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EVIDENCE FOR THE INVOLVEMENT OF G PROTEINS IN THE MODULATION OF ^{22}Na AND ^{45}Ca FLUXES IN MYOGENIC L6 AND AORTIC SMOOTH MUSCLE CELLS

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SUMMARY: Preincubation of rat skeletal muscle derived L6 myoblasts with 10 mM NaF increased, the activity of Na^+/H^+ exchanger, and the uptake of ^{45}Ca by 2 and 5 folds, respectively. In cultured vascular smooth muscle cells (CVSMC) of rat aorta, NaF increased the activity of Na^+,K^+-pump, Na^+/H^+ exchanger, passive permeability for Na^+, and ^{45}Ca uptake by 1.6, 9, 2, and 9 folds, respectively. Both, in CVSMC and L6 cells, the effect of NaF on the Na^+/H^+ exchanger and ^{45}Ca uptake were significantly augmented by 20 μM $AlCl_3$. No effect of $AlCl_3$ on the NaF dependent changes in ion flux was seen in rat erythrocytes. The results suggest that in L6 and CVSMC cells, the Na^+/H^+ exchanger and Ca^{++} uptake pathway(s) are activated by GTP-binding proteins.

GTP (guanosine triphosphate)- binding proteins (Gp) are involved in the regulation of the activity of several enzyme (*e.g.*, adenylate cyclase, phospholipase C, cyclic GMP, phosphodiesterase) mediated intracellular signalling. Ion fluxes across the plasma membrane, especially those involving the receptor-modulated K^+ channels (in heart and nerve tissue), L-type Ca^{++} channels (in heart and skeletal muscles), and Na^+/Cl^- channels (kidney and epithelial cells) are regulated by Gp (see 1-3). In recent years it has been shown that NaF, a potent activator of Gp, in presence of Al^{3+} induces ^{45}Ca influx and increases intracellular free calcium concentration in human platelets (4), human umbelical endothelial cells (5), rat parotid acini (6), rat hepatocytes (7), cultured vascular smooth muscle cell [CVSMC] of rat aorta (8), 3T3 L1 fibroblasts (9), avian supra-orbital nasal gland cells (10), and rat brain synaptosomes (11). NaF also augments amiloride or amiloride-analogue inhibited ^{22}Na influx and cytoplasm

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alkanization in hamster fibroblasts (12) and CVSMC (13). The abovementioned reports suggest the involvement of Gp in the regulation of Na^+/H^+ exchanger and unidentified Ca^{++} pathway(s) in the respective type of cells. The hypothesis of the involvement of Gp is based on the assumption that fluoride anions (F^-) penetrate the plasma membrane and in the presence of endogenous Al^{3+} induces the activation of Gp perhaps by the ligand AlF_4^-. AlF_4^- is thought to circumvent the need for GDP dissociation by binding alongside GDP and mimicing the terminal phoshate group of GTP thereby causing activation of Gp. Since NaF is a non-specific agonist of Gp and might also inhibit phosphatases and other enzymes by Al^{3+}-independent mechanisms, we compared the effect of NaF alone and in combination with $AlCl_3$. The $AlCl_3$-dependent effect on ^{86}Rb, ^{22}Na and ^{45}Ca fluxes in skeletal muscle derived L6 myoblasts, CVSMC and rat erythrocytes was interpreted as the effect caused by the activation of Gp.

MATERIAL AND METHODS

Fetal calf serum (FCS) and other reagents for cell culture were purchased from Gibco laboratories (Grand Island, NY, USA). FCS required for the culture of CVSMC was obtained from Fakola AG (Basel, Switzerland). The radioisotpes $^{86}RbCl$, $^{22}NaCl$, and $^{45}CaCl_2$ were obtained from Amersham (Bucks, England). Ethylisopropylamiloride (EIPA) was obtained from Ciba Geigy (Basel). Bumetanide was a kind gift from Prof. J. Duhm of the Institute of Physiology, Munich University, FRG. Ouabain and bovine serum albumin (BSA) were obtained from Serva (FRG). HEPES, Tris, NaF and $AlCl_3$ were purchased from Sigma Chemical (MO, USA). All other chemicals were of the highest purity available.

Rat skeletal muscle derived L6 cells were obtained from American Type Cell Culture (ATCC, Rockville, MD). Monolayer cultures were grown in DMEM supplemented with 10% FCS, 5 mM glutamine, 0.3% D-glucose, 50 U/ml of penicillin, and 50 µg/ml of streptomycin, in humidified air containing 10% CO_2 at +37°C. All experiments were carried out with undifferentiated mononucleated cells (myoblast) of the 4th-10th passage. Prior to 12-14 hours of ion flux studies, cells were seeded in either 24 well ($4-5 \times 10^5$ cells per well; for the measurements of ^{86}Rb and ^{45}Ca influx) or 6 well ($1.5-2 \times 10^6$ cells per well; for the measurement of ^{22}Na influx) culture dishes as required. All experiments were carried out at 37°C. Prior to the study of the rates of cation fluxes, cells (in each well) were washed with 2 x 1 ml aliquots of medium D (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in 10 mM Tris-HEPES buffer, pH 7.4). For the determination of ^{86}Rb influx, washed cells were incubated in 0.5 ml of medium E (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM D-glucose in 10 mM Tris-HEPES buffer, pH 7.4) containing 1-2 µCi *86*RbCl per ml. In part of the experiments, medium E also contained inhibitors of Na^+,K^+-pump (1 mM ouabain) or $Na^+,K^+,2Cl^-$-cotransport (10 µM bumetanide). The rate of ^{22}Na influx was determined by incubating the cells in medium E containing ouabain, bumetanide, and 3-4 µCi $^{22}NaCl$ per ml. In part of the experiments, medium E also contained 10 µM EIPA as an inhibitor of Na^+/H^+ exchanger. The rate of ^{45}Ca influx was determined by incubating the cells in medium E containing 2-3 µCi $^{45}CaCl_2$ per ml. To terminate isotope fluxes, dishes were kept on ice and each well washed with 5 x 2 ml aliquots of an ice-cold medium containing 100 mM $MgCl_2$ in 10 mM Tris-HEPES buffer, pH 7.4. The cells were then lysed by the addition of a solution containing 1% sodium dodecylsulfate and 2 mM EDTA. One ml of the cell lysate followed by 4 ml of OptiPhase 'HiSafe'3 (LKB Wallac, Finland) was then added to scintillation vials. Radioactivity in the vials was determined by a 1214 RACKBETA liquid scintillation counter (LKB Wallac, Finland). The kinetics of ^{86}Rb, ^{22}Na and ^{45}Ca influx were linear upto 35-45, 3-5, and 7-10 mins of incubation respectively. To determine the rate of ^{86}Rb, ^{22}Na and ^{45}Ca influx, the incubation time was limited to 20, 2 and 3 mins, respectively.

The rate of cation influx was calculated as $(\underline{A}).(\underline{a}.\underline{n}.\underline{t})^{-1}$, where $\underline{A}$ is the radioactivity in cell lysate (cpm), $\underline{a}$ is the specific radioactivity of the incubation medium ($cpm.nmol^{-1}$), $\underline{n}$ is the number of 10^6 cells per well, and $\underline{t}$ is the time interval (in mins) of incubating the cells with the isotope.

Aortic smooth muscle cells from 20 week old male Kyoto Wistar rats were isolated, phenotypically characterized and propagated as described previously (19). Before the influx experiments, cells were preincubated at +37°C for 10 mins in either medium C (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose in 20 mM Tris-HEPES buffer, pH 7.4) alone, medium C with 10 mM NaF, or medium C with 10 mM NaF and 20 μM $AlCl_3$. The rates of ^{86}Rb and ^{22}Na influx were determined as described previously (19). The rate of ^{45}Ca influx was measured as described for L6 cells.

Erythrocytes were obtained from 12-16 week old Kyoto Wistar female rats and kept on ice in phosphate buffered saline (PBS, 150 mM NaCl in 5 mM sodium phosphate buffer), pH 7.4. Preparation of erythrocytes and the measurement of ^{22}Na and ^{86}Rb influx in intact erythrocytes was performed as described previously (17). Quin 2 loaded erythrocytes were used to study the kinetics of ^{45}Ca influx. One volume of packed erythrocyte was mixed with four volumes of medium A (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 10 mM D-glucose in 20 mM Tris-HEPES buffer, pH 7.4). To the mixture, 1% (w/v) BSA and 150 μM quin 2AM (from 40 mM stock solution prepared in dimethylsulfoxide) was added. Erythrocytes were incubated for 90 mins at +37°C, sedimented and washed with 4 vols of medium A without BSA and quin 2AM. Prior to the measurement of ion fluxes, erythrocytes were incubated for 30 mins at +37°C in 10 vols of either medium A alone, medium A with 10 mM NaF, or medium A with 10 mM NaF and 20 μM $AlCl_3$. Following 30 mins of such preincubation, erythrocytes were sedimented and washed with 20 aliquots of medium A without NaF or $AlCl_3$ supplementation. Packed erythrocytes (50 μl) were mixed with 4 vols (200 μl) of medium A containing 4 μCi/ml $^{45}CaCl_2$. The rate of uptake of ^{45}Ca was studied at +37°C. To terminate the uptake of ^{45}Ca, 1 ml of ice-cold PBS containing 0.1 mM EDTA (medium B) was added. Subsequently, erythrocytes were sedimented, washed with 2 x 1 ml aliquots of medium B and lysed with 0.5 ml of 0.5% Triton X100 and 0.5 ml of tricloroacetic acid. The radioactivity of the protein-free supernatant was determined. ^{45}Ca uptake was calculated as $(A_Q-A_O)/a.m$, where A_Q and A_O are the radioactivity (cpm) of the quin 2 loaded and intact (quin 2 unloaded) erythrocytes respectively; a is the specific radioactivity (cpm/μmol) of the incubation medium; and m is the volume (litres) of packed erythrocytes used for the experiment.

The activities of Na^+,K^+-pump and $Na^+,K^+,2Cl^-$-cotransport were determined as 1 mM ouabain-inhibited and 1 mM ouabain-insensitive 10 μM bumetanide-inhibited components of ^{86}Rb influx respectively. Activity of Na^+/H^+ exchanger was determined as (1 mM ouabain + 10 μM bumetanide)-insensitive 10 μM EIPA inhibited component of ^{22}Na influx.

Results presented in Tables 1-3 are mean ± S.E.M of data obtained in 4 or 6 experiments. Where indicated, means were compared using Students t test for unpaired data.

RESULTS

Skeletal muscle derived L6 myoblasts. No significant effect of either NaF or NaF+$AlCl_3$ on the activity of Na^+,K^+-pump, $Na^+,K^+,2Cl^-$-cotransport or passive permeability of the L6 cell membrane for ^{86}Rb or ^{22}Na was observed (Table 1). Preincubation of the cells with NaF increased the activity of the Na^+/H^+ exchanger and ^{45}Ca influx by 2 and 4-5 folds, respectively. In the presence of $AlCl_3$, the NaF-caused increase in the activity of Na^+/H^+ exchanger and ^{45}Ca uptake were further increased by ~90% and ~25%, respectively.

Vascular smooth muscle cells. Preincubation of CVSMC with 10 mM NaF for 10 mins increased the activity of the Na^+,K^+-pump by 55-65% without any significant

TABLE 1. The effect of NaF and $AlCl_3$ on ^{22}Na, ^{86}Rb and ^{45}Ca fluxes in rat skeletal muscle derived L6 myoblasts

Addition in preincubation medium	None (control[¶])	NaF 10 mM	10 mM NaF + 20 μM $AlCl_3$
Cation flux systems	(pmol per 10^5 cells per minute)		
^{86}Rb influx			
i. Na^+,K^+-pump [ouabain-inhibited]	47±6	33±8	32±6
ii. ouabain-insensitive bumetanide-inhibited component	172±12	204±18	218±15
iii. K^+ leakage [(ouabain+bumetanide) -insensitive]	51±3	53±4	48±2
^{22}Na influx			
i. Na^+/H^+ exchanger [(ouabain+bumetanide)-insensitive EIPA-inhibited]	93±45	205±47*	386±52[§]
ii. Na^+ leakage [(ouabain+bumetanide +EIPA)-insensitive]	384±49	360±53	398±80
^{45}Ca influx	1.6±0.1	7.6±0.2**	9.4±0.6[§]

Data are mean ± S.E.M of 4 experiments.
*$P< 0.01$, **$P< 0.0001$ (compared to the corresponding control values). [§]$P< 0.05$ (compared to the corresponding values obtained from NaF preincubated cells). [¶]original data on ^{22}Na and ^{86}Rb fluxes in L6 myoblast have been submitted for publication by C.K.S *et al.* 1993.

effect upon other mechanisms of potassium (^{86}Rb) influx (Table 2). Preincubation with NaF also increased the activity of Na^+/H^+ exchanger, passive membrane permeability for sodium, and the rate of ^{45}Ca influx by 9, 3, and 9 folds, respectively. Th addition of $AlCl_3$ to the preincubation medium already containing NaF, augmented the NaF caused increase in the activity of Na^+/H^+ exchanger and ^{45}Ca influx by 35-45%. No significant effect of $AlCl_3$ was revealed in the other ion transport systems studied (Table 2).

Erythrocytes. Table 3 illustrates that 30 min preincubation of rat erythrocytes with NaF caused a 50 % decrease in the activity of the Na^+,K^+-pump and two-fold increase in the rate of $Na^+,K^+,2Cl^-$-cotransport. Under the same conditions, NaF did not have any effect upon the passive permeability (leakage) of the erythrocyte membrane for potassium (^{86}Rb). No significant effect of EIPA on the (ouabain + bumetanide)-insensitive ^{22}Na was observed. The observation is in accordance with previous results suggesting that under basal conditions the activity of Na^+/H^+ exchanger in mammalian erythrocytes is negligible (17,20-22). NaF did not modify the activity of the Na^+/H^+ exchanger but increased the passive permeability [(ouabain+bumetanide+EIPA)-insensitive ^{22}Na influx] of the erythrocyte membrane for sodium by 40-50%. The intracellular calcium pool of mammalian erythrocytes is small and the rate of ^{45}Ca uptake by these cells is

TABLE 2. The effect of NaF and $AlCl_3$ on ^{22}Na, ^{86}Rb and ^{45}Ca fluxes in cultured vascular smooth muscle cells of the rat aorta

Addition in preincubation medium	None (control)	NaF 10 mM	10 mM NaF + 20 μM $AlCl_3$
Cation flux systems	(pmol per 10^5 cells per minute)		
^{86}Rb influx			
i. Na^+,K^+-pump [ouabain-inhibited]	142±27	225±31*	244±35*
ii. $Na^+,K^+,2Cl^-$-cotransport [ouabain-insensitive bumetanide-inhibited]	89±16	62±14	60±9
iii. K^+ leakage [(ouabain+bumetanide) -insensitive]	69±9	68±12	68±6
^{22}Na influx			
i. Na^+/H^+ exchanger [(ouabain+bumetanide)-insensitive EIPA-inhibited]	74±12	657±63**	901±88§
ii. Na^+ leakage [(ouabain+bumetanide +EIPA)-insensitive]	80±13	237±43**	270±51**
^{45}Ca influx	9.5±0.9	85±11**	116±10§

Data are mean ± S.E.M of 6 experiments.
*$P< 0.05$, **$P< 0.001$ (compared to the corresponding control values). §$P< 0.001$ (compared to the corresponding values obtained from NaF preincubated cells).

negligible (23). Data illustrated in Fig. 1 show that NaF accelerated ^{45}Ca uptake in Quin 2 loaded erythrocytes by 20-30%. Table 3 and Fig. 1 shows that $AlCl_3$ did not modify the effect of NaF on the rate of cation fluxes in rat erythrocytes.

TABLE 3. The effect of NaF and $AlCl_3$ on ^{22}Na and ^{86}Rb fluxes across the rat erythrocyte membrane

Addition in preincubation medium	None (control)	NaF 10 mM	10 mM NaF + 20 μM $AlCl_3$
Cation flux systems	(mmol per liter of cells per hour)		
^{86}Rb influx			
i. Na^+,K^+-pump [ouabain-inhibited]	1.37±0.05	0.61±0.06*	0.64±0.04*
ii. $Na^+,K^+,2Cl^-$-cotransport [ouabain-insensitive bumetanide-inhibited]	0.36±0.03	0.73±0.1*	0.76±0.08*
iii. K^+ leakage [(ouabain+bumetanide) -insensitive]	1.21±0.08	1.09±0.11	1.07±0.12
^{22}Na influx			
i. Na^+/H^+ exchanger [(ouabain+bumetanide)-insensitive EIPA-inhibited]	0.03±0.06	0.01±0.03	0.02±0.05
ii. Na^+ leakage [(ouabain+bumetanide +EIPA)-insensitive]	1.13±0.12	1.63±0.07*	1.6±0.09*

Data are mean ± S.E.M of 4 experiments.
*$P< 0.001$ (compared to the corresponding control values).

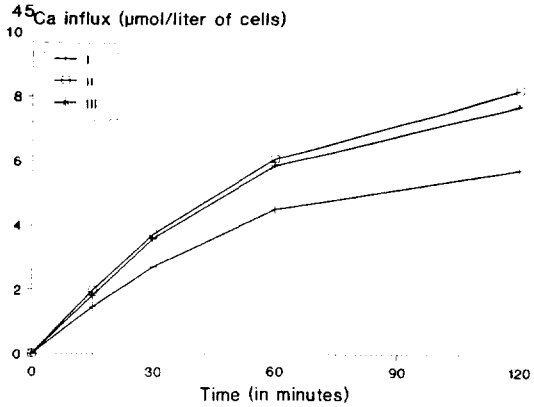

Fig. 1.
Kinetics of ^{45}Ca influx in rat erythrocytes. I, control; II, the effect of 10 mM NaF; and III, the effect of 10 mM NaF in combination with 20 μM $AlCl_3$. No effect of NaF alone or in combination with $AlCl_3$ was observed.

DISCUSSION

The observations revealed that NaF modified the activity of several ion transport systems including Na^+/H^+ exchanger of L6 and CVSMC cells, Na^+,K^+-pump of CVSMC and erythrocytes, $Na^+,K^+,2Cl^-$-cotransport of erythrocytes, passive permeability of CVSMC and erythrocyte plasma membrane for sodium, and the rate of ^{45}Ca influx in all the three cell types investigated. In the presence of $AlCl_3$, the NaF-dependent component of the activity of Na^+/H^+ exchanger and the rate of ^{45}Ca uptake were further increased both in L6 and CVSMC cells only (Tables 1 and 2). Potentiation of the effect of NaF by $AlCl_3$ may be viewed as an evidence for the involvement of GTP binding proteins in the modulation ion flux mechanisms related to intracellular signalling. Results presented in Tables 1 and 2 show that $AlCl_3$ augments the NaF induced increase in the activity of the Na^+/H^+ exchanger and the rate of ^{45}Ca influx in L6 and CVSMC cells. Thus, it may be understood that Gp-mediated pathways modulate the functioning of these ion transporting systems.

NaF is a non-specific agonist of Gp and may also inhibit the activity of phosphatases and some glycolytic enzymes (16). It may be assumed that the ~25% and ~55% decrease in the activity of Na^+,K^+-pump observed in NaF treated L6 myoblasts and erythrocytes, respectively, was caused by a decrease in intracellular ATP. Unlike that in the L6 cells and erythrocytes, pretreatment of CVSMC with NaF caused a ~60% increase in the activity of the Na^+,K^+-pump (Table 2). It may be assumed that this phenomenon was a consequence of increased intracellular sodium concentration which may have occured due to the drastic (~200%) increase in the passive permeability of the CVSMC membrane for sodium (Table 2). The mechanisms underlying the $AlCl_3$-independent effect of NaF on $Na^+,K^+,2Cl^-$-cotransport in rat erythrocytes and passive permeability of the CVSMC membrane for sodium are yet unknown.

The basal activity of the Na^+/H^+ exchanger in L6 and CVSMC cells is about the same (Tables 1 and 2). However, the effect of AlF_4^- on the activity of this exchanger was observed to be almost 3-fold higher in CVSMC compared to that in the L6 cells. Although $AlCl_3$-dependent augmentation of the effect of NaF was almost twice in L6 cells compared to that in the CVSMC, F^- alone caused a dramatic (~800%) increase in the activity of the Na^+/H^+ exchanger of the CVSMC membrane. The activity of Na^+/H^+ exchanger of the erythrocyte membrane was negligible and no effect of either NaF alone or NaF + $AlCl_3$ could be observed (Table 3). Previous reports have shown that NaF does not have any effect upon the activity of the Na^+/H^+ exchanger either in rat thymocytes (24) or in human platelets (25). Recently it has been shown that the Na^+/H^+ exchanger of the barnacle muscle fiber is elicited by dialyzing the cells with GTP itself or cholera toxin which is an activator of GTP (26). Thus it may be understood that Gp-mediated mechanisms of the modulation of the Na^+/H^+ exchanger is tissue specific, and this specificity is probably dependent upon the set of GTP-binding proteins or/and properties of the Na^+/H^+ exchanger expressed in tissues. It is known that cell shrinkage and activation of protein kinase C are among the most powerful mechanisms which may activate the function of the Na^+/H^+ exchanger (27, 28). However, it should be noted that we failed to reveal any significant effect of NaF on the volume of CVSMC as well as on the effect of staurosporin (an inhibitor of protein kinase C) on the NaF-dependent increase in the activity of Na^+/H^+ exchanger (our unpublished observation). Such an observation suggests that the NaF-caused striking (~900%) increase in the activity of the Na^+/H^+ exchanger of the CVSMC membrane is probably not dependent upon changes in cell volume or activity of protein kinase C. Subsequently, it may be hypothesized that Gp-dependent modulation of the ion exchange mechanism is caused by a direct coupling of Gp with the Na^+/H^+ exchanger.

Data presented in Tables 1 and 2 show that, both in L6 and CVSMC cells, NaF activated the rate of ^{45}Ca influx by almost ~900% and ~500%, respectively. Such drastic effects of NaF upon ^{45}Ca influx has been also observed in endothelial cells (5) and in brain synaptosomes (11). Both in L6 cells and CVSMC, NaF-dependent increase in the rate of ^{45}Ca influx were further increased by 25-40% upon addition of $AlCl_3$ indicating the involvement of Gp in the modulation of the related ion transport pathway(s). $AlCl_3$-dependent augmentation of the NaF-induced increase in intracellular free calcium has been also observed in quin 2AM loaded rat hepatocytes (29) and in indo-1 loaded duckling supra-orbital nasal gland (10) thus indicating the presence of Gp-activated pathway for Ca^{++} influx in the plasma membrane of these cells. This pathway, though evidently present, is yet to be characterized. It is tempting to suspect that AlF_4^--induced influx of ^{45}Ca is mediated by intracellular Na^+/extracellular Ca^{++} exchange. This mechanism of ion exchange is likely to be activated under conditions of high intracellular Na^+

concentrations as may occur following exposure to NaF when the activity of the Na^+/H^+ exchanger increases remarkably (Tables 1 and 2). It may be noted that both in L6 and CVSMC, the AlF_4^--caused proportional increase in the rate of ^{45}Ca influx is almost the same compared to the proportional increase in the activity of the Na^+/H^+ exchanger (Table 2).

In conclusion, this study presents strong evidence that in CVSMC and L6 cells, Na^+/H^+ exchange mechanism and Ca^{++} uptake pathway(s) are activated by GTP-binding proteins. However, the exact mechanisms of modulation are yet to be characterized.

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