Src Family Tyrosine Kinase Regulates Intracellular pH in Cardiomyocytes

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Abstract. The Anion Cl^-/HCO_3^- Exchangers AE1, AE2, and AE3 are membrane pH regulatory ion transporters ubiquitously expressed in vertebrate tissues. Besides relieving intracellular alkaline and CO_2 loads, the AEs have an important function during development and cell death and play a central role in such cellular properties as cell shape, metabolism, and contractility. The activity of AE(s) are regulated by neurohormones. However, little is known as to the intracellular signal transduction pathways that underlie this modulation. We show here that, in cardiomyocytes that express both AE1 and AE3, the purinergic agonist, ATP, triggers activation of anion exchange. The AE activation is observed in cells in which AE3 expression was blocked but not in cells microinjected with neutralizing anti-AE1 antibodies. ATP induces tyrosine phosphorylation of AE1, activation of the tyrosine kinase Fyn, and association of both Fyn and FAK with AE1. Inhibition of Src family kinases in vivo by genistein, herbimycin A, or ST638 prevents purinergic activation of AE1. Microinjection of either anti-Cst.1 antibody or recombinant CSK, both of which prevent activation of Src family kinase, significantly decreases ATP-induced activation of AE. Microinjection of an anti-FAK antibody as well as expression in cardiomyocytes of Phe397 FAK dominant negative mutant, also prevents purinergic activation of AE. Therefore, tyrosine kinases play a key role in acute regulation of intracellular pH and thus in cell function including excitation–contraction coupling of the myocardium.

Intracellular pH (pH_i)[1] regulates various cellular functions including Ca^{2+} homeostasis, ionic channels (Busa and Nuccitelli, 1984), gene expression (Isfort et al., 1993), and cell death (Reynolds et al., 1996). In cardiac cells, pH_i is a major regulator of the excitation–contraction coupling. pH_i modulates excitability (e.g., ionic conductances), contractility (e.g., Ca-sensitivity of myofilaments), and conduction (e.g., gap–junction conductance; Orchard and Kentish, 1990). Besides proton pumps and channels, most cells possess three membrane ionic transporters that closely regulate their pH_i. Under acid loads, both a Na^+/H^+ antiport and a Na^+-dependent HCO_3^- transporter are switched on to restore basal pH_i. In contrast, a Na-independent Cl^-/HCO_3^- exchanger that operates as a HCO_3^- extruder alleviates intracellular alkaline and CO_2 loads (Madshus, 1988).

The anion Cl^-/HCO_3^- exchangers (AE) belong to a multigenic family that comprises AE1, AE2, and AE3. The AEs are ubiquitously expressed in vertebrate tissues (Alper, 1991; Kay et al., 1995). AE1 is expressed in erythrocytes and encodes the Band3 protein, a major membrane protein that has been extensively studied (Wang, 1994). Truncated forms of AE1 are also present in kidney (Brosius et al., 1989) and heart (Puceat et al., 1995). AE2 is an housekeeping gene that is expressed in most tissues at least at the level of mRNA (Kudrycki et al., 1990). AE3 is expressed in brain (bAE3) as a protein of 160 kD (Kopito et al., 1989) and as a shorter polypeptide (cAE3, 120 kD) in heart (Linn et al., 1992).

Besides cytosolic pH regulation, the presence of AEs in nuclear, golgi, and mitochondrial membranes (Kay et al., 1990) suggests that they may play a role in regulation of pH of these intracellular compartments. The AEs also feature non-pH regulatory functions. They have critical functions in embryogenesis and morphogenesis during devel-

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1. *Abbreviations used in this paper*: AA, amino acids; AE, anion Cl^-/HCO_3^- exchangers; FAK, focal adhesion kinase; GFP, green fluorescent protein; ODNAS, oligodeoxynucleotide antisense; PI3K, phosphatidylinositol-3-kinase; pH_i, intracellular pH; PKC, protein kinase C.

opment (Zhao et al., 1995). AE1 participates in cell death; it generates a surface senescent cell antigen, a protein that appears on old cells and marks them for removal by the immune system (Kay, 1981). The AE proteins also play a central role in such cellular properties as cell shape by anchoring the cytoskeleton to the plasma membrane (Jay, 1996), metabolism by binding glycolytic enzymes (Low et al., 1993), and cell contractility (Puceat et al., 1995).

The anion exchange performed by the AEs proteins is modulated by pH_i and by hormones or neurotransmitters in various tissues (Ganz et al., 1990; Muallem and Loessberg, 1990; Puceat et al., 1991; Desilets et al., 1994). However, no insight into the mechanism of this neuromodulation has been yet provided in any cell type.

Thus, our goal was to identify the particular AE protein that is activated by a neurohormonal stimulus and to uncover the signal transduction pathway that mediates this effect. To this end, we took advantage of ATP, a purinergic agonist that strongly activates the Cl^-/HCO_3^- exchanger in cardiac cells (Puceat et al., 1991*a*), in osteoclasts (Yu and Ferrier, 1995) and likely in neurons (de Souza et al., 1995) to investigate both the AE isoform involved and the signal transduction pathway that links the purinergic receptor to activation of AE. It was found that the purinergic agonist, ATP, triggered concomitant and specific tyrosine phosphorylation of AE1 and activation of anion exchange. ATP induced activation of the tyrosine kinases Src and Fyn and association of both Fyn and FAK with AE1. Specific inhibition of Src family kinases in vivo prevented purinergic activation of AE1. Thus, these results suggest that tyrosine kinases play a major role in acute regulation of intracellular pH and exert an important function in excitation–contraction coupling of the myocardium, a concept that could also be extended to other excitable or nonexcitable cells in which AE functions have been shown to be modulated by neurohormones.

Materials and Methods

Monitoring of pH_i in Cardiac Cells

Cardiac cells were isolated from hearts of 200–250-g male Wistar rats using collagenase (CLS4; Worthington, Freehold, NJ) as described previously (Puceat et al., 1995). 6–10 $\times$ 10^6 cells/heart were routinely obtained. Cardiomyocytes were isolated from 2–3-d-old neonatal rats and kept in culture for 5 d (Puceat et al., 1994). Isolated ventricular cells were loaded for 20 min at 37°C with 5 μM Snarf-1/AM (Molecular Probes, Eugene, OR). The cells were transferred to the stage of an epifluorescence microscope and superfused with a medium containing 117 mM NaCl, 5.7 mM KCl, 1.2 NaH_2PO_4, 24 mM $NaHCO_3$, 1.7 mM $MgCl_2$, 1.0 mM $CaCO_3$ bubbled with O_2/CO_2, 95%/5%. In Cl-free solution containing 19 mM $NaHCO_3$, Na-gluconate, $KHCO_3$, and $MgSO_4$ replaced NaCl, KCl, and $MgCl_2$, respectively. The field was illuminated at 514 nm with a Xenon lamp. The images were recorded at 580 and 640 nm using a Wwiever and a CCD camera (Hamamatsu Phototonics, Hamamatsu City, Japan). The ratio of fluorescence intensity of images acquired at each wavelength was calculated on line by a computer (Argus software, Hamamatsu). An external calibration using nigericin allowed for the calculation of pH_i as described previously (Puceat et al., 1991*a*). The experiments were performed at 35 ± 1°C. The Cl^-/HCO_3^- activity was estimated from the slope of alkalinization calculated within the first two min after the removal of Cl^- ions from the cell superfusing buffer. The equivalent H^+ flux (dpH/dt) was calculated assuming a cell buffering capacity of 35 and 40 mM in neonatal (Korichneva et al., 1995) and adult (Puceat et al., 1995) rat cardiomyocytes, respectively.

Transfection and Transferrinfection of Neonatal Rat Cardiomyocytes

Neonatal rat cardiomyocytes maintained in culture for 2–3 d were transfected with 250 nM AE3 oligodeoxynucleotide sense (AGGGGAATGACAAG) or antisense (CTTGTCATTCCCCT) corresponding to nucleotides −6 to +7 of rat AE3 cDNA (Linn et al., 1992) using 3 μg/ml DOSPER (Boehringer, Meylan, France) for 6 h in serum- and antibiotic-free medium. This protocol allows for 95% of transfection efficiency as assessed by FACS® analysis of cells transfected with fluorescein-conjugated ODN. Confocal microscopy of the transfected cells further showed the intracellular distribution of the ODN. The high yield of transfected cells favored the use of neonatal rat cardiac cells in this set of experiments. After washing, the cells were kept in medium added with 5% heat-inactivated fetal calf serum for 36 h and pH_i was monitored as described above.

To transfect plasmids encoding wild-type FAKY397 or the dominant negative mutant FAKF397 in neonatal rat cardiomyocytes, a transferrinfection protocol using the replication-deficient Adl5dl312 adenovirus was used (Curiel et al., 1991; Wagner et al., 1992). The virus was amplified in HEK293 cells. In brief, HEK393 cells were infected with 1 PFU/cell of Adl5dl312 virus. Cells were scraped 24 or 48 h later and lysed by three cycles of freezing and thawing in phosphate-buffered solution. The virus was then purified by density centrifugation on a two step CsCl gradient. The band containing the viral particles was collected and dialyzed at 4°C for 2 h against 10 mM Tris, 1 mM $MgCl_2$, and 10% glycerol.The virus preparation was then pooled and frozen in aliquot at −80°C. 4 $\times$ 10^{10} viral particles were incubated with 1.2 μg poly-L-lysine in 400 μl serum-free medium for 30 min at 20°C. Then 0.5 μg of FAK plasmid and 0.5 μg of pGREEN LANTERN-1 plasmid (GIBCO BRL, Eragny, France) containing the reporter gene green fluorescent protein (GFP) were added and the mixture was further incubated for 30 min. 0.3 μg of poly-L-lysine was added and the complex adenovirus-poly-L-lysine-DNA was added to the cells in a final volume of 1 ml. Cells were infected for 1 h at 37°C and then extensively washed. The experiments were carried out 24 h after transferrinfection. The fluorescence of GFP allowed us to recognize the transferrinfected cells to record pH_i. To estimate the level of expression of FAK Y397 or F397, cells were immunostained with the antibody directed against the NH_2-terminal domain of FAK and observed using confocal microscopy as previously described (Pucéat et al., 1995).

Immunoprecipitation of Proteins

Whole cell lysates were prepared by incubation of adult rat cardiac cells for 20 min at 4°C in NET-N buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 50 mM NaF, 1 mM Na_3VO_4, 1% NP40, 1 mM PMSF, and 10 μg/ml leupeptin. The cell sample was spun at 12,000 *g* for 15 min and precleared for 1 h at 4°C with protein A–Sepharose. The lysates were spun and the supernatant was used for immunoprecipitation. The primary antibody was added for 2 h at 4°C. Protein antibody complex bound to protein A–Sepharose was pelleted by spinning down the lysates. The pellets were washed twice with NET-N buffer, once with NET buffer and boiled in the presence of Laemmli electrophoresis buffer. The samples were run in 7.5% SDS PAGE and electrophoretically transferred to nitrocellulose. The blots were treated as previously described (Puceat et al., 1995) and probed with the antibody recognizing the protein of interest and a secondary peroxidase-conjugated antibody. The proteins were revealed using ECL reagent. The bands on the films were quantified by an imaging system (SCION IMAGE software; NIH, Bethesda, MD). The Swiss protein database revealed that the amino acid sequence of the peptides (RKFKNSTYFPGK and FKPKYHPDVPF amino acids (AA) 607–618 and 814–826, respectively (second and fifth intracellular loop of mouse AE1), used to raise the anti-rat AE1 antibodies do not share homology with any other known protein sequence (accession number of mouse AE1 P04919). The antibody did not cross-react with AE2 or AE3 (data not shown). The anti-AE3 antibodies were raised against the cardiac-specific NH_2-terminal domain (AA 18–30) and the COOH-terminal domain (AA 999–1010) that is shared by both brain and cardiac rat AE3 (Linn et al., 1992; Puceat et al., 1995). The preimmune AE1 serum and/or the antiserum incubated in the presence of the peptide used to raise it was used as a control in the immunoprecipitation and coimmunoprecipitation experiments.

Measurement of Tyrosine Kinase Activity

Cell lysates were subjected to immunoprecipitation using an anti-Cst.1, a specific anti-Src (clone 2:17) or anti-Fyn (anti-Fyn.2) antibody. The im-

mune complex was washed with NET-N buffer three times, once with TBS, and once with kinase buffer (10 mM Pipes and 10 mM $MnCl_2$, pH7). The kinase reaction was run for 10 min at 30°C in the presence of 5 μCi γ-[^{32}P]ATP and 6 μg acid-denatured enolase or no substrate for autophosphorylation. The reaction was stopped by adding Laemmli buffer. The complex was run in SDS-PAGE. After staining and destaining, the gel was dried and exposed to autoradiography films. In the autophosphorylation experiments, the gels were treated with KOH (1 N) for 1 h at 60°C before autoradiography. Phosphorylation of enolase was quantified using SCION IMAGE software. In some experiments, after the in vitro kinase assay, the anti-Src or anti-Fyn immunocomplex was electrophoresed and transferred to a nitrocellulose membrane. The blot was cut, the upper part was probed with the anti-Src or -Fyn antibody, and the lower part was subjected to autoradiography to reveal enolase phosphorylation.

Microinjection of Cardiomyocytes

Microinjection of neonatal rat cardiac cells was performed according to Shubeita et al. (1994). The pipette concentration of antibodies was 500 μg/ml (unless otherwise mentioned) in 150 mM KCl, 0.025 mM EGTA, and 1 mM Pipes, pH 7.2. Antibodies were affinity-purified and concentrated as described in Roche et al. (1995*a*). Polyclonal anti-Yes.6 antibody was raised against the AA 15–35 of mouse Yes. CSK purified from Sf-9 insect cells expressing human CSK (Koegl et al., 1994) was used at 100 μg/ml and boiled CSK (3 min, 100°C) served as a control. AE activity was recorded on cells 1 h after microinjection. Neonatal rat cells were chosen in this set of experiments because diffusion of antibodies (microinjected close to the nucleus) was facilitated by the small volume of the cytosol and by the shape of the myocytes.

Results

ATP Activates the Cl^-/HCO_3^- Exchanger in Neonatal and Adult Rat Cardiac Cells

We previously demonstrated in adult rat cardiac cells that ATP activated the Cl^-/HCO_3^- exchange when this latter was silent when cells feature a resting pH_i around 7.1; that resulted in an intracellular acidosis (Puceat et al., 1991). We now investigated whether the purine could exert the same stimulatory effect when the anion exchanger was working, namely when chloride transmembrane gradient and in turn resting pH_i was changed. Adult and neonatal cardiomyocytes featured a resting pH_i of 7.18 ± 0.04 (n = 72) and 7.27 ± 0.05 (n = 392), respectively. ATP, applied to isolated cardiac cells strongly accelerated the Cl^-/HCO_3^- exchange that worked either under normal physiological direction or in the reverse mode (i.e., exchanging intracellular Cl^- for extracellular HCO_3^- ions). In the latter case, ATP increased by 2.20 ± 0.15-fold the rate of alkalinization that developed when cells were superfused with a Cl^--free buffer. On adding back Cl^- ions, the recovery of cell pH_i towards basal value was also accelerated to the same extent (2.18 ± 0.09-fold) by the presence of extracellular ATP (Fig. 1). The magnitude of purinergic stimulation of AE activity was the same in adult and neonatal rat cardiomyocytes measured in 72 and 392 cells, respectively. Thus, we confirm that ATP is a strong activator of the cardiac Cl^-/HCO_3^- exchange both in neonatal and adult rat cardiomyocytes as previously shown (Puceat et al., 1991*a*).

Prevention of AE3 Expression and Blocking Anti-AE1 Antibodies Reveal that AE1 Is the Target of the Purinergic Stimulation

The next question we addressed was the identification of the AE isoform that mediates the purinergic activation of

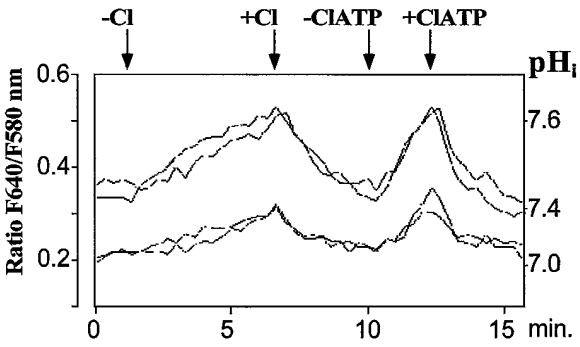

Figure 1. ATP enhances AE1-mediated Cl^-/HCO_3^- exchange activity. Cardiomyocytes isolated from adult rat hearts were superfused with a Cl-free buffer (*−Cl*) that reverses the exchanger leading to HCO_3^- influx and thus to an increase in pH_i. Cl^- ions were added back (*+Cl*) to the buffer and the same protocol was repeated in the presence of 50 μM ATP. pH_i was monitored in cardiac cells as described in Materials and Methods. The four traces represent individual cells.

the Cl^-/HCO_3^-. Both AE1 and AE3 proteins are expressed in cardiac cells (Korichneva et al., 1995; Puceat et al., 1995). AE2 is also expressed at the level of mRNA in whole heart (Kudrycki et al., 1990; Wang et al., 1996). Using RT-PCR, we detected AE2 mRNA in isolated neonatal and adult rat cardiomyocytes. However, despite several attempts using specific anti-AE2 antibodies that recognized well the protein in stomach membrane or MDCK cells, we failed to detect the protein in cardiac cell (not shown). Thus, AE1 and AE3 remained the putative proteins, responsible for cardiac Cl^-/HCO_3^- exchange. To determine which AE is stimulated by the purinergic agonist, we transfected neonatal rat cardiomyocytes with an oligodeoxynucleotide antisense (ODNAS) targeted at the translation initiation codon of cardiac AE3 (Linn et al., 1992). The ODNAS specifically decreased the level of the 120-kD cardiac AE3 protein by 70 ± 9% (n = 4), whereas AE1 expression was not affected; we also observed a significant decrease in the level of the 160-kD bAE3 protein (by 59 ± 6%, n = 4; Fig. 2 *A*, *inset*). However, the magnitude of purinergic stimulation of the Cl^-/HCO_3^- exchange activity was not affected in ODNAS-transfected cells when compared with ODN sense–transfected cells (Fig. 2 *A*) or nontransfected cells (see Fig. 2 *B*). Because cardiac truncated AE1 is not cloned and, thus, the translation initiation site of this gene not known, we were unable to use the same approach to knockout AE1 expression. Rather, specific anti-AE1 antibodies were raised against the second and fifth cytoplasmic loops of rodent AE1, regions close to the region presumed to be the pore of the transporter (Muller-Berger et al., 1995). Microinjection into neonatal rat cardiomyocytes of a mixture of the two purified antibodies that specifically recognize the 80-kD cardiac AE1 as previously shown (Korichneva et al., 1995; Puceat et al., 1995) (Fig. 2 *B*, *inset*) significantly decreased the anion exchange (by 74 ± 8%, n = 85) and fully prevented purinergic activation of the Cl^-/HCO_3^- exchange whereas injection of the same antibodies preincubated in the presence of the peptides used to immunize the rabbits did not exert any significant effect (Fig. 2 *B*). We also re-

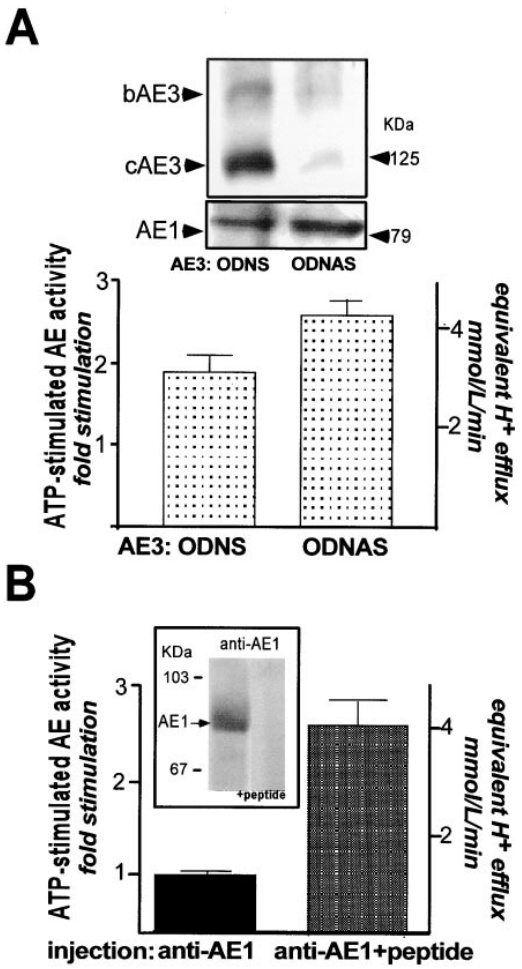

Figure 2. AE1 is the target for ATP-induced activation of the anion exchange. (*A*) pH_i was measured in neonatal rat cardiomyocytes 36 h after transfection of AE3 ODNS or ODNAS. ATP-induced activation of AE was calculated from the slopes of alkalinization observed when Cl^- ions are removed from the cell superfusing solution as shown in Fig. 1 *A*. Data are expressed as the mean ± SEM of at least 30 cells from four separate transfection experiments. The inset shows representative anti-AE3 and anti-AE1 immunoblots of proteins (150 μg) from a crude membrane fraction prepared the day of pH measurement from ODNS- or ODNAS-transfected cells. The anti-AE3 blot was probed using the antibody raised against the COOH-terminal domain of AE3 shared by both cardiac and brain AE3. The anti-AE1 blot was probed using the anti AE1 antibody raised against the AA 607–618 or mouse AE1 (*B*) a mixture of two purified anti-AE1 antibodies (1 mg/ml) preincubated or not in the presence of 10 μg/ml of the two peptides used to immunize the rabbits were microinjected into neonatal cardiomyocytes and the purinergic activation of AE (with 50 μM ATP) activity was tested in Snarf-loaded cells 1 h after injection. The bar graph represents the mean ± SEM of at least 75 cells from three separate cultures in each experimental condition. The inset shows a Western blot of membrane protein (150 μg) indicating the specificity of the preparation of anti-AE1 antibodies used in the microinjection experiments.

corded ATP-induced activation of the DIDS-sensitive Cl^-/HCO_3^- exchange activity in MDCK cells that possess endogenous AE1 and AE2 but not AE3 proteins (2.2 ± 0.3-fold stimulation; $n = 17$; $P = 0.001$). Therefore, AE1 mediates purinergic activation of the Cl^-/HCO_3^- exchange, which strengthens our previous proposal that AE1 plays the role of anion exchanger in cardiac cells (Puceat et al., 1995).

Purinergic Stimulation of Cells Induces Phosphorylation of AE1 on Tyrosine Residues

We then investigated the signal transduction pathway that underlies ATP-induced activation of AE. AE1 expressed in the heart as a protein of 80 kD (Puceat et al., 1995), features several potential tyrosine phosphorylation sites (Yannoukakos et al., 1991) and has been shown to be an in vitro substrate for several tyrosine kinases including Src, Abl, the EGF receptor, Syk (Harrison et al., 1994), and ZAP-70 (Watts et al., 1996). Furthermore, although ATP activates protein kinase C (PKC; Pucéat et al., 1994) we failed to detect any effect of PKC inhibitors (chelerythrine or staurosporine) on ATP-induced activation of Cl^-/HCO_3^- exchanger (data not shown). Thus, we examined whether tyrosine kinases could play a role in ATP-triggered anion exchange activation. The antiphosphotyrosine immunoblot of immunoprecipitated AE1 showed a basal tyrosine phosphorylation of the protein. AE1 phosphorylation was dramatically increased during the first minute of purinergic stimulation, a time of maximal ATP-induced activation of the anion exchange (Puceat et al., 1991; Fig. 1). A 60-kD tyrosine phosphorylated protein also coimmunoprecipitated with AE1. The phosphorylation of the later protein showed the same early time course as AE1 phosphorylation. Using the same immunoprecipitation approach, we failed to detect any phosphorylation of AE3 (Fig. 3 *A*). Maximal AE1 phosphorylation (average of 2.8-fold) was reached at 3 min (Fig. 3, *A* and *B*).

ATP Activates Src Family Kinases

The cytoplasmic tyrosine kinases Src and Fyn were found expressed in both neonatal and adult cardiomyocytes. Their kinase activity was measured with an in vitro kinase assay after immunoprecipitation using the anti-Cst.1 antibody that recognizes both Src and Fyn (Roche et al., 1995). Extracellular ATP quickly and strongly activated the kinases as assessed from both phosphorylation of an exogenous substrate and autophosphorylation of kinases (Fig. 4 *A*). Kinase activation reached threefold after 0.5 min, reached a peak at 3–5 min and was sustained for at least 15 min (Fig. 4 *B*). Using a specific anti-Src or anti-Fyn antibody to selectively immunoprecipitate each kinase, we observed that Fyn was three times (3.5 ± 0.6, $n = 3$) as active as Src; such an observation could have been expected from the level of expression of these kinases assessed by Western blotting of proteins from a whole cell lysate (data not shown). However, both kinases were further activated in cells stimulated with ATP for 1 or 5 min (Fig. 4 *C*). It should be noted that the same amount of either Src or Fyn were immunoprecipitated in control and ATP-stimulated cells as assessed by Western blot. Furthermore, the comparison of the immunoreactive Src or Fyn after immunoprecipitation with the immunoreactive kinases on a Western blot of a whole cell lysate proteins showed that a large fraction of the kinases was pulled down. Because AEs are localized in cardiac costameres (Korichneva et al., 1995; Puceat et al., 1995), regions simi-

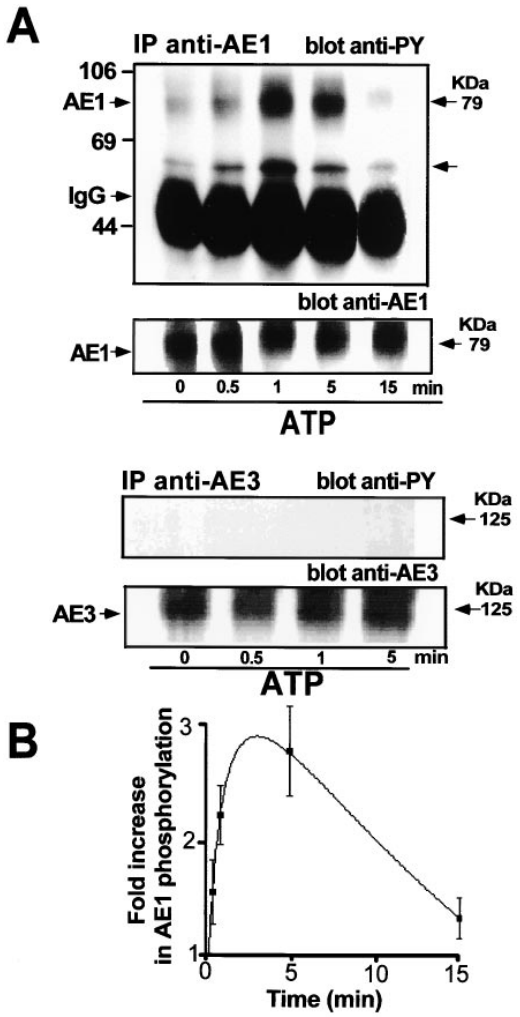

Figure 3. Purinergic stimulation of cardiomyocytes induces tyrosine phosphorylation of AE1. (*A*) Adult rat cardiomyocytes were stimulated for 0.5–15 min with ATP (50 μM), lysed, and then the whole cell lysate was subjected to immunoprecipitation using a specific anti-rat AE1 antiserum (raised against AA 608–618; *top blots*) or anti-AE3 antiserum (directed against the NH_2-terminal domain of rat AE3; *bottom blots*). The blots were probed with a monoclonal anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY), stripped, and then reprobed with the anti-AE1– or -AE3–purified antibody. Similar results were obtained in earlier experiments using the anti-whole erythroid band3 antibody (Puceat et al., 1995). (*B*) Graph summarizing the data illustrated in *A*. The results shown in this figure are normalized to the amount of immunoprecipitated AE1 and are the means ± SEM of at least four separate experiments.

lar to focal adhesion contacts, we examined whether focal adhesion kinase (FAK), another cytoplasmic tyrosine kinase (Schaller et al., 1992) was expressed in cardiomyocytes and if so, phosphorylated in ATP-stimulated cells. FAK was found expressed in both adult and neonatal rat cardiomyocytes and a marked tyrosine phosphorylation of FAK was observed within 1–5 min of purinergic stimulation. Maximal phosphorylation reached 3.1 ± 0.3-fold at 5 min (n = 5; Fig. 4 *D*). Despite numerous attempts, we failed to detect a stimulation of FAK activity when using an in vitro kinase assay after immunoprecipitation.

Src family kinases have been proposed to be activated by G protein-coupled receptor through activation of phosphatidylinositol-3-kinase (PI3K), through subunits of Gi protein or both (Luttrell et al., 1996; Lopez-Ilasaca et al., 1997). To test each of these possibilities, cells were first incubated for 15 min with 0.5 μM wortmannin. Src and Fyn were immunoprecipitated together by the anti-Cst.1 antibody and their activities measured using enolase as a substrate. ATP still induced a twofold stimulation of kinase activity (2.0 ± 0.1-fold vs 2.5 ± 0.2 in control in this series of experiments, n = 4). Treatment of cells with pertussis toxin (0.3 μg/ml for 4 h or overnight) did not affect ATP-induced activation of Src/Fyn either. Under this experimental condition, kinases were stimulated by 2.6 ± 0.3-fold (n = 4). PI3K or Gi protein did not participate in purinergic stimulation of AE activity since neither wortmannin or pertussis toxin pretreatment of cells affected the purinergic effect on the Cl^-/HCO_3^- exchange (2.1 ± 0.1-fold stimulation in control vs 2.1 ± 0.1 and 2.0 ± 0.1 in wortmannin and pertussis toxin-treated cells, respectively, n = 19 cells in each experimental condition).

ATP Induces AE1 Association with Fyn and FAK

We then tested whether physical interactions between tyrosine kinases and AE1 occur in vivo. AE1 and Fyn coimmunoprecipitated from both unstimulated and ATP-stimulated cells. More immunoreactive Fyn and AE1 was detected in the AE1 and Fyn immunocomplex, respectively, from ATP-stimulated cells. (Fig. 5, *A* and *B*). To assess whether Fyn was activated in the AE1 immunocomplex, we investigated whether AE1 immunocomplex displayed a tyrosine kinase activity using enolase as a substrate. After AE1 immunoprecipitation, we observed a basal kinase activity that was further enhanced by purinergic stimulation of cells as early as one minute. Kinase activity was not detectable when the immunoprecipitation was done with the anti-AE1 antibody preincubated with the peptide used to raise this antibody (Fig. 5 *C*). In line with AE1 and Fyn association, we also observed a coimmunoprecipitation of Fyn and FAK in ATP-activated cells. We failed to detect any coimmunoprecipitation of Src with AE1 or FAK (not shown), although we cannot exclude that it was due to the low level of this kinase in cardiac cells. Moreover, the 60-kD tyrosine phosphorylated protein coimmunoprecipitated with AE1 (Fig. 2 *A*) was recognized by the anti Fyn antibody (data not shown). AE1 was found associated with FAK in unstimulated cells and the association was increased upon ATP-stimulation (Fig. 5 *D*).

Activation of Src Family Kinases Are Required for Stimulation of Anion Exchange by ATP

Two strategies were used to determine whether purinergic activation of the tyrosine kinases pathway mediates increase in AE activity. First, adult rat cardiomyocytes were treated with either genistein or ST638, two mechanistically dissimilar tyrosine kinase inhibitors (Akiyama et al., 1987; Shiraishi et al., 1989). Both drugs significantly prevented ATP-induced acidification after activation of the Cl^-/HCO_3^- exchanger (Fig. 6 *A*) but did not affect isoproterenol-induced decrease in pH_i when tested on the same cells

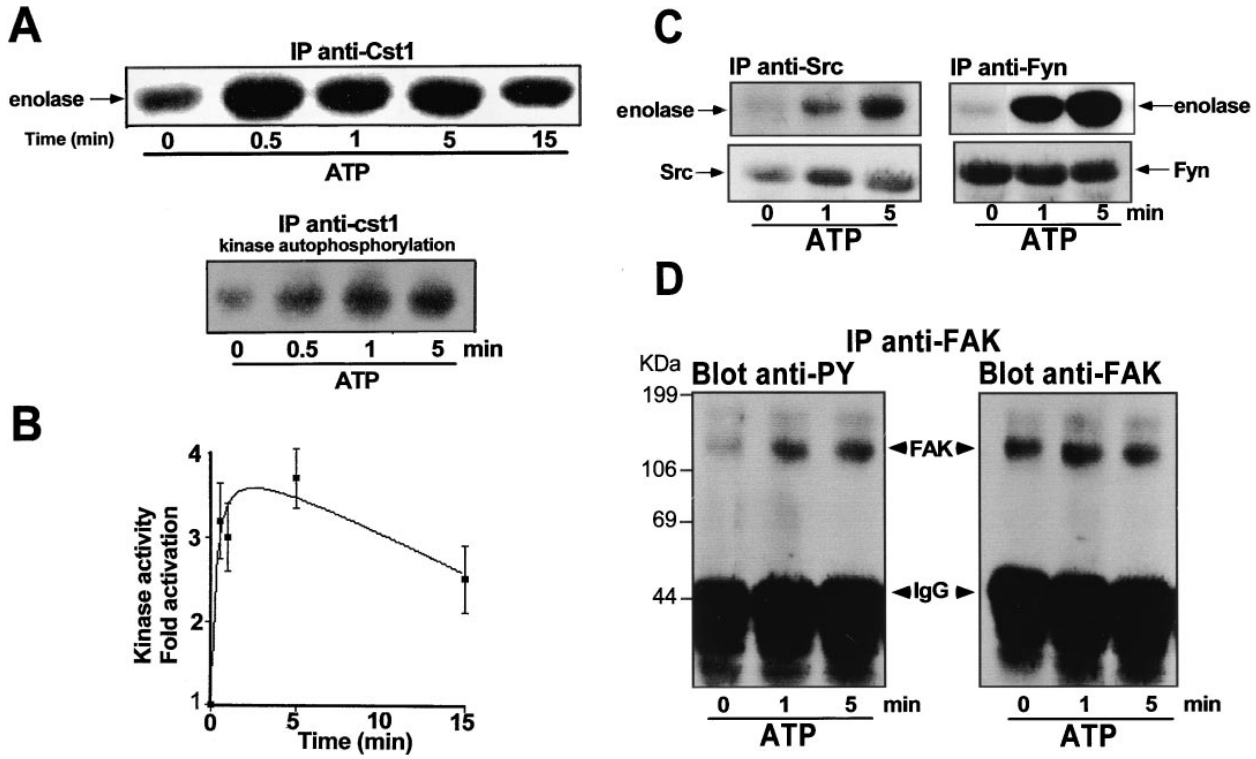

Figure 4. ATP activates Src and Fyn and phosphorylates FAK. (*A*) Adult rat cardiomyocytes were stimulated with 50 μM ATP for 0.5–15 min. The anti-Cst.1 antibody was used to immunoprecipitate the Src family tyrosine kinases. kinase activity was measured in the immune complex using acid-denaturated enolase as a substrate or no substrate(autophosphorylation). (*B*) Graph summarizing the results obtained in at least four experiments. (*C*) Cells were stimulated for 1 or 5 min with 50 μM ATP and specific anti-Fyn (Fyn2) or anti-Src (clone 2:17) antibodies were used for immunoprecipitation. (*D*) FAK was immunoprecipitated from a whole cell lysate prepared from unstimulated or ATP-stimulated cells. The Western blot was probed with an anti-phosphotyrosine antibody, stripped, and then reprobed with an anti-FAK antibody.

(not shown). Pretreatment of cardiomyocytes with herbimycin A, which acts through a third specific inhibitory mechanism (Fukazawa et al., 1991). also prevented the ATP-induced increase in Cl^-/HCO_3^- exchange activity (Fig. 6 *B*). It should be noted that cell treatment for 30 min with either genistein (20 μg/ml) or herbimycin A (2 μg/ml), abolished AE1 phosphorylation induced by extracellular ATP (Fig. 6 *A*, *inset*). To further identify the kinase that mediates purinergic activation of AE1, we microinjected into neonatal rat cardiomyocytes the affinity-purified anti-Cst.1 antibody that blocks the activity of both Src and Fyn (Roche et al., 1995). The stimulatory effect of ATP on AE activity was then abolished. Intracellular microinjection of an antibody raised against Yes, a Src family kinase that is not expressed in cardiomyocytes also failed to block the purinergic activation of AE when compared with IgG-injected cell. Furthermore, the extent of purinergic activation of AE (twofold) was about the same in microinjected and noninjected cells (compare Fig. 2 with Fig. 6, *A–C*). Interestingly, injection of an anti-Src antibody, known to inhibit cell division in some fibroblasts (Roche et al., 1995*b*), did not prevent ATP-induced activation of AE. As an alternative approach to inhibit Src family kinases, and to investigate the mode of activation of these kinases in cardiac cells, we microinjected a purified fraction of CSK (COOH-terminal Src Kinase). CSK specifically phosphorylates the key regulatory tyrosine 527 in the COOH-terminal domain of Src, Fyn and Yes (Cooper et al., 1986), which in turn suppresses their kinase activity. In CSK-injected cells, AE activity was no longer stimulated by extracellular ATP. Heat-denaturated CSK was ineffective indicating that the kinase activity of CSK is mandatory to inhibit purinergic activation of AE. To determine the role of FAK in purinergic activation of AE1, we microinjected an anti-FAK antibody raised against the NH_3-terminal domain of rat FAK (residues 1–376; Burgaya et al., 1997), a domain that contains the sequence that functions as an SH3-binding site for Src and Fyn (Thomas et al., 1997). In the microinjected cells, the purinergic activation of AE1 was significantly reduced (Fig. 7 *A*), whereas it was not affected in cells microinjected with the antibody preincubated with the immunogenic hexahistidine fusion protein (Burgaya et al., 1997; Fig. 7 *A*). To further investigate the participation of FAK in ATP-stimulated AE activity, we transferrinfected into cardiomyocytes plasmids encoding a wild-type FAK (FAKY397) or a mutated FAK (FAKF397) that should act in a dominant negative manner (Schlaepfer and Hunter, 1997). The wild-type and the mutated FAK was expressed to the same extent and did not show any differences in the intracellular localization as assessed by immunostaining combined to confocal microscopy (data not shown). AE activity was measured 24 h after transferrinfection. In cells transferrinfected with the wild-type FAK, ATP still strongly enhanced AE activity whereas in myocytes infected with the

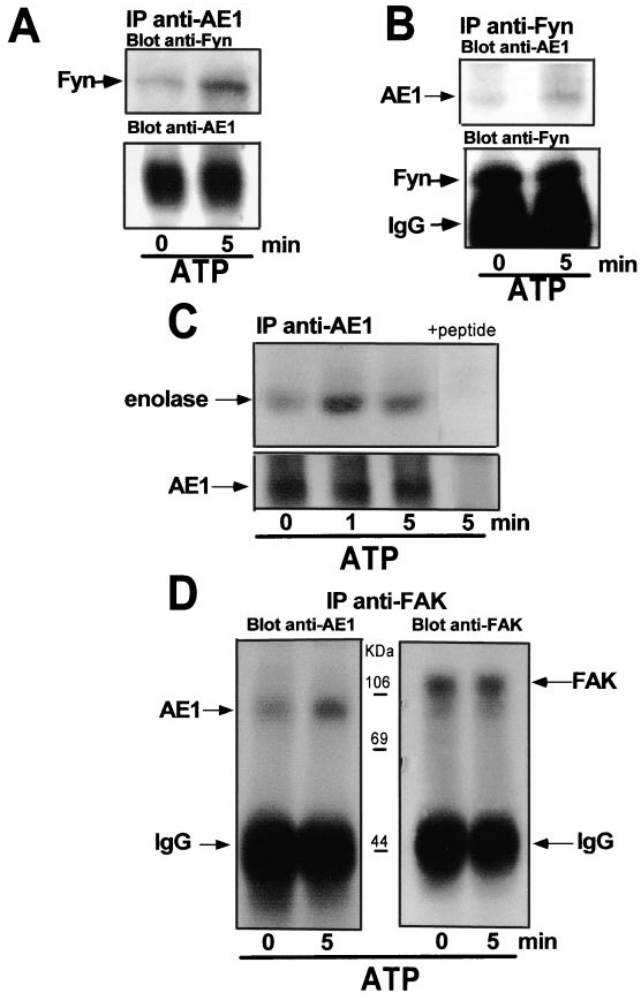

Figure 5. ATP induces association between Fyn, FAK, and AE1 in cardiomyocytes. (*A*) AE1 was immunoprecipitated from a whole cell lysate of unstimulated or ATP-stimulated cells. The blot was probed with the anti-Fyn antibody, stripped, and then reprobed with the anti-AE1 antibody. (*B*) Fyn was immunoprecipitated from a whole cell lysate of unstimulated or stimulated cells. The blot was probed with an anti-AE1 antiserum, stripped, and then reprobed with the anti-Fyn antibody. (*C*) AE1 was immunoprecipitated from a whole cell lysate of unstimulated or cells stimulated with 50 μM ATP for 1 or 5 min. The kinase activity was measured in the immune complex using enolase as a substrate. No enolase phosphorylation was detected when the preimmune AE1 serum was used (not shown) or with the immune serum in the presence of the peptide used to raised the anti-AE1 antibody. (*D*) The anti–FAK antibody was used to immunoprecipitate the kinase from a whole cell lysate prepared from unstimulated or ATP-stimulated cells. The blot was then probed with the anti-AE1 antiserum, stripped and reprobed with the anti-FAK antibody. The blots *A–D* are representative of three experiments giving similar results.

mutant FAKF397, purinergic activation of AE1 was dramatically reduced (Fig. 7 *B*).

Discussion

In this study, we have shown that purinergic stimulation of cardiac cells activates the Cl^-/HCO_3^- exchange and that this is associated with and dependent upon phosphorylation of the AE1 isoform of the AE family by Src family tyrosine kinases.

Cardiac cells express two isoforms of the AE family, namely AE1 and AE3 (Korichneva et al., 1995; Puceat et al., 1995). Although AE2 mRNA could be detected by RT-PCR or Northern blot by us and others (Kudrycki et al., 1990; Wang et al., 1996), we could never detect the AE2 protein. Furthermore, the poor DIDS sensitivity of AE2 protein (EC_{50},150 μM; Lee et al., 1991) makes it an unlikely candidate for the cardiac Cl^-/HCO_3^- exchange that is fully inhibited by low concentrations of DIDS (EC_{50}, 3 μM; Korichneva et al., 1995; Puceat et al., 1995). The involvement of AE1 and AE3 was tested in experiments designed to block their expression or function. The knock out of expression of cardiac AE3 by the antisense oligonucleotide targeted to the translation initiation codon did not affect the purinergic stimulation of the anion exchange. The lack of effect on AE1 expression demonstrated that the cAE3 oligonucleotide was AE3 specific although not fully specific of cAE3. Indeed, it did also decrease bAE3 expression. The latter effect could be attributed to the binding of the ODNAS to the sequence nt 306–332 and nt 1320–1326 of bAE3 cDNA (Kudrycki et al., 1990) that share 66% homology with the initiation translation site of cAE3 and to the low level of bAE3 protein in isolated rat ventricular cardiomyocytes. Although the antisense strategy resulted in a 70–80% decrease in AE3 expression, it could be argued that the 20–30% of cAE3 left after treatment of cells with antisense could be sufficient to mediate the purinergic stimulation of the anionic transport. Thus, we also used an alternative approach in which we microinjected anti-AE1 antibodies into neonatal cardiomyocytes. The antibodies dramatically decreased the anionic exchange activity as previously observed with an anti-whole band3 protein antibody AE3 (Puceat et al., 1995); they further abolished the purinergic stimulation of the anion exchange. Altogether, our data demonstrate that AE1 is the particular isoform that mediates the purinergic stimulation of the anion exchange. We also recorded ATP-induced activation of the DIDS-sensitive Cl^-/HCO_3^- exchange activity in MDCK cells that possess endogenous AE1 and AE2 but not AE3 proteins which suggests that purinergic activation of AE1 is not a mechanism restricted to cardiac cells.

The main question we addressed in this study was to uncover the signal transduction pathway that underlies the purinergic activation of the Cl^-/HCO_3^- exchanger. We found that ATP strongly and rapidly activates Src and Fyn and induces tyrosine phosphorylation of FAK. That AE1 is a target for these kinases can be seen both from the concomitant phosphorylation on tyrosine residues of AE1 and from Fyn and FAK associations with this anion exchanger. Using enolase as a substrate, we could also demonstrate a tyrosine kinase activity that was further activated in ATP-stimulated cells in AE1 immunocomplex. Since FAK was phosphorylated and found associated with AE1 in ATP-stimulated cells, this kinase may also be involved in the process. Microinjection of an anti-FAK antibody directed against the NH_2-terminal domain of the kinase or expression of a dominant negative FAKF397 mutant that are likely to prevent or displace endogenous Fyn/FAK or FAK/AE1 interaction, respectively, significantly inhibited

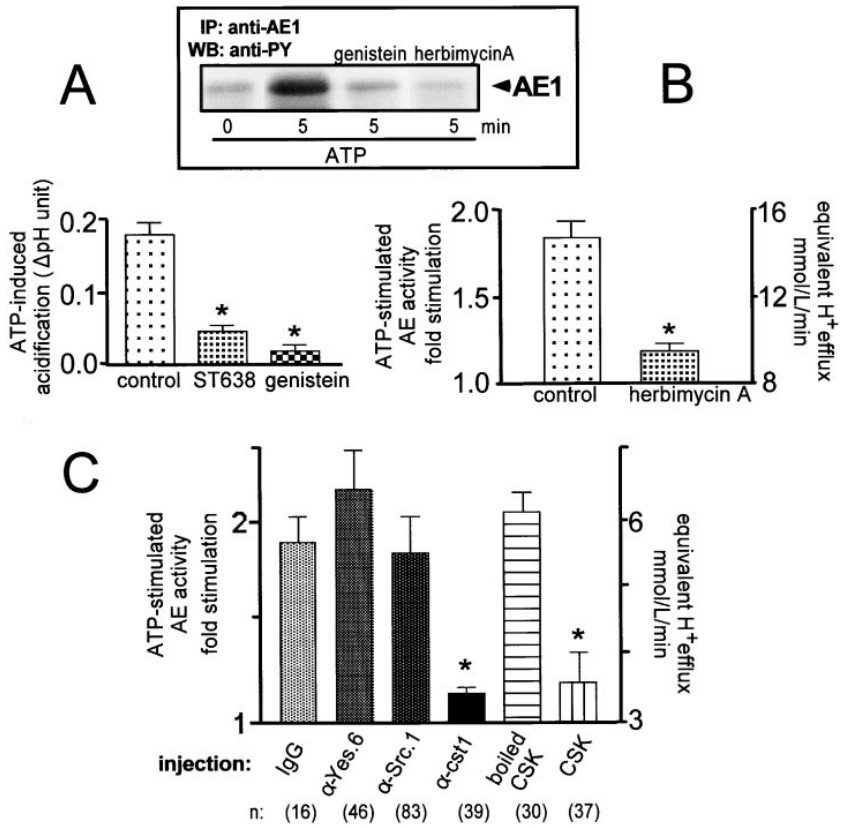

Figure 6. The Src family of tyrosine kinases mediates ATP-induced activation of the Cl^-/HCO_3^- exchanger. (*A*) Tyrosine kinase inhibitors prevented ATP-induced acidification and AE1 phosphorylation. Adult rat cardiomyocytes were incubated for 30 min at 37°C in the presence of 20 μg/ml of genistein or 100 μM ST638. Cells were then loaded with Snarf1/AM and the effect of ATP (50 μM) on pH_i was measured. The inset shows an antiphosphotyrosine blot after AE1 immunoprecipitation from control or ATP-stimulated cells preincubated or not with genistein or herbimycin A. (*B*) Herbimycin A prevented ATP-induced activation of the exchanger. The adult rat cardiomyocytes were treated for 30 min at 37°C with 2 μg/ml herbimycin A. (*C*) Microinjection of the anti-Cst.1 antibody and of purified CSK abolished ATP-induced activation of AE in neonatal rat cardiomyocytes. Purified IgG, anti-Yes.6, anti-Src.1, anti-Cst.1, native purified CSK, or boiled CSK were injected into the cells. The AE activity was measured 1 h later. In *B* and *C*, AE activation was determined as described in Fig. 1. In *A* and *B*, the results are expressed as means ± SEM of at least 20 cells from three different cell preparations. *Significantly different from control (Student's *t* test, $P < 0.01$). In *C*, results are expressed as means ± SEM (*n*, numbers of microinjected cells investigated from at least three different cell cultures are indicated below the bars). *Significantly different from the respective control, rabbit affinity-purified IgG for the antibodies, boiled CSK for CSK (Student's *t* test, $P < 0.01$).

ATP-induced activation of AE1.We could not detect FAK activation in ATP-stimulated cells. Dissociation of tyrosine phosphorylation from activation of PYK2, a tyrosine kinase that belongs to the same family as FAK, has previously been observed in PC12 overexpressing activated Src (Dikic et al., 1996). FAK was also recently suggested to play a role of adaptor protein rather than the one of a kinase (Burgaya et al., 1997). Thus, we rather suggest that Fyn is responsible for AE1 phosphorylation and that FAK serves as a docking protein for Fyn.

We also attempted to understand how Fyn was activated after purinergic stimulation of cardiac cells. Wortmannin, used at a concentration high enough to inhibit PI3K including the G protein regulated *P* 110 PI3K isotype highly expressed in the heart (Stephens et al., 1994; Stoyanov et al., 1995), did not significantly prevent Src/Fyn or AE activation. Pertussis toxin treatment also did not affect kinase or AE activity. Thus, our findings rule out the participation of a Gi protein and of PI3K in the purinergic signaling pathway.

Three mechanisms for Src/Fyn activation have been proposed: first, dephosphorylation of the inhibitory tyrosine 527, second, displacement of the C-tail from the SH2 domain, and third, displacement of the SH2-SH1 linker from the SH3 domain (Shalloway and Taylor, 1997). Microinjection of CSK prevented purinergic activation of the AE, strongly suggesting that a dephosphorylation of tyrosine 527 of Fyn is necessary for its activation and in turn stimulation of AE. Thus, we predict that the earliest event in the purinergic signal transduction pathway is activation of a tyrosine phosphatase.

To test whether activation of Src family kinases is mandatory for ATP-induced activation of AE, we used three pharmacological agents (e.g., genistein, herbimycin, ST638) that each displays different inhibitory mechanisms to prevent ATP-induced activation of tyrosine kinases in cardiac cells. Whatever their mechanism of action (e.g., blocking of ATP binding site, catalytic site, or reduction of sulfhydryl groups), they all blocked ATP-mediated stimulation of anion exchange activity. To further characterize the tyrosine kinase involved in the phosphorylation of AE1, we used a more specific approach. We microinjected blocking antibodies into cardiac cells. The anti-Cst.1 antibody that inhibits Src, Fyn, and Yes but not IgG or anti-Yes antibody prevented ATP-induced stimulation of anion exchange showing the specificity of the anti-Cst.1 antibody inhibition. The anti-Src antibody did not significantly affect the purinergic effect suggesting a role of Fyn in AE1 activation; this is in agreement with the association of Fyn but not Src with AE1 and with the similar time courses of Fyn autophosphorylation and AE1 phosphorylation. Intracellular injection of a purified fraction of CSK that phosphorylates the inhibitory tyrosine residue 527 of Src family kinases also strongly prevented ATP-triggered anion exchange activation. Purinergic stimulation of cardiomyocytes induces the tyrosine phosphorylation of many pro-

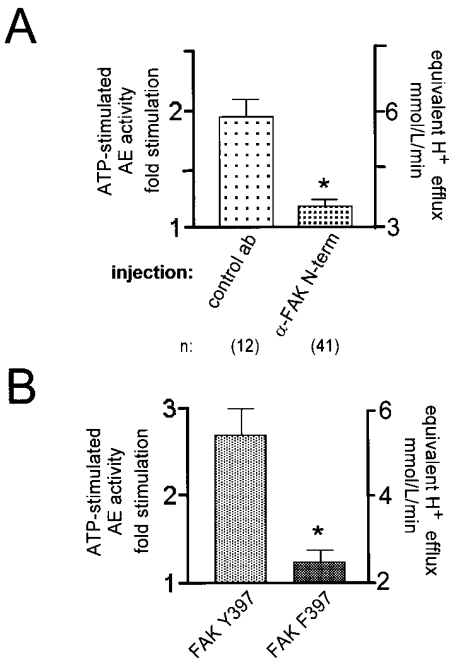

Figure 7. (*A*) Microinjection of the anti-FAK antibody directed against the NH_2-terminal domain prevented ATP-induced activation of AE in neonatal rat cardiomyocytes. A purified antibody anti–NH_2-terminal (*α-FAK N-term*) domain of FAK was microinjected into cardiomyocytes and AE activity was measured 1 h later. The antibody preincubated with the hexahistidine fusion protein bound to Ni-agarose was microinjected as a control (*control ab*). Results are expressed as means ± SEM (*n*, numbers of microinjected cells investigated from at least three different cell cultures are indicated below the bars). (*B*) Neonatal rat cardiac cells were transferrinfected with plasmids encoding wild-type FAK (*FAKY397*) or the dominant negative FAK mutant (*FAKF397*). AE activity was measured 24 h after transferrinfection in 14 and 13 infected cells (GFP positive), respectively. Results are expressed as means ± SEM.

teins (Puceat and Vassort, 1996) in addition to AE1. Whether direct AE1 phosphorylation is required for activation of the anion exchange is likely but cannot be ascertained from our data. Phosphorylation on tyrosine residue of a regulatory protein or of cytoskeleton proteins may also result in AE1 activation. When cardiac truncated AE1 will be cloned, mutation of the tyrosine phosphorylation, and/or Fyn-binding sites will be helpful to bring further information as to the role of AE1 phosphorylation in the neurohormonal activation of the anion exchange. Nevertheless, we can conclude that activation of Src family tyrosine kinase is a critical required step for ATP-induced stimulation of anion exchange.

How does AE1 get activated after ATP stimulation? We postulate that ATP-induced phosphorylation of FAK provides a docking site for Fyn through its SH2 and SH3 domains (Cobb et al., 1994; Thomas et al., 1997). FAK then recruits Fyn to the costameres where it phosphorylates AE1. Interestingly, the role of tyrosine kinases in cardiac cell homeostasis is not restricted to AE. Tyrosine phosphorylation of PLCγ accounts for ATP-induced IP_3 generation (Puceat and Vassort, 1996) and this phenomenon is likely to modulate Ca release from intracellular stores. The later process is facilitated by AE-mediated acidosis that contributes to a large extent to purinergic arrhythmogenesis (Puceat et al., 1991*b*). Thus, rapid activation of tyrosine kinases of the Src family by neuromediators allows for an acute regulation of both H^+ and Ca^{2+} homeostasis in cardiac cells. Tyrosine kinases are indeed expected in this fashion to play a major role in excitation–contraction coupling of the myocardium and to be involved in cardiovascular diseases such as arrhythmia. In other excitable or nonexcitable cells that all express AE family proteins, tyrosine kinases activated by purinergic or other G-protein–coupled receptor agonists may play an important regulatory role in H^+ homeostasis, a major modulator of cell function.

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