monomeric Kir2.1 is <10% as intense as that for nearby endogenous phosphorylated proteins.



**Figure 4.12** Failure to detect Kir2.1 phosphorylation in membranes dissected from oocytes expressing the channel. Western blot of membranes from oocytes expressing epitope-tagged Kir2.1 shows positive staining with anti-HA antibody, but not with the anti-phosphotyrosine antibody 4G10. The left panel is stained with Covance HA.11. Lane 1: Uninjected oocytes. Lane 2: Kir2.1-HA. Lane 3: Kir2.1Y242F-HA. Lane 4: Kir2.1-HA prepared by *in vitro* translation from wheat germ extract. Lanes 1-3 contain protein from membranes dissected from 21-23 oocytes.

In addition to these experiments, a number of other immunoprecipitation and immunodetection schemes were examined. Immunoprecipitation of phosphoproteins from oocyte homogenates with anti-PY antibodies followed by detection with anti-HA antibodies was undertaken, as well as the reverse. A completely different assay was also attempted, namely, autoradiography. Even though this technique is often considered to be more sensitive than Western blotting, it has the disadvantage of not distinguishing between phosphorylation on tyrosine, serine, or threonine without phosphopeptide mapping. Oocytes were supplied with <sup>32</sup>P either by co-injection with mRNA encoding the channel, or by incubation in  $\gamma$ -[<sup>32</sup>P]ATP. HA-tagged channels were immunoprecipitated from whole-cell membrane preparations and from physically dissected membranes by both anti-HA and anti-PY antibodies and subjected to SDS PAGE. No differences between the Kir2.1-HA and Kir2.1-Y242F-HA channels were ever observed in <sup>32</sup>P autoradiography, and further mapping was not attempted. That we did not detect tyrosine phosphorylation of Kir2.1 is not conclusive, but it leads us to the explanation that other protein(s) may be phosphorylated. Interactions among these proteins, or between these proteins and Kir2.1, may lead to downregulation of K<sup>+</sup>current and endocytosis after Y242 is de-caged.

### 4.3.3 Discussion

### 4.3.3.1 Caged tyrosine allows study of ion channel modulation

Evidence for the regulation of ion channels by phosphorylation has become increasingly difficult to ignore in recent years.<sup>84,85</sup> In most cases, it has been impossible to establish the molecular basis for this regulation. In principle, phosphorylation could

directly influence channel properties by inducing conformational changes in ion channel proteins themselves. Alternatively, phosphorylation may promote, inhibit, or alter protein-protein interactions between the channel and other potential modulators of channel function. In the latter case, information about the kinetics of these events may be useful preliminary knowledge in helping to identify what kinds of processes may lie downstream of phosphoryl transfer. The use of caged compounds for determining the kinetics of cellular events is well-precedented.<sup>4-6</sup> Through the use of nonsense suppression to introduce unnatural amino acids, functional groups on the ion channel itself may be caged. The photolytic decaging of protected tyrosine has been demonstrated in the nicotinic acetylcholine receptor, but for the study of ion channel phosphorylation, caged tyrosine represents a new tool.<sup>43</sup> A particular tyrosine is rendered unsuitable as a substrate for phosphorylation until the moment it is released by UV irradiation provided by the experimenter.

Here, Kir2.1 was chosen as an experimental system, because there is a clearly detectable current reduction dependent upon a single tyrosine in the C-terminal intracellular tail of the receptor, Y242. Mutation of this residue to phenylalanine abolishes the effects of tyrosine kinases on whole-cell channel conductance.<sup>76</sup> (Figure 4.13)



**Figure 4.13** Whole-cell current from oocytes expressing Kir2.1 (left panel) and Kir2.1-Y242F (right panel). Data columns represent normalized currents (mean  $\pm$  SEM, 4-5 oocytes). Where indicated, oocytes were co-injected with cRNA for tyrosine kinases v-Src or Pyk2 and incubated with 10  $\mu$ M PAO for at least 30 min prior to recording. 100% corresponds to 13.3  $\mu$ A for WT and 31.4  $\mu$ A for Y242F.

When this tyrosine is replaced with Tyr(ONb), the channel becomes insensitive to the activity of v-Src, in the same way that the Y242F mutant fails to respond to tyrosine kinases. However, irradiation reveals the wild-type residue, rendering the channel sensitive to tyrosine kinase-mediated current modulation. Over the course of the next thirty minutes or so, the whole-cell current decreases by about 50%. (Figure 4.14)



**Figure 4.14** Inhibition of current in oocytes expressing Kir2.1-Y242TAG suppressed with Tyr(ONb), shown by typical current traces from individual oocytes. Left panel: The effect of irradiating an oocyte (3 s) that is neither co-expressing v-Src nor exposed to PAO. Center panel: Effect of co-expression of v-Src and exposure to PAO, but no irradiation of the oocyte. Right panel: Traces from an oocyte co-expressing v-Src, exposed to PAO, and irradiated. Oocytes were clamped at 0 mV and stepped to -80 mV to elicit inward current. Bath solutions contained 96 mM KCl. Time after irradiation is indicated.

This time course seems slow for a conformational change resulting in altered channel properties. It is possible that current reduction results from protein-protein interactions arising as a result of phosphorylation-induced conformational changes, however. In order to understand the nature of these interactions, it is necessary to consider the various pathways which could account for the phenomenon.

As in any mutagenesis experiment on an integral membrane protein, it is difficult to be certain that the conformation of the mutant protein is identical to that of the wild-type protein. The nitrobenzyl group adds steric bulk to the tyrosine side chain and prohibits the tyrosyl hydroxyl from engaging in hydrogen bonding. It could be the case that photolysis simply allows the protein to adopt the wild-type conformation, perhaps permitting the binding of another protein which is blocked by Tyr(ONb). The caged Kir2.1 behaves identically to the wild-type channel by most measures, and the necessity for both a tyrosine kinase and a phosphatase inhibitor to induce current inhibition point to a role for phosphorylation. However, we cannot rule out the possibility that a phosphoprotein which would normally bind to the C-terminal region of Kir2.1 and decrease its current is able to do so after removal of the protecting group.

### 4.3.3.2 Kir2.1 current decrease could result from endocytosis

Another possibility to account for the current reduction we observe upon liberating the tyrosine residue is that the tyrosine itself is an necessary component of some proteinprotein interaction motif. In fact, the sequence in which Y242 occurs, YIPLD, is a consensus sequence for clathrin-mediated endocytosis. The motif YXX $\Phi$ , where X is any amino acid and  $\Phi$  is a hydrophobic residue, has been proposed as the recognition signal for AP-2, an adapter protein which targets polypeptides to clathrin-coated vesicles. Crystallographic evidence confirms that the AP-2  $\mu$  subunit is able to bind peptides containing the YXX $\Phi$  motif.<sup>74,86</sup> A study of the ENaC channel in *Xenopus* oocytes has demonstrated that a tyrosine residue in such a motif is functionally important in targeting an ion channel for removal from the plasma membrane.<sup>77</sup>

An obvious explanation for the phenomenon we observed with Kir2.1 would be that caged tyrosine protects Kir2.1 from incorporation into clathrin-coated vesicles. Once photolysis liberates the wild-type residue in a tyrosine-based endocytotic motif, however, the channel is removed from the plasma membrane by a clathrin-mediated pathway. This would neatly account for the observed loss in current. In addition, an endocytotic mechanism could easily be regulated by the phosphorylation state of tyrosine. For example, negative regulation of endocytosis by tyrosine kinases could occur if phosphorylation of the tyrosine in this motif precludes binding, as suggested by the crystal structure of a YXXΦ-containing peptide bound to AP-2 and a number of functional studies.<sup>78,87,89</sup> Alternatively, endocytosis could be positively regulated by a kinase if components of the endocytotic machinery required tyrosine phosphorylation for full activity.

### 4.3.3.3 Current decrease is not due solely to endocytosis

In order to establish the relative contribution of endocytotic and alternative phosphorylation-based mechanisms, experiments were performed to address the issue of receptor turnover. If Kir2.1 were being removed from the plasma membrane, this should be detectable in a number of ways. The two which we chose to examine are changes in whole-cell membrane capacitance, which is related to cellular surface area, and changes in surface fluorescence of thiol-labeled Kir2.1. First of all, there is a decrease in

membrane capacitance associated with current inhibition, as shown in Figure 4.15. Oocytes which are expressing receptors for which there is no current reduction upon irradiation also show no capacitance change. Conversely, oocytes expressing Kir2.1 suppressed with Tyr(ONb) at position 242 show capacitance decrease if and only if they are co-injected with tyrosine kinase, treated with a tyrosine phosphatase inhibitor, and are irradiated to remove the nitrobenzyl protecting group.



**Figure 4.15** Inhibition of current and decrease of capacitance in oocytes expressing Kir2.1-Y242TAG suppressed with Tyr(ONb). Typical current traces from individual oocytes are shown. Left panel: The effect of irradiating an oocyte (3 s at arrow) that is neither co-expressing v-Src nor exposed to PAO. Center panel: Effect of co-expression of v-Src and exposure to PAO, but no irradiation of the oocyte. Right panel: Traces from an oocyte co-expressing v-Src, exposed to PAO, and irradiated. Oocytes were clamped at 0 mV and stepped to -80 mV to elicit inward current. Bath solutions contained 96 mM KCl.

The second experiment corroborates this. In this case, the oocyte surface was labeled with a membrane-impermeant thiol-reactive fluorescent compound (Figure 4.16). By viewing the fluorescence decrease over time, a similar trend to that seen by capacitance measurements was observed. Although the time course of the capacitance change and the extent are slower and not as great as those of the whole-cell current change, it appears that endocytosis is stimulated by kinase-mediated events subsequent to uncaging a tyrosine at position 242.



**Figure 4.16** Fluorescence analysis of membrane retrieval. Left panel: Confocal image of two oocytes labeled with 10  $\mu$ M tetramethylrhodamine-5-maleimide, a membrane-impermeant thiol-reactive fluorophore. The oocyte at left was injected with v-Src and Kir2.1-Y242TAG suppressed with Tyr(ONb). Oocyte on the right was not injected. Right panel: Fluorescence measurements for individual oocytes co-expressing v-Src and Kir2.1 Y242Tyr(ONb) and exposed to PAO. Oocytes were labeled with 10  $\mu$ M tetramethylrhodamine maleimide for 30 min prior to recording. Fluorescence is measured as PMT output voltage. As indicated, controls included omission of co-injected v-Src and omission of irradiation.

Both of these experiments measured changes which would arise from any endocytotic mechanism. However, since the caged tyrosine makes up part of a tyrosine-based clathrin-mediated endocytosis motif, experiments to further delineate the role of specific endocytotic mechanisms are possible. The assembly of clathrin-coated vesicles is driven by the small GTPase dynamin. Previous work in other labs has shown that a K44A mutation in dynamin renders it unable to hydrolyze GTP.<sup>81,82</sup> More importantly, for the purposes of the experiments described here, this K44A dynamin is capable of exerting a dominant negative effect over wild-type dynamin. Indeed, dominant-negative dynamin has been used in *Xenopus* oocytes to suppress the activity of endogenous dynamin and largely shut down the clathrin-mediated endocytotic pathway.<sup>77</sup> Co-injection of dominant negative dynamin should render the tyrosine-based endocytosis motif of Kir2.1 ineffectual in removing channels from the plasma membrane. If this is the primary mechanism by which current is inhibited subsequent to the de-caging of Tyr(ONb) at position 242, dominant negative dynamin should remove the observed current reduction.

What was observed instead is that dominant negative dynamin diminishes the degree of current inhibition, but does not abolish it. (Figure 4.17)



**Figure 4.17** Dynamin expression distinguishes inhibition of the channel from endocytosis. Current and capacitance data from representative oocytes co-expressing v-Src and Kir2.1-Y242TAG suppressed with Tyr(ONb) and treated with PAO. Data were recorded 48 h after injection. Left panel: An oocyte without co-injected dynamin cRNA. Center panel: An oocyte co-injected with 10 ng WT dynamin cRNA. The capacitance decrease for this cell was unusually large. Right panel: An oocyte co-injected with 10 ng dominant negative dynamin-K44A.

In these experiments, the mean current reduction with the clathrin-dependent pathway intact was 41%. When the pathway is shut down (as evidenced by the 4% capacitance change, relative to 3% for uninjected oocytes), the current reduction was 28%. (Table 4.5 and Table 4.6) Thus, the current decrease appears to be partially dependent on endocytosis. Oocytes which are defective in their ability to remove membrane proteins from the cell surface do not undergo the full extent of current reduction. However, some current reduction is seen, in spite of the fact that plasma membrane retrieval has been practically eliminated by dominant negative dynamin. The conclusion from this set of experiments is that clathrin-mediated endocytosis is operative in reducing whole-cell K<sup>+</sup> currents, but that it is not responsible for all of the current inhibition observed after decaging Y242. A non-clathrin pathway has been reported for ligand-gated ion channel endocytosis that operates even in the presence of dominant-negative dynamin.<sup>90</sup>

Alternatively, pathways acting directly on the channel may operate along with clathrinmediated endocytosis.

In fact, endocytosis and phosphorylation are not completely independent phenomena in these experiments. Figure 4.15 shows that endocytosis appears to be stimulated by phosphorylation in an interesting way. Simply revealing the native  $YXX\Phi$  endocytotic motif by photolysis does not cause the whole-cell capacitance change associated with endocytotic activity. Apparently, the clathrin-mediated pathway is unable by itself to cause endocytosis with its attendant change in membrane capacitance. Only when the tyrosine is revealed and tyrosine kinase is present is there an observable capacitance change. At present, it is difficult to know with certainty why native Kir2.1 and v-Src are required for whole-cell capacitance decrease. As indicated above, the crystal structure of AP-2  $\mu$  co-crystallized with YXX $\Phi$  peptide suggests that phosphotyrosine would be unable to make the requisite binding contacts.<sup>78</sup> Thus, it would be expected that v-Src might inhibit endocytosis. There are a number of reasons which might explain why our observation is at variance with this. First of all, there is presumably some regulation of AP-2 binding to tyrosine-based endocytosis motifs, which quite commonly occur multiple times in a single protein.<sup>91</sup> Kir2.1 contains two more C-terminal YXXΦ sequences, YEPV from 326-329 and YSRF, comprising residues 340-344 as well as a potential dileucine motif at 231-232. (Figure 4.18)

MGSVRTNRYSIVSSEEDGMKLATMAVANGFGNGKSKVHTRQQCR SRFVKKDGHCNVQFINVGEKGQRYLADIFTTCVDIRWRWMLVIF CLAFVLSWLFFGCVFWLIALLHGDLDTSKVSKACVSEVNSFTAA FLFSIETQTTIGYGFRCVTDECPIAVFMVVFQSIVGCIIDAFII GAVMAKMAKPKKRNETLVFSHNAVIAMRDGKLCLMWRVGNLRKS HLVEAHVRAQLLKSRITSEGEYIPLDQIDINVGFDSGIDRIFLV SPITIVHEIDEDSPLYDLSKQDIDNADFEIVVILEGMVEATAMT TQCRSSYLANEILWGHRYEPVLFEEKHYYKVDYSRFHKTYEVPN TPLCSARDLAEKKYILSNANSFCYENEVALTSKEEEEDSENGVP ESTSTDSPPGIDLHNQASVPLEPRPLRRESEI

**Figure 4.18** Primary sequence of Kir2.1 showing the presence of numerous endocytosis motifs. The motif including Y242 is indicated in red, the others in blue.

It may be that phosphorylation of Y242 positively regulates binding to the more Nterminal endocytotic motifs. Second, the method presented here relies on functional interactions between a multiple-subunit, multiple transmembrane-domain integral membrane protein in a living cell. It could be that the evidence collected thus far for downregulation of endocytosis via tyrosine phosphorylation represents only one of a number of important interactions for regulating endocytosis via tyrosine-based motifs.

### 4.3.3.4 Phosphorylation is implicated in Kir2.1 modulation

In addition, phosphorylation state may well regulate endocytosis at the level of the cycling of endocytotic vesicles. Just as there is an equilibrium between kinases and phosphatases, there must be an equilibrium between endocytosis and exocytosis. The signals by which integral membrane proteins are targeted to endocytotic vesicles are just beginning to be discovered, and even less is known about the signals for retrieval.<sup>74,86</sup> Progress in this area is likely to come from the study of G-protein coupled receptors, where the role of internalization and recycling in causing agonist-dependent desensitization is rapidly being elucidated.<sup>92</sup> Based on this work, it seems possible, even probable, that phosphorylation of ion channels determines whether they are recycled to

the plasma membrane or targeted for degradation. Since vesicle internalization and refusion modulate whole-cell capacitance changes, the mediation of their targeting by the phosphorylation state of their membrane protein contents could explain the apparent requirement for Kir2.1 to be phosphorylated at Y242.

### 4.3.3.5 Attempts to detect phosphorylation

Since the available evidence points strongly toward a role for tyrosine phosphorylation in the observed current reduction of Kir2.1, it would be valuable to know whether or not the channel is phosphorylated directly. Both anti-phosphotyrosine antibodies and <sup>32</sup>P were employed in an effort to detect the presence of a phosphoryl group on wild-type and de-caged Kir2.1. These experiments failed to detect such phosphorylation. The oocyte system has been reported to be difficult to work with in this way, so it is unwarranted to conclude that failure to detect phosphorylation implies that the protein is not phosphorylated. Experiments are ongoing to attempt to detect a role for phosphorylation of Y242 in *Xenopus* oocytes. Given the difficulty of biochemically detecting phosphorylation, the alternative technique of introducing non-hydrolyzable phosphotyrosine analogs at position 242 is being considered. Progress toward this goal will be discussed further in Chapter 5.

As noted earlier, a variety of tyrosine kinases are able to induce the current inhibition we observed. Since Y242 is unlikely to be a consensus phosphorylation site for all of these kinases, the implication is that a downstream kinase is ultimately responsible for the effect. Identification of this kinase represents a long-term goal for studies on the molecular detail of phosphorylation-mediated ion channel regulation. Introduction of a mechanism-based inhibitor as an amino acid side chain is a potentially useful method for trapping the kinase responsible for phosphorylation. Alternatively, antisense mRNA to a series of tyrosine kinases may reveal that the diminished activity of a particular kinase ameliorates the current inhibition resulting from phosphorylation.

# 4.3.4 Summary

Active tyrosine kinases decrease the activity of WT Kir2.1, are required for the decreases in both current and membrane area following tyrosine decaging, and produce decreases in active channel density per patch. None of these effects is observed for the Y242F mutant channel. Usually, these data would be interpreted to suggest that phosphorylation of Y242 causes these effects, as proposed in earlier work on this system. However, the involvement of tyrosine 242 in an endocytosis motif suggests an alternative explanation. All of these observations are consistent with tyrosine-based endocytosis of the channel. The requirement for tyrosine kinases could reflect the need for components of the endocytotic machinery to be activated by tyrosine phosphorylation. The requirement for a native tyrosine at position 242 of Kir2.1 could be explained by the apparent involvement of this residue in binding of adapter proteins targeting the channel for endocytosis. Thus, the question arises of whether our observations result from phosphorylation of Y242 or from endocytosis of the channel subsequent to unmasking of a tyrosine-based sorting motif.

Biochemical evidence that Kir2.1 is phosphorylated at position 242 in cells which exhibit current decrease would permit a compelling claim that phosphorylation is the mechanism responsible for channel modulation subsequent to photolysis. We were unable to obtain such evidence. (Figure 4.12) Unfortunately, failure to detect phosphorylation is itself somewhat difficult to interpret. As a positive control for tyrosine phosphorylation, the auto-phosphorylated receptor tyrosine kinase TrkB was expressed in oocytes. As expected, both the 4G10 anti-PY and the C14 anti-TrkB antibodies show easily observable staining and co-localization under standard Western blotting conditions. (Figure 4.11) It is evident that the technique is capable of directly detecting tyrosine phosphorylation of integral membrane proteins expressed in *Xenopus* oocytes. However, there are complications in extending this result to the detection of phosphorylated Kir2.1. The level of expression of the two proteins, the extent of tyrosine phosphorylation, the duration of protein phosphorylation, and the sensitivity of particular phosphotyrosine residues to de-phosphorylation may well differ. As a result, the inference that Kir2.1 is not phosphorylated is not conclusive.

Any proposed mechanism to account for the decrease in whole-cell current and capacitance subsequent to photolysis must take into account the following observations. First, both a free tyrosine side chain at position 242 and tyrosine kinases are required. (Figure 4.14) Second, the presence of kinases and Y242 brings about decreases in whole-cell current, membrane capacitance, and surface fluorescence. (Figure 4.14, Figure 4.15, Figure 4.16) Third, dominant-negative dynamin blocks the changes in capacitance and fluorescence and causes a partial block of current decrease. (Figure 4.17) Fourth, the presence of v-Src and PAO decreases the number of channels per patch without affecting the single-channel properties of individual channels. (Figure 4.9) Experiments by other laboratories have suggested also that phosphotyrosine is not competent for recognition by adapter proteins responsible for targeting membrane proteins for endocytosis.<sup>78,87-89</sup>

All of these observations (and our inability to detect phosphotyrosine) fit neatly with a model in which the role of Y242 is in targeting Kir2.1 for endocytosis, with one exception. This exception is that a significant component of current decrease is insensitive to dominant-negative dynamin. It is clear that dominant-negative dynamin suppresses changes in membrane capacitance. (Figure 4.7) If current decrease were solely a result of channel endocytosis, the predicted result of halting membrane internalization would be to eliminate current decrease. Since this is not the observed result, it is tempting to speculate that multiple mechanisms of channel modulation may be involved. Cells almost certainly regulate channels through both phosphorylation and endocytotic targeting. However, both of these effects would have to be in the same direction, have the same time course, and result in no alteration of single-channel properties. We therefore prefer a model in which interaction with the endocytotic machinery is the sole regulatory mechanism acting on Y242, which is consistent with the similarity of the observed kinetics in the presence and absence of dominant-negative dynamin.

In this model, a protein-protein interaction which is responsible for targeting of the channel to endocytotic vesicles occurs, even though the downstream pinching off of clathrin vesicles may be prohibited by dominant-negative dynamin. Channels in these invaginations are unable to contribute to the whole-cell current, possibly as a result of restricted flow of the external high-potassium recording solution through the dynamin collar which constricts the neck of the vesicle. As a result, the whole-cell current decreases even though the cell's surface area and capacitance remain the same. The extent of current decrease may be less than before because the arresting of vesicles may down-regulate the assembly of dynamin collars, or perhaps because some of the vesicle-bound channels remain active. As indicated above, it is difficult to exclude the

possibility that Y242 may be phosphorylated. In the absence of direct evidence, though, we feel the most parsimonious explanation of the data is that presented above.

Undoubtedly, further details will be forthcoming on the modulation of ion channels by both phosphorylation and endocytosis. At the currently achievable level of molecular detail, this study represents a delineation of the roles of these two processes in modulating Kir2.1 current through the common intermediate of a C-terminal tyrosine residue. Because of the complexity of multiple regulatory processes involving a single residue, we introduce in the next chapter a technique involving caged phosphoamino acids. Through the use of this methodology, we hope to control directly, for the first time, the phosphorylation state of a particular residue in a functioning channel inside a living cell.

### 4.3.5 Experimental methods

### 4.3.5.1 Chemical synthesis

The 4PO-protected amino acid Tyr(ONb) was prepared as reported.<sup>61</sup> Briefly, L-tyrosine was complexed with copper(II) to protect the amino and carboxyl termini. To this complex was added nitrobenzyl chloride under basic conditions, yielding Try(ONb). Addition of 4-pentenoic anhydride gave the N-protected 4-PO-Tyr(ONb). The amino acid was coupled to the dinucleotide dCA by established procedures.<sup>93</sup> In summary, the carboxyl terminus was converted to the cyanomethyl ester by treatment with chloroacetonitrile. The active ester was condensed with dCA under basic conditions to give the aminoacylated dinucleotide, which was enzymatically ligated to a 74-base tRNA precursor with T4 RNA ligase.

### 4.3.5.2 DNA / RNA constructs

tRNA THG73 was modified from eukaryotic *Tetrahymena thermophila* tRNAGln(CUA) as described and inserted into pUC19 giving the plasmid pTHG73.<sup>93</sup> pTHG73 was linearized by *Fok* I and transcribed *in vitro* using the MegaShortscript kit (Ambion).

The tyrosine codon at position 242 of the mouse Kir2.1 cDNA in pcDNA I was mutated to phenylalanine or to the nonsense codon TAG, giving two mutants called Kir2.1(Y242F) and Kir2.1(Y242TAG), respectively. Mutagenesis was carried out using the QuikChange kit (Stratagene). Mutations were verified by sequencing of both strands through the affected regions. The hemagglutinin antigenic sequence YPYDVPDYA was added to the C-terminus of Kir2.1 using PCR amplification, giving Kir2.1-HA. The Kir2.1 plasmids were linearized by *Not* I.

The cDNA for v-Src kinase (a gift of Dr. I. B. Levitan), PyK2 (a gift of Dr. J. Schlessinger), human WT dynamin I and dynamin-I-K44A (gifts of Dr. Alex van der Bliek and Dr. Thomas Moss), and rat TrkB (gift of G. Yancoupolis) were linearized by *XbaI*, *SspI*, *SalI* and *SalI*, and *XbaI* or *NotI*, respectively. All mRNAs were transcribed *in vitro* using the SP6 or T7 mMessage mMachine kit (Ambion) as appropriate.

### 4.3.5.3 Suppression of Kir2.1-Y242TAG with Tyr(ONB) in Xenopus oocytes

The 4PO-Tyr(ONb)-tRNA in 1 mM NaOAc, pH 4.5, was de-protected just prior to injection by mixing with equal volume of a saturated aqueous solution of iodine (1.2 mM) for 10 min at ambient temperature. 10–15 ng of Kir2.1(Y242TAG) mRNA and 20-25 ng of tRNA-Tyr(ONb) in a total volume of 32.2 nL per oocyte were co-injected into stage V and VI oocytes by a Drummond automatic injector. The cRNA for WT Kir2.1 cRNA, the conventional mutant Kir2.1-Y242F, or Kir2.1-HA were injected at 1

ng/oocyte. Oocytes were incubated in 50% L-15 medium supplemented with 7.5 mM HEPES, 0.8 mM of glutamine and 10  $\mu$ g/ml of gentamycin sulfate, pH 7.5, at 18-20°C. After incubation for 24-48 h, functional measurements were made.

# 4.3.5.4 Electrophysiology

For macroscopic recordings, oocytes were voltage clamped at 0 mV in a high K<sup>+</sup> solution (96 mM KCl, 2 mM NaCl, 1 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub> and 5 mM HEPES, pH 7.5) with two electrodes (filled with 3 M KCl, resistance 0.5-3 M $\Omega$ ) and then stepped to a test potential of -80 mV using a GeneClamp 500 (Axon Instruments). Membrane capacitance was measured on oocytes clamped at 0 mV in response to a train of 10 mV pulses delivered at 2.9 Hz with 50% duty cycle. Voltage command signals were generated, and membrane capacitance measured, by the algorithms in pCLAMP 8.0 software (Axon Instruments). In preliminary experiments, capacitance measurements were verified manually by integrating the response to 10 mV test pulses.

For cell-attached single-channel recordings, the pipette solution and the bath contained the same high K<sup>+</sup> solution. The patch channel of the GeneClamp 500 was employed, with 2 kHz filtering. The signals were recorded and analyzed using FETCHEX, FETCHAN, and pSTAT in the pCLAMP 6 suite.

# 4.3.5.5 Decaying optics

The apparatus for Tyr(ONb) decaging was reported previously.<sup>43</sup> Light from a 300 W Hg arc lamp was filtered through a Schott UG11 filter to provide 300-350 nm light and was focused through a 50 mm quartz lens (Oriel) onto a liquid light guide (Oriel, 1 m long and 3 mm in diameter) connected to the recording chamber. At the chamber, the end of

the liquid light guide contacted a Pyrex cover slip placed at the bottom surface of the chamber, upon which the oocyte rested. Miller *et al.* reported that 3 s irradiation was sufficient for photolysis of over 90% of caged tyrosine on the oocyte surface.<sup>43</sup>

### 4.3.5.6 Fluorescence labeling and measurements

For non-specific labeling of the cell surface, oocytes were incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub> and 5 mM HEPES, pH 7.5) containing 10  $\mu$ M tetramethylrhodamine-5-maleimide for 30 min at 20-22 °C. The labeled oocytes were then washed five times with ND96 solution to remove unbound fluorescent dye. The labeling intensity was examined at the animal pole. Confocal images were taken with a Bio-Rad MRC 600 microscope with a 10x objective lens, with 10 average scans.

Surface fluorescence from labeled oocytes was measured using an Olympus IX-70-FLA inverted reflected-light fluorescence microscope.<sup>83</sup> Exciting light was delivered from a stabilized 100 W Hg lamp. We used a 40X, NA1.3 objective lens. A photomultiplier tube (PMT) attached at the side port recorded the fluorescence. To avoid bleaching, a shutter in the excitation pathway was opened and the fluorescence was measured for only 10 to 20 sec at intervals of 2 to 2.5 min. The emitted signal, as voltage from the PMT output, was appropriately amplified and filtered, then sent to an Axon Digidata interface for collecting data by pCLAMP 7 (Axon Instruments, Foster city, CA).

# 4.3.5.7 Immunochemical detection

Oocytes were injected with Kir2.1-HA. Plasma membranes were isolated by physical dissection. The hypotonic solution used to prepare the oocytes for dissection was

modified to contain 5 mM PAO and 1 mM sodium orthovanadate, one EDTA-free Boehringer Complete tablet per 40 ml, and 0.08% SDS (which expedites dissection). PAO was dissolved in DMSO and stored at -80 °C. Yolk and pigment granules were removed by a 2 min 14,000 rpm spin at 4 °C and the supernatant dissolved in 15 ml 2x SDS gel loading buffer. Samples were boiled for 5 min or heated to 55 °C for 10 min prior to loading. SDS PAGE was performed using 10% polyacrylamide (40T:1C) gels or 10% Tris-Cl ReadyGels (BioRad, Hercules CA).

Proteins were transferred to nitrocellulose overnight at 30 V. Blots were blocked for 1 hr in 1x TPBS [PBS containing 0.1% (v/v) Tween-20] and 5% (w/v) non-fat dried milk (Carnation, Columbus OH). Blots were exposed to primary antibody for one h in TPBS with 5% milk, washed three times 10 min each in TPBS, then incubated one h in TPBS containing 5% milk and secondary antibody. Detection employed ECL reagents from Amersham.

Primary antibodies included monoclonal HA.11 ascites fluid; monoclonal antiphosphotyrosine antibodies 2G8-D6, 6G9, 1G2, PY20, PY72 (Covance, Berkeley CA), and 4G10 (Upstate Biotechnology, Lake Placid NY); and anti-TrkB antibody C14 (Santa Cruz Biotechnology, Santa Cruz CA). All Covance antibodies and 4G10 were diluted 1:1000 for Western blotting; C14 was diluted 1:200. Of the phosphotyrosine antibodies, 4G10 had the highest affinity under our conditions. PY72 also performed well. HRPconjugated secondary antibodies included goat anti-mouse (Jackson Immunologicals) and donkey anti-rabbit (Amersham), diluted 1:5000 and 1:200, respectively. The experimental data were analyzed using ORIGIN (Microcal Software, Inc.) and

CLAMPFIT 8 software (Axon Instruments, Foster City, CA).

### 4.3.5.9 Reagents

BDNF was kindly provided by Dr. Andy Welcher (Amgen, Inc., Thousand Oaks CA).

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