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pp60^{c-src} KINASE ACTIVITY IN BOVINE CORONARY EXTRACTS IS STIMULATED BY ATP

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SUMMARY: pp60^{c-src} kinase is believed to participate in regulating key cellular mechanisms including signal transduction and differentiation of smooth muscle during early embryogenesis. In this study, pp60^{c-src} kinase activity was demonstrated in extracts from adult bovine coronary arterial smooth muscle. Activity, reflected by autophosphorylation of pp60^{c-src}, phosphorylation of exogenous substrates, and phosphorylation of several endogenous substrates, was enhanced about 2 fold when added Mg^{2+} was replaced by Mn^{2+}. Unexpectedly, activity was dramatically stimulated 20-50 fold by prior incubation with ATP. Such stimulation appears to be mediated through a novel mechanism which is independent of ATP-induced phosphorylation of reaction components. These new observations strongly suggest that a unique mechanism exists for regulation of coronary arterial pp60^{c-src} kinase activity. Conceivably, this mechanism may serve important roles in modulating signal transduction and contractility of vascular smooth muscle.

pp60^{c-src} is a 60 kDa phosphoprotein which exhibits tyrosine kinase activity: it is encoded by the proto-oncogene c-src, the cellular homolog of the Rous sarcoma viral oncogene called v-src (1-3). Expression of c-src is regulated in a developmental and tissue-specific manner, whereas post-translational regulation of pp60^{c-src} kinase activity appears to involve ATP-mediated changes in its level and sites of phosphorylation. Although the functional roles of pp60^{c-src} are uncertain, recent studies suggest that changes in expression and/or activity of pp60^{c-src} participate in regulating signal transduction and differentiation (4-6). In this context, Simon et al. (6) reported that high levels of c-src in Drosophila visceral smooth muscle were expressed only during early embryogenesis. However, whether or not c-src is expressed in other types of smooth muscle or in adult terminally differentiated mammalian smooth muscle is unknown.

In this communication we show pp60^{c-src} kinase activity is present in extracts from adult bovine coronary arterial smooth muscle, an observation suggesting that the

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functions of pp60$^{c\text{-}src}$ in mammalian smooth muscle extend beyond early embryogenesis. We also report that the kinase activity of pp60$^{c\text{-}src}$ in arterial extracts is dramatically increased by ATP through a novel mechanism which does not appear to involve a phosphotransferase reaction. Conceivably, this unusual and enormous potential for widely altering pp60$^{c\text{-}src}$ kinase activity may be linked to mechanisms underlying arterial signal transduction and contractility.

MATERIALS AND METHODS

The intimal surface of bovine coronary artery was rubbed to remove endothelial cells (7), and the medial layer of smooth muscle cells was homogenized at 4°C in 5 vols of homogenization buffer containing 20 mM 3-[N-Morpholino] propanesulfonic acid (MOPS) pH 7.0, 150 mM NaCl, 1% Na deoxycholate, 5% nonidet P-40, 0.1% Na dodecyl sulfate (SDS), 1 mM Na_2 ethylenediaminetetraacetic acid (EDTA), 100 μg/ml of aprotinin (Sigma), 100 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (Sigma), 100 μg/ml of phenylmethylsulfonyl fluoride (Sigma), and 100 μg/ml of N-p-tosyl-L-lysine chloromethyl ketone (Sigma). Homogenates were kept on ice for 30 min, and centrifuged for 10 min at maximum speed in a Beckman microfuge at 4°C. Protein in the supernatant fraction was determined by the Bradford Method (8), using bovine serum albumin (Sigma) as standard, and adjusted to 8 mg/ml by dilution in homogenization solution.

The method for determination of pp60$^{c\text{-}src}$ kinase activity in arterial extracts employed an immunoprecipitation assay which was adapted from procedures described by Courtneidge (9) and Bolen et al. (10) for cultured cells and different types of tissues. These procedures are based on the use of a specific monoclonal antibody (MAb 327), developed by Lipsich et al. (11), which recognizes pp60$^{c\text{-}src}$ without altering its enzymic activity.

Routinely, a mixture containing 250 μl of extract (2 mg protein), 5 μl of the appropriate dilution of MAb 327 (Oncogene Sciences), and 95 μl of homogenization buffer (with or without specific additives) were incubated overnight at 4°C. Following incubation, immune complexes were precipitated by adding 10 μl of 10% formalin-fixed Staphylacoccus aureus (Pansorbin, CalBiochem) which was precoated with rabbit anti-mouse immunoglobulin (Zymad). The mixture was kept on ice for 30 min and centrifuged to sediment the immunoprecipitate. Each immunoprecipitate was washed 3 times with 500 μl of a solution containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% triton, 1% Na deoxycholate, 0.1% SDS and 1 mM dithiothreitol, and 2 times with 20 mM Tris pH 7.5, 150 mM NaCl, and 1 mM dithiothreitol.

Kinase assays were performed in a reaction mixture (20 μl) containing the immunoprecipitate, 20 mM MOPS pH 7.0, 0.5 mg/ml α casein (Sigma, a mixture of α_1 and α_2 caseins), 5 mM $MgCl_2$ and 20 μM ATP supplemented with 20 μCi [$^{32}P_\gamma$]-ATP (NEN). Reactions were started by addition of ATP, and stopped after 10 min of incubation at 30°C by adding 10 μl of Laemmli SDS-buffer (12) and heating in a boiling water bath for 3 min. After cooling to room temperature, 25 μl of the mixture was subjected to SDS electrophoresis on a 7.5% polyacrylamide gel (9-11). Quantitation of pp60$^{c\text{-}src}$ kinase activity was achieved by autoradiography of destained gels and subsequent densitometric scanning of the autoradiograms (LKB ultroscan), as well as by counting of incorporated ^{32}P by liquid scintillation spectroscopy of bands cut from electrophoretic gels (9,10,13-15).

RESULTS AND DISCUSSION

To our knowledge, these studies provide the first evidence for the presence of pp60$^{c\text{-}src}$ kinase activity in extracts prepared from adult, terminally differentiated bovine coronary artery (Fig. 1). Such activity was reflected by autophosphorylation of pp60$^{c\text{-}src}$, and by phosphorylation of exogenously supplied casein α_1 and casein α_2. Several unidentified polypeptides in the arterial immunoprecipitates were also phosphorylated. These copurifying endogenous substrates were of Mr 97,000, 83,000, 45,000, 42,000, and 26,500. However, as shown in the autoradiogram, no phosphorylation of any of these proteins was apparent when anti-src monoclonal antibody was omitted from immunoprecipitation mixtures: this confirms that measured kinase activities were ascribable to pp60$^{c\text{-}src}$. Though not shown, phosphoamino acid analysis revealed that autophosphorylation of pp60$^{c\text{-}src}$ or phosphorylation of α_1 and α_2 casein occurred on tyrosine residues.

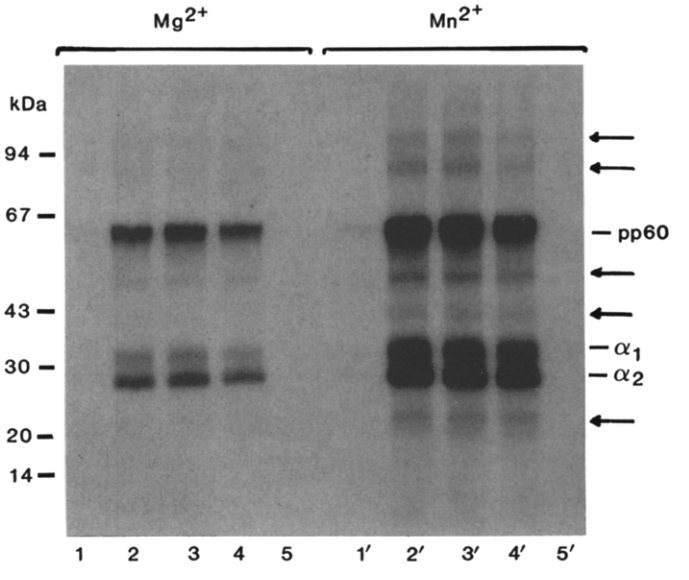

Figure 1. A homogenate of the smooth muscle layer from a bovine coronary artery was processed and assayed for pp60$^{c\text{-}src}$ kinase activity in the presence of either 5 mM $MgCl_2$ (lanes 1-5) or 5 mM $MnCl_2$ (lanes 1'-5'), and subjected to electrophoresis and autoradiography as described in **MATERIALS AND METHODS.** The autoradiogram shows results obtained with immunoprecipitates prepared under the following conditions: lanes 1 and 1', no added ATP or AMP·PNP; lanes 2 and 2', with 1 mM ATP; lanes 3 and 3', with 1 mM AMP·PNP; lanes 4 and 4', no added ATP or AMP·PNP but washed with 1 mM AMP·PNP; and lanes 5 and 5' in the absence of anti-src monoclonal antibody. pp60$^{c\text{-}src}$, α_1 and α_2 caseins are identified at right. Arrows at right point to endogenous substrates which are present in the immunoprecipitates. The electrophoretic mobilities of Mr marker proteins are shown at left and included phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

Enhancement of pp60^{c-src} kinase activity by Mn^{2+} has been well-documented for a variety of cell types (9,13,16). In this study, substitution of Mn^{2+} for Mg^{2+} resulted in á 2 fold increase in autophosphorylation of pp60^{c-src}, and phosphorylation of endogenous and exogenous substrates. Similar results were obtained when data was expressed as incorporation of ^{32}P (Table 1) or in arbitrary units of autoradiographic band areas (Table 2).

A huge and unexpected increase in pp60^{c-src} kinase activity in arterial extracts occurred in response to ATP. That is, inclusion of 1 mM ATP in immunoprecipitation mixtures produced about a 20 fold increase in autophosphorylation of pp60^{c-src}, and 20-40 fold increases in phosphorylation of exogenous and endogenous substrates (Fig. 1, Tables 1

TABLE 1. pp60^{c-src} kinase activity in coronary arterial extracts expressed as incorporation of ^{32}P

			^{32}P Incorporated			
TEST CONDITION	**pp60^{c-src}**		**α_1 Casein**		**α_2 Casein**	
	CPM	**R**	**CPM**	**R**	**CPM**	**R**
Mg^{2+}, Control	2,632		97		1,059	
+ATP	53,110	20.2	20,694	22.6	37,667	35.6
+AMP·PNP	61,595	23.4	23,388	25.5	42,784	40.4
+AMP·PNP Wash	45,451	17.3	20,815	22.7	44,380	41.9
-MAb	202	0.08	155	0.12	13	0.01
Mn^{2+}, Control	5,164		2,728		2,832	
+ATP	174,617	35.8	96,396	35.3	150,270	53.1
+AMP·PNP	171,761	33.3	87,968	32.3	150,758	53.2
+AMP·PNP Wash	160,364	31.1	88,137	32.3	148,984	52.6
-MAb	84	0.02	74	0.03	67	0.02

Each value for incorporation of ^{32}P (CPM, counts per minute) is given as the mean for 4 determinations. R, a measure of the extent of stimulation, is the ratio of mean activity obtained following immunoprecipitation in the presence of ligand (1 mM ATP or 1 mM AMP·PNP) to the activity obtained following immunoprecipitation in the absence of ligand (Control). The R for **-MAb** is the ratio obtained after immunoprecipitation without anti-src monoclonal antibody to activity measured with antibody. Values for **AMP·PNP wash** represent assays of immunoprecipitates which were collected in the usual way (control) but treated with wash solution containing 1 mM AMP·PNP.

TABLE 2. pp60^{c-src} kinase activity in coronary arterial extracts measured autoradiographically

TEST CONDITION	pp60^{c-src}		α_1 Casein		α_2 Casein	
	Area	R	Area	R	Area	R
Mg^{2+}, Control	0.28		0.09		0.12	
+ATP	5.37	19.2	1.88	20.1	4.14	34.5
+AMP·PNP	5.88	21.0	2.09	23.2	4.34	36.2
+AMP·PNP Wash	5.20	18.6	1.99	22.1	4.82	40.2
-MAb	0	0	0.03	0.3	0	0
Mn^{2+}, Control	0.44		0.22		0.24	
+ATP	16.33	37.0	7.39	33.6	13.92	58.0
+AMP·PNP	14.98	34.1	7.42	33.7	12.52	52.2
+AMP·PNP Wash	13.94	36.2	7.18	32.6	13.64	56.8
-MAb	0.04	0.1	0.03	0.14	0	0

Data is from the same experiments shown in Table 1. Test conditions and calculations for R are as described in Table 1. Areas of autoradiographic bands are expressed in arbitrary units (LKB, ultroscan XL).

and 2). This response was more pronounced in the presence of Mn^{2+}, resulting in a 30 fold increase in autophosphorylation of pp60^{c-src}, and a 30-50 fold increase in phosphorylation of substrates. Thus, the potential pp60^{c-src} kinase activity in coronary arterial extracts is probably comparable to the distinctively high activity present in neural tissues (16,17) and in certain transformed cells (1-3,9,18).

We considered the possibility that the stimulatory response of arterial pp60^{c-src} to ATP might be due to ATP-mediated phosphorylation because pp60^{c-src} kinase activity can be altered by phosphorylation (2,3,13,19). Surprisingly, however, the same degree of stimulation was achieved with AMP·PNP, a non-metabolizable analog of ATP which cannot serve as a phosphoryl donor in phosphotransferase reactions. Moreover, stimulation with AMP·PNP also could be induced after immunoprecipitation of the pp60^{c-src}. Thus, marked stimulation of activity was apparent when immunoprecipitates were prepared in the usual way (i.e., without ATP or AMP·PNP included in the immunoprecipi-

tation mixture) and treated with wash solution containing AMP·PNP (Fig. 1, lanes 4 and 4', Tables 1 and 2). This demonstrates that enhanced activity is due to ligand-induced stimulation of pp60$^{c\text{-}src}$ rather than enhanced immunoprecipitation. Similarly, stimulation of activity in response to either ATP or AMP·PNP persisted when potential phosphotransferase activity was blocked by chelation of Mg^{2+} with 20 mM EDTA (20). Taken together, these data indicate the stimulatory response of arterial pp60$^{c\text{-}src}$ kinase activity to ATP is ascribable to a novel mechanism which does not involve phosphorylation. Whether pp60$^{c\text{-}src}$ extracted from other tissues is also subject to stimulation by ATP is unknown. However, unpublished studies in our laboratory show that such stimulation does not occur in bovine brain extracts.

The current demonstration of pp60$^{c\text{-}src}$ kinase activity in extracts from adult coronary arteries strongly suggests the enzyme participates in regulating specific functions of terminally differentiated vascular smooth muscle cells. Such regulatory mechanisms, perhaps involving signal transduction related to contraction and relaxation of blood vessels, may be fine-tuned by the ATP-mediated stimulation of pp60$^{c\text{-}src}$ kinase activity described in this study.

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REFERENCES

1. Rohrschneider, L.R. (1986) In Oncogenes and Growth Control (P. Kahn, and T. Graf, Eds), pp 27-31, Springer-Verlag, Heidelberg, FRG.
2. Sefton, B.M., and Hunter, T. (1986) Cancer Surveys 5:159-172.
3. Jove, R., and Hanafusa, H. (1987) Ann. Rev. Cell Biol. 3:31-56.
4. Schartl, M., and Barnekow, A. (1984) Dev. Biol. 105:415-422.
5. Golden, A., Nemeth, S.P., and Brugge, J.S. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:852-856.
6. Simon, M., Dress, B., Kornberg, T., and Bishop, J.M. (1985) Cell 42:831-840.
7. Hickey, K.A., Rubanyi, G., Paul, R.J., and Highsmith, R.F. (1985) Am. J. Physiol. 248:C550-C556.
8. Bradford, M.M. (1976) Anal. Biochem. 72:248-254.
9. Courtneidge, S.A. (1985) EMBO J. 4:1471-1477.
10. Bolen, J.B., Veillette, A., Schwartz, A.M., DeSeau, V., and Rosen, N. (1987) Oncogene Res. 1:149-168.
11. Lipsich, L.A., Lewis, A.J., and Brugge, J.S. (1983) J. Virol. 48:352-360.
12. Laemmli, U.K. (1970) Nature (London) 227:680-685.
13. Cooper, J.A., and King, C.S. (1986) Mol. Cell. Biol. 6:4467-4477.

14. Silver, P.J., and Di Salvo, J. (1979) J. Biol. Chem. 254:9951-9954.
15. Kranias, E.G., and Di Salvo, J. (1986) J. Biol. Chem. 261:10029-10032.
16. Brugge, J.S., Cotton, P.C., Queral, A.E., Barrett, J.N., Nonner, D., and Keane, R.W. (1985) Nature (London) 316:554-557.
17. Brugge, J., Cotton, P., Lustig, A., Yonemoto, W., Lipsich, L., Coussens, P., Barrett, J.N., Nonner, D., and Keane, R.W. (1987) Genes and Develop. 1:287-296.
18. Rosen, N., Bolen, J.B., Schwartz, A.M., Cohen, P., DeSeau, V., and Isreal, M.A. (1986) J. Biol. Chem. 261:13754-13759.
19. Cooper, J.A., Gould, K.A., Cartwright, C.A., and Hunter, T. (1986) Science 231:1431-1434.
20. Di Salvo, J., Gifford, D., and Kokkinakis, A. (1986) Biochem. Biophys. Res. Commun. 136:789-796.