Phosphorylation of the salivary Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter

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Phosphorylation of the salivary Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, NKCC1 (also known as BSC2) plays a central role in the secretion of salt and water by many exocrine epithelia (5, 8). In these tissues NKCC1 is localized to the basolateral membrane of the secretory cell, where it concentrates Cl\(^-\) in the cytoplasm above electrochemical equilibrium. The apical membrane typically contains a secretagogue-activated Cl\(^-\) channel. During stimulation, Cl\(^-\) thus enters the cell via NKCC1 and exits via the apical Cl\(^-\) channel. The resulting trans-epithelial Cl\(^-\) secretion then leads to the secretion of salt and osmotically obliged water. There is now good evidence from a number of these tissues that NKCC1 fluxes also are dramatically increased as a result of secretagogue action and that this effect is not simply due to the changes in electrochemical driving forces for Na\(^+\), K\(^+\), and Cl\(^-\) that accompany stimulation (3, 4, 6, 15, 18). Rather, this upregulation appears to arise directly from the increased activity of individual cotransporters.

In previous studies from our laboratories we have examined this phenomenon in some detail in rat parotid acinar cells (3, 9, 17–19). In particular, we have characterized the upregulation of NKCC1 activity induced by both muscarinic and β-adrenergic stimulation (Ca\(^{2+}\)-mobilizing and cAMP-generating stimuli, respectively, in these cells). The case of β-adrenergic stimulation, which mainly concerns us here, we have shown that there is a close correlation between isoproterenol-induced increases in NKCC1 transport activity and NKCC1 phosphorylation (18, 19). More specifically, we found that the isoproterenol dose responses for both of these phenomena were essentially identical, with a half-maximal effect at ~20 nM agonist in each case, and that isoproterenol stimulation was accompanied by the increased phosphorylation of a 17-kDa peptide resulting from NKCC1 digestion by V8 protease. In addition, these effects are paralleled by an increase in the number of high-affinity binding sites for the NKCC1 inhibitor bumetanide in membranes prepared from stimulated acini (9). These results are consistent with the hypothesis that β-adrenergic stimulation and the accompanying phosphorylation result in the activation of previously quiescent, or near-quiescent, transporters.

In contrast to many other exocrine epithelia, salivary glands secrete fluid in response to Ca\(^{2+}\)-mobilizing rather than cAMP-generating agonists (the increased NKCC1 activity resulting from the latter is thought to account for the increased fluid secretion observed when β-adrenergic stimulation is superimposed on muscarinic stimulation in these tissues; see, for example, Ref. 18). Thus β-adrenergic stimulation of salivary acini does not result in the intracellular Cl\(^-\) loss and cell...
shrinkage that typically accompany fluid secretion by exocrine cells (14, 18), and the involvement of these effects in the isoproterenol-induced upregulation of the acinar NKCC1 can therefore be excluded. In addition, we have shown that the effect of isoproterenol on NKCC1 can be prevented by protein kinase inhibitors and mimicked by permeant cAMP analogs as well as maneuvers known to increase cytosolic cAMP levels (18, 19), suggesting the involvement of protein kinase A (PKA). However, somewhat surprisingly we also have found that the site of isoproterenol-induced phosphorylation is found in the NH2-terminal cytosolic tail of NKCC1 (9) which does not contain the sole PKA consensus site in this cotransporter.

Here we continue our studies of the phosphorylation events associated with the rat parotid NKCC1. Briefly stated, we show that NKCC1 is phosphorylated by endogenous kinases associated with isolated rat parotid acinar basolateral membranes (BLMs). Additional NKCC1 phosphorylation is seen in the presence of added PKA, but our data suggest that this is due to an effect of PKA on endogenous membrane kinase or phosphatase activities rather than its direct phosphorylation of NKCC1. Moreover, these phosphorylations do not take place at the site associated with the up-regulation of NKCC1 by isoproterenol stimulation. Finally, we show that the phosphorylation of NKCC1 induced by isoproterenol treatment can be mimicked by the addition of cAMP to permeabilized acini and that this effect can be blocked by a specific peptide inhibitor of PKA. Taken together, these results provide strong evidence that PKA is indeed involved in the phosphorylation of NKCC1 induced by isoproterenol treatment but that an additional, non-membrane associated factor (for example, a cytosolic kinase activated by PKA) is required to effect the phosphorylation event.

**METHODS**

**Materials.** [γ-32P]ATP (3,000 Ci/mmol) was obtained from NEN. Molecular weight standards and prepped SDS-PAGE and tricine gels were from Novex. Protein G/Septagel and tricine gels were from Novex. Protein G/Sephadex and tricine gels were from Novex. Molecular weight standards and prepoured SDS-PAGE gels were from Novex. Protein G/Sephadex and tricine gels were from Novex. Protein G/Sepha-NEN. Molecular weight standards and prepoured SDS-PAGE gels were from Novex. Protein G/Sephadex and tricine gels were from Novex.

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preconjugated protein G beads (slurry) and incubated for 4 h at 4°C. The beads were then collected by centrifugation, washed six times with 500 μl of SS (changing the tube on the last wash), and extracted with SDS-PAGE sample buffer.

**Phosphorylation studies using permeabilized rat parotid acini.** Rat parotid acini were prepared as previously described (19). Phosphorylation reactions were carried out at 37°C in buffer I containing a 20% suspension of acini, 0.1 mM sodium orthovanadate, and other additions as indicated (final volume 100 μl). The reaction was initiated by the addition of 40 μM \(^{32}\text{P}\text{ATP}\) and 0.1 mM digitonin and was stopped by the addition of 500 μl of SS. After the addition of SS, insoluble debris was removed by centrifugation at 10,000 g for 10 min, and the resulting supernatant was combined with 30 μl (slurry) of preconjugated protein G beads for immunoprecipitation of NKCC1 as described in Phosphorylation studies using intact rat parotid BLMs.

**Digestion with V8 protease.** Digestion with V8 protease was carried out as previously described (9, 19). Briefly, bands corresponding to immunoprecipitated rat NKCC1 were cut from dried SDS-PAGE gels, rehydrated in 50 mM NH₂HCO₃ (pH 8.0) plus 1 mM DTT, and incubated with V8 protease (20 U/ml) for 6 h. The digested material recovered from the gel fragments was then dried, taken up in sample buffer, and subjected to tricine-SDS electrophoresis.

**Gel electrophoresis and autoradiography.** SDS-PAGE, tricine-SDS electrophoresis, autoradiography, and densitometry were carried out as previously described (9). Densitometric results are given as means ± SE for the number of replicates indicated. For SDS-PAGE we employed 4–20% gradient gels. For tricine-SDS electrophoresis we used 16% gels. Unless otherwise noted, the results shown are from SDS-PAGE gels.

**RESULTS**

The two left-hand lanes of Fig. 1 show the results of an experiment in which rat parotid BLMs were incubated with \(^{32}\text{P}\text{ATP}\) for 30 s or 30 min, as described in METHODS, and then analyzed by SDS-PAGE and autoradiography. After 30 s of incubation, a number of proteins, particularly in the molecular mass range 50–200 kDa, were labeled with \(^{32}\text{P}\). Because the BLMs used in these experiments were extensively washed during their preparation (see METHODS), this result indicates the presence of one or more protein kinases tightly associated with the rat parotid BLM. In control experiments (not shown) we have demonstrated that overall BLM protein phosphorylation reaches a maximum ~30 s after the addition of \(^{32}\text{P}\text{ATP}\) under our experimental conditions (as indicated in METHODS, the half-time of \(^{32}\text{P}\text{ATP}\) hydrolysis was ~10 s under these conditions). After 30 min of incubation, much of the membrane phosphorylation is lost (Fig. 1), likewise demonstrating the presence of significant membrane-associated protein phosphatase activity (as indicated below, this loss of phosphorylation can be blocked by inhibition of protein phosphatases, confirming that it is not due to nonspecific effects such as proteolysis). As illustrated in the two right-hand lanes of Fig. 1, when NKCC1 was immunoprecipitated from these membranes, we found that it was one of the phosphoproteins detected. Analysis by scanning densitometry indicated that phosphorylation of NKCC1 at 30 s was 3.40 ± 0.08 times that observed at 30 min (n = 7).

Figure 2 illustrates the effects of various protein kinase inhibitors on the ATP-induced phosphorylation of NKCC1 in rat parotid BLMs. The effects of 10 nM staurosporine and 100 nM K252a (general serine/threonine kinase inhibitors with IC\(_{50}\) values in the range 1–10 nM and 20–200 nM, respectively), 1 μM H-89 (a relatively specific inhibitor of PKA with IC\(_{50}\) ~0.05 μM), 3 μM lavendustin A (a relatively specific inhibitor of tyrosine kinases with IC\(_{50}\) ~0.01 μM), and 2 μM bisindolylmaleimide I (a relatively specific inhibitor of protein kinase C with IC\(_{50}\) <0.1 μM for most isotypes) are shown. Marked inhibition of phosphorylation is seen with staurosporine and K252a, which were used in their usual effective range, but there is little or no effect of the other kinase inhibitors, all of which were used at concentrations at least an order of magnitude above their IC\(_{50}\) values. When we examined the effects of these protein kinase inhibitors on the ATP-induced phosphorylation of all BLM proteins (cf., 2 left-hand lanes of Fig. 1), qualitatively similar results were found (not shown).

Figure 3 shows the effects of various protein phosphatase inhibitors on the dephosphorylation of NKCC1 by membrane-associated protein phosphatase(s). The effects of calyculin A, okadaic acid, and microcystin-LR, all used at 100 nM, are shown in Fig. 3, left. Both calyculin A and microcystin-LR are known to inhibit serine/threonine protein phosphatase type 1 at concentrations of ~1 nM, and all three compounds inhibit serine/threonine protein phosphatase type 2A at concentrations of ~1 nM. However, even at 100 nM concentration these compounds have only modest effects on the dephosphorylation of NKCC1 by membrane-associated phosphatases; we suspect that this is be-
cause there are relatively small quantities of these predominantly cytosolic phosphatases that copurify with the BLM. In contrast, the more general phosphatase inhibitor, sodium orthovanadate, whose effects are shown in Fig. 3, right, markedly inhibits the dephosphorylation of NKCC1 at 0.1 mM concentration. Qualitatively similar results were found for the effects of these protein phosphatase inhibitors on overall dephosphorylation of all BLM proteins by membrane-associated phosphatases (not shown).

As already discussed, our previous studies demonstrate that the activity of NKCC1 is dramatically upregulated in response to β-adrenergic stimulation of rat parotid acini and indicate that this effect is the result of NKCC1 phosphorylation. Our results also suggest that PKA may be involved in this effect. To explore this possibility further, we tested the effects of adding cAMP or cPKA to rat parotid BLMs in the presence of [γ-32P]ATP. The results of such an experiment are shown in Fig. 4. The top, left-hand panel of Fig. 4 shows an autoradiograph of immunoprecipitated NKCC1 showing apparent increases in phosphorylation by both treatments relative to an untreated control. The top, right-hand panel of Fig. 4 shows an autoradiograph of V8 protease digests of these samples run on a tricine-SDS gel; in these digests we see a single phosphopeptide with molecular mass ~7 kDa.

The bottom panel of Fig. 4 presents a densitometric analysis confirming that the phosphorylation of this 7-kDa peptide is increased by the presence of cAMP or cPKA. The effect of cAMP in this experiment is presumably due to the presence of PKA bound to A-kinase anchoring proteins (AKAPs) (1, 16) in the BLM and thereby copurified with them. We have, in fact, previously presented evidence for the presence of endogenous PKA and its cAMP-dependent phosphorylation of Na+/K+-ATPase in this BLM preparation (10).

However, as also stated earlier, the phosphorylation of NKCC1 that accompanies isoproterenol stimulation of intact acini and correlates with functional NKCC1 upregulation is associated with a 17-kDa peptide resulting from V8 protease digestion. Although an ~7-kDa phosphopeptide also was seen in these previous V8 protease digests (9, 19), its phosphorylation was not significantly affected by isoproterenol treatment. To better understand the effect of cPKA on the 7-kDa phosphopeptide shown in Fig. 4, we carried out the experiment shown in Fig. 5. Here BLMs were preincubated with unlabeled ATP in the presence or absence of cPKA, and then additions were made to both reactions to bring them to the same levels of cPKA and the PKA inhibitor H-89 (final concentration 1 μM), before adding [γ-32P]ATP and measuring NKCC1 phosphorylation. As can be readily appreciated from Fig. 5, significantly more incorporation of 32P into NKCC1 is seen in the membranes preincubated with cPKA. However, because cPKA was present in both reactions with [γ-32P]ATP, this increase in phosphorylation could not be due to a direct phosphorylation of NKCC1 by cPKA. Instead, the results of this experiment are consistent
with the hypothesis that cPKA treatment of acinar BLMs results in the activation of endogenous membrane-associated kinase(s) or the inhibition of membrane phosphatases, which in turn leads to increased phosphorylation of NKCC1.

To be able to evaluate this result in the context of our earlier experiments with intact acini, we carried out the experiment illustrated in Fig. 6. Here digitonin-permeabilized acini were incubated with \[^{32}\text{P}]\text{ATP}\) alone, with \[^{32}\text{P}]\text{ATP}\) in the presence of cAMP, or with \[^{32}\text{P}]\text{ATP}\) plus cAMP and PKI(5–24), a specific peptide inhibitor of PKA. After the phosphorylation reaction was terminated, immunoprecipitated NKCC1 (top left) and immunoprecipitated NKCC1 digested with V8 protease (top right) were examined by autoradiography after SDS-PAGE or tricine-SDS electrophoresis, respectively. Combined results are shown (bottom) of densitometric analyses of 4 independent experiments after V8 protease digestion (see text for details). All experimental procedures are described in METHODS.

![Fig. 4. Effects of cAMP and the catalytic subunit of protein kinase A (cPKA) on the phosphorylation of NKCC1 in isolated rat parotid BLMs. Rat parotid BLMs were incubated for 30 s in the presence of 40 \(\mu\text{M}\) \[^{32}\text{P}]\text{ATP}\) and 1.0 mM dithiothreitol (DTT) (C), 1.0 mM DTT plus 10 \(\mu\text{M}\) cAMP, or 1.0 mM DTT plus 16 U of cPKA as indicated. After the phosphorylation reaction was terminated, immunoprecipitated NKCC1 (top left) and immunoprecipitated NKCC1 digested with V8 protease (top right) were examined by autoradiography after SDS-PAGE or tricine-SDS electrophoresis, respectively. Combined results are shown (bottom) of densitometric analyses of 4 independent experiments after V8 protease digestion (see text for details). All experimental procedures are described in METHODS.](https://example.com/image4)

**DISCUSSION**

We have demonstrated here that isolated rat parotid BLMs possess significant membrane-associated protein kinase and protein phosphatase activities and that...
of PKA, tyrosine kinase, and protein kinase inhibitors staurosporine and K252a; however, to be of the serine/threonine type, because its effect on membrane-associated protein kinase activity appears mediated by these enzymes is NKCC1 (Figs. 1–3).

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The phosphorylation of NKCC1 produced by the membrane-associated protein kinase activity was on a 7-kDa peptide resulting from V8 protease digestion (Fig. 4). Additional phosphorylation of this peptide was seen when isolated BLMs were incubated with ATP in the presence of cAMP (Fig. 4). This effect is presumably due to the presence of endogenous PKA in parotid BLMs. It has been shown that membrane-bound AKAPs bind PKA via its regulatory subunit RII and thereby anchor it near specific target proteins (1, 16). These target proteins are then rapidly phosphorylated in response to increased cellular cAMP, which releases cPKA from RII. Phosphorylation of Na\(^{+}\)-K\(^{+}\)-ATPase in response to cAMP addition has, in fact, previously been demonstrated in this same parotid BLM preparation and the involvement of AKAPs confirmed (10). As already emphasized, however, the upregulation of NKCC1 function associated with isoproterenol treatment and increased acinar cAMP levels correlates with the phosphorylation of a 17-kDa peptide resulting from V8 protease digestion. Initially we wondered if PKA was actually anchored near NKCC1 via AKAPs but had been stripped off during the BLM preparation, thus accounting for the absence of phosphorylation of the 17-kDa peptide with cAMP treatment. However, attempts to reload membrane-bound AKAPs with PKA, followed by centrifugation and cAMP plus \(\gamma^{32}\)P-ATP to BLM did not result in phosphorylation of the 17-kDa peptide, although NKCC1 phosphorylation of the 7-kDa peptide was increased (Fig. 4).

We did find, however, that preincubation of BLMs with cPKA before the addition of \(\gamma^{32}\)P-ATP resulted in markedly higher levels of NKCC1 phosphorylation than observed in untreated controls (Fig. 5). This result suggests that the effects of cAMP and cPKA on phosphorylation of NKCC1 are due to activation of the endogenous membrane-associated kinase activity or inhibition of membrane phosphatases, rather than a direct phosphorylation of NKCC1 by cPKA. Thus it appears as if PKA may be unable to directly phosphorylate NKCC1 at any site in isolated BLMs.

We previously observed a 7-kDa phosphopeptide in V8 protease digests of immunoprecipitated NKCC1 from intact rat parotid acini (9, 19). Because the endogenous protein kinase activity of the rat parotid BLM appears to be quite high and is clearly able to overpower the endogenous protein phosphatase activity (Fig. 1), we speculate that phosphorylation of NKCC1 on the 7-kDa peptide site is maintained near saturation levels in the intact acinar cell. This could account for the observation that no increase in phosphorylation at this site is observed with isoproterenol stimulation despite the resulting activation of PKA.

one of the proteins phosphorylated and dephosphorylated by these enzymes is NKCC1 (Figs. 1–3). The membrane-associated protein kinase activity appears to be of the serine/threonine type, because its effect on NKCC1 was blocked by the general serine/threonine kinase inhibitors staurosporine and K252a; however, specific inhibitors of PKA, tyrosine kinase, and protein kinase C had little or no effect on this kinase activity even at high concentrations (Fig. 2). The membrane-associated phosphatase activity was inhibited by the nonspecific phosphatase inhibitor sodium orthovanadate, but more specific inhibitors of protein phosphatases 1 and 2A were relatively ineffective (Fig. 3).

Moreover, because our experiments were carried out in essentially Ca\(^{2+}\)-free medium (1 mM EGTA), the involvement of protein phosphatase 2B (calcineurin) also can be excluded. Further experiments designed to identify the specific protein kinases and phosphatases involved in these effects were not attempted.
The significance of this phosphorylation of NKCC1 in rat parotid acinar cells remains to be determined.

The fact that the phosphorylation of NKCC1 induced by isoproterenol treatment can be mimicked by the addition of cAMP to permeabilized acini and that this effect can be blocked by a specific peptide inhibitor of PKA (Fig. 6) provides strong evidence for the involvement of PKA in this effect. Because this phosphorylation cannot be produced by the addition of PKA to BLM, we conclude that some factor present in intact cells but missing in isolated BLMs may be involved. Further experiments are required to identify this (putative) participant in the phosphorylation reaction; however, an obvious candidate would be a cytosolic kinase that is activated by PKA. In this regard, Forbush and colleagues have presented evidence that NKCC1 can be upregulated as a result of phosphorylation by an as yet unidentified Cl−-dependent kinase (7, 11) and that this phosphorylation can be reversed by protein phosphatase type 1, which is directly targeted to NKCC1 (2). Whether these same enzymes are involved in β-adrenergic stimulation-dependent NKCC1 phosphorylation in salivary glands remains to be determined. If this is the case, however, then this kinase must be activated by a non-Cl−-dependent mechanism in these tissues because, as already stressed, intracellular Cl− levels in salivary acinar cells are not affected by β-adrenergic stimulation.

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