ORIGINAL ARTICLE

Differential and Brain Region–Specific Regulation of Rap-1 and Epac in Depressed Suicide Victims

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Context: Depression is a major public health problem. Despite many years of research, the molecular mechanisms associated with depression remain unclear. Rap-1, activated in response to many extracellular stimuli, is one of the major substrates of protein kinase A, which participates in myriad physiologic functions in the brain, including cell survival and synaptic plasticity. Rap-1 is also activated directly by cyclic adenosine monophosphate through Epac, and thus participates in mediating physiologic functions independent of protein kinase A.

Objective: To examine whether the pathogenesis of depression is associated with altered activation and expression of Rap-1, as well as expression of Epac, in depressed suicide victims.

Design: Postmortem study.

Setting: Tissues were obtained from the Lenhossek Human Brain Program, Semmelweis University, Budapest, Hungary, and the Brain Collection Program of the Maryland Psychiatric Research Center, Baltimore.

Participants: Postmortem brains of 28 depressed suicide victims and 28 nonpsychiatric control subjects.

Intervention: Examination of brain tissues.

Main Outcome Measures: Rap-1 activation as well as messenger RNA and protein levels of Rap-1 and Epac in prefrontal cortex, hippocampus, and cerebellum.

Results: Rap-1 activation was significantly reduced ($P<.001$) in prefrontal cortex and hippocampus in the suicide group. This was associated with significant reductions in Rap-1 messenger RNA and protein levels ($P<.001$). In contrast, protein level of only Epac-2 ($P<.001$) but not Epac-1 ($P=.89$) was significantly increased in prefrontal cortex and hippocampus of these subjects. These changes were present whether the 2 cohorts were analyzed together or separately. None of the measures showed any significant change in cerebellum in the suicide group.

Conclusion: Given the importance of Rap-1 in neuroprotection and synaptic plasticity, our findings of differential regulation of Rap-1 and Epac between brain regions suggest the relevance of these molecules in the pathophysiology of depression.

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MAJOR DEPRESSION IS ONE of the most serious threats to mental and physical health.[1,2] It affects about 17% of the population at some point in life and is often a chronic and lifelong illness[3,4] associated with significant morbidity and a high risk of mortality and suicide.[5,6] In recent years, significant progress has been made in elucidating the neurobiology of depression[7]; however, the precise molecular mechanisms are still unclear.

Rap-1, a Ras family of small guanine nucleotide triphosphate (GTP)–binding proteins, was initially found in a screen for Ras-homologous proteins[8,9] and was independently identified as an antioncogenic protein that efficiently reverses the morphologic transformation of v-K-Ras–expressing cells.[10,11] Rap-1 is one of the important substrates of protein kinase A (PKA), a crucial enzyme in the cyclic adenosine monophosphate (cAMP) signaling pathway, and is phosphorylated at its C-terminal Ser-180 residue in a wide variety of cells,[12-16] including neurons and glia.[17-19] Activation of Rap-1 leads to its participation in a variety of important physiologic functions: cell proliferation and survival, cell adhesion, and differentiation,[11,20] as well as plasticity.[21] The activation mechanism of Rap-1 involves guanine nucleotide exchange factors (GEFs) that substitute the bound guanine nucleotide diphosphate (GDP) for GTP. The GTP-bound form is active; the GDP-bound form is inactive. The GTPase-activating proteins induce the hydrolysis of the bound GTP to complete the cycle.[11]

Rap-1 is activated not only by PKA; cAMP also plays an important role in such activation.[22,23] This happens through a protein called *Epac* (derived from "exchange

protein directly activated by cAMP"). Epac is a guanine-nucleotide-exchange factor that is activated both in vitro and in vivo by direct binding to cAMP.[24] This is a multidomain protein containing an autoinhibitory cAMP-binding domain that inhibits the catalytic region and a disheveled, Egl-10, and pleckstrin homology domain, which is involved in membrane localization. The presence of the cAMP-binding domain facilitates direct activation of Rap-1 by cAMP, independent of its activation of PKA.[22,23,25] This provides additional mechanisms by which cAMP controls physiologic functions.[26] In a recent study, Kaneko and Takahashi[27] reported that cAMP facilitates transmitter release via activating the PKA-independent Epac pathway in the nerve terminal, indicating direct involvement of cAMP in synaptic potentiation. Interestingly, we have demonstrated altered cAMP signaling in depressed subjects, such that catalytic activity of PKA, as well as tritiated cAMP binding to regulatory subunits of PKA, is decreased in prefrontal cortex (PFC) of depressed suicide victims.[28,29] This is accompanied by selective reductions in catalytic and regulatory subunits of PKA. The role of PKA in depression has also been demonstrated by other investigators.[30-33]

The present study was undertaken to test the hypothesis that the pathogenesis of depression is associated with altered activation and expression of Rap-1. Given that Rap-1 is directly activated by cAMP through Epac, we examined expression of Epac in depressed subjects. Our study provides evidence of differential regulation of Rap-1 and Epac in depressed subjects.

METHODS

SUBJECTS

The study was performed in PFC (Brodmann area 9), hippocampus, and cerebellum obtained from the right hemisphere of depressed suicide victims (suicide group) and nonpsychiatric control subjects (control group). Samples of PFC and cerebellum from 28 depressed suicide victims and 28 normal controls and hippocampus from 22 depressed suicide victims and 22 normal controls were used. Brain tissues were collected from the Lenhossek Human Brain Program, Semmelweis University, Budapest, Hungary (Budapest cohort; 17 depressed suicide victims and 17 control subjects), and the Brain Collection Program of the Maryland Psychiatric Research Center, Baltimore (Maryland cohort; 11 depressed suicide victims and 11 control subjects). Tissues were collected only after a family member gave informed consent. Toxicology data were obtained by the analysis of urine and blood samples. Brain pH was measured in cerebellum in all cases.

The psychiatric diagnostic criteria applied were detailed in our earlier publications.[29,34,35] Of 28 subjects, at least 17 were diagnosed as having depression during their lifetime, but all subjects had a firm diagnosis of depression at the time of death. This study was approved by the institutional review board of the University of Illinois at Chicago.

DETERMINATION OF MESSENGER RNA LEVELS OF Rap-1, Epac, AND CYCLOPHILIN

The quantitation of Rap-1, Epac, and cyclophilin messenger RNA (mRNA) was determined by competitive reverse transcriptase–polymerase chain reaction using internal standards as described previously.[34,35] The primer pairs were designed to allow amplification of 211 to 590 base pairs (bp): forward, 5′GATATGGCTGGACATCCCTTC 3′ and reverse, 5′CTATTGGTGGTAGGGCGTTTC for Rap-1 (GenBank accession No. NM016544); 1115 to 1480 bp: forward, 5′CAGAGACATTCCTCAGCGACT 3′, and reverse, 5′AGTTCCGGGCCTTCATCTGAG 3′ for Epac (GenBank Accession No. NM006105); and 118 to 421 bp: forward, 5′AGCACTGGAGAGAAAGGATTTG 3′, and reverse, 5′CCTCCACAATATTCATGCCTTC 3′ for cyclophilin (GenBank accession No. XM371409). The internal primer sequences for Rap-1, Epac, and cyclophilin were 5′GTGCCAACAAGAT***CT***ATTGTACCA 3′ (395-418 bp), 5′CCTGTATGG<u>CTC*G*AG</u>GCTCCACACT 3′ (1296-1320 bp), and 5′GGTGGCAAGTCCATCTAT/AAATGCTGGACCCAACAC 3′ (220-320 bp), respectively. The underlined bases indicate the restriction sites (*Bgl*II for Rap-1 and *Xho*I for Epac), whereas bold and italicized bases indicate the mutation sites. To ensure that amplified sequences of cyclophilin, Rap-1, and Epac matched the corresponding sequences reported in GenBank, the PCR products were sequenced with M13 primer. In all assays, as a control, one reverse transcriptase reaction was performed in the absence of RNA.

Rap-1 ACTIVATION ASSAY

Tissues were homogenized according to Baldassa et al,[36] and lysates were clarified by centrifugation at 13 000 rpm for 10 minutes at 4°C. Rap-1 activation was determined by a Rap-1 activation kit (Stressgen Bioreagents, Victoria, British Columbia). This assay uses a glutathione-*S*-transferase (GST) fusion protein containing a Rap-1 binding domain (RBD) of human guanine-nucleotide-exchange factor specific for the Ras protein Ral (Ra1GDS) to affinity precipitate active Rap-1 (GTP–Rap-1) from lysate. Equal volumes of samples containing 500 µg of protein were incubated with GST-RalGDS-RBD and a glutathione disk. After 1 hour of incubation at 4°C, beads were pelleted and rinsed with ice-cold lysis buffer. Protein was eluted from the beads by 2× sodium dodecyl sulfate sample buffer and boiled for 5 minutes. The pulled-down active GTP–Rap-1 was detected by Western blot analysis using a specific Rap-1 antibody as described in the next section. For control experiments, lysates were treated with guanosine-5′-O-(3-thio)-triphosphate (GTPγS) or GDP to activate or inactivate Rap-1. Lysates were subsequently incubated with GST-RalGDS-RBD and an immobilized glutathione disk.

WESTERN BLOT

Immunolabeling of Rap-1 and of Epac-1 and Epac-2 was determined in the same tissue fraction in which Rap-1 activation was determined. Protein samples (80 µg of protein) were resolved onto 12% (wt/vol) sodium dodecyl sulfate–polyacrylamide gel and blotted on enhanced chemiluminescence membrane as described previously.[29] Each gel contained samples from 3 control subjects and 3 depressed suicide victims, and all membranes were processed simultaneously, starting from gel loading to development of the film. A pooled brain sample from control and suicide group subjects was run on each gel to reduce the interblot variability. Membranes were incubated with Rap-1 (1:1000), Epac-1 (1:250), or Epac-2 (1:1000) antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C, followed by appropriate secondary antibody. β-Actin antibody (Sigma Chemical Co, St Louis, Mo) was used as a housekeeping protein.[29] The optical density (OD) of each band was corrected by the OD of the corresponding β-actin band. The specificity of each antiserum was checked by using a 100-fold excess of blocking peptide (relative to the mo-

larity of the antiserum) corresponding to the epitope used to generate Rap-1, Epac-1, or Epac-2. The variability within and between blots was 6% and 8%, respectively. We observed that Rap-1 migrated to 22 kDa, whereas Epac-1 and Epac-2 migrated to 120 and 126 kDa, respectively. These molecular weights are in agreement with previous reports in the literature.[23,37-40]

STATISTICAL ANALYSIS

The data analyses were performed with SPSS 8.0 software (SPSS Inc, Chicago, Ill). To test the variation in selected measures between the 2 different cohorts of brain samples, a 2-way analysis of variance was performed; no significant variations were found in any of the measures. In subsequent analyses, an independent-sample *t* test was applied to test for differences in total depressed suicide victims and total normal controls, and separately in each cohort of brain samples. Since we did not predict the changes in specific Rap-1 or Epac measures, the *t* test was exploratory. Bonferroni-adjusted *P* values (α level .05/6=.008) were considered significant. To examine whether various measures of Rap-1 and of Epac were affected by postmortem interval (PMI), age, or pH of the brain, we determined their interrelationships by Pearson Product moment analysis. The effects of sex on various PKA measures were determined by an independent-sample *t* test comparing men and women. Similarly, an independent-sample *t* test was used to compare depressed subjects in whom toxicology studies showed no indication of antidepressant use at the time of death with depressed subjects who did not.

RESULTS

As indicated in **Table 1**, there were no significant differences in PMI (t_{53}=1.0, P=.32) or pH of the brain (t_{54}=0.49, P=.63) between the control and suicide groups. Age was slightly but significantly lower in the suicide group than the control group (t_{54}=2.3, P=.03).

Rap-1 ACTIVATION

The specificity of Rap-1 activation was determined in the presence of GTPγS or GDP. Lysates were subsequently incubated with GST-RalGDS-RBD fusion protein in the presence of an immobilized glutathione disk. Samples were eluted and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed with Rap-1 antibody. A representative Western blot illustrating the specificity of Rap-1 activation is shown in **Figure 1**A. We observed that, in the presence of GTPγS, the band was intense, whereas in the presence of GDP, no band was observed. Western blots showing Rap-1 activation in PFC of 3 patients in the suicide group and 3 subjects in the control group are provided in Figure 1B, and mean OD values are represented as a bar diagram in Figure 1C. The level of active Rap-1 was significantly decreased in PFC (t_{54}=4.7, P<.001) and hippocampus (t_{42}=6.87, P<.001) of the total suicide group as compared with the total control group. This decrease was present in both cohorts of brain samples when analyzed separately (Maryland cohort: PFC, t_{20}=3.45, P=.003; hippocampus, t_{20}=4.63, P<.001; Budapest cohort: PFC, t_{32}=3.26, P=.003; hippocampus, t_{20}=5.39, P<.001). No significant change in Rap-1 activation was noted in cerebellum of depressed subjects (t_{54}=0.11, P=.91).

PROTEIN LEVELS OF RAP-1

Western blots showing Rap-1 and β-actin in PFC of 3 patients in the suicide group and 3 subjects in the control group are presented in **Figure 2**A. As can be seen in the bar diagram (Figure 2B), the immunolabeling of Rap-1 as a ratio to β-actin was significantly decreased in PFC (t_{54}=5.10, P<.001) and hippocampus (t_{42}=5.0, P<.001) of patients in the suicide group. The magnitude of the decrease was similar in the 2 cohorts (PFC: Maryland cohort, t_{20}=3.03, P=.007; Budapest cohort, t_{32}=4.20, P<.001; hippocampus: Maryland cohort, t_{20}=3.02, P=.007; Budapest cohort, t_{20}=3.96, P=.001) (**Table 2**). No significant change in the protein level of Rap-1 was observed in cerebellum (t_{54}=0.85, P=.37). Similar results were obtained in all 3 brain areas when OD ratios of Rap-1 were calculated with respect to pooled samples loaded on the same gel (data not shown).

mRNA LEVELS OF RAP-1

The mRNA levels of Rap-1 were determined only in PFC and hippocampus because those were the areas in which significant changes were observed in depressed subjects. The mRNA level of housekeeping gene cyclophilin was determined, and ratios of Rap-1 to cyclophilin were calculated. We observed that the amplification products for Rap-1 and cyclophilin arose from the mRNA template at 380 and 304 bp, and the digestion products arose from complementary RNA at 196 + 184 and 239 bp, respectively (data not shown).

The mRNA level of cyclophilin (attomoles per microgram of total RNA) was unaltered between the control and suicide groups in both PFC (control group, 777±99 attomoles; suicide group, 745±72 attomoles; t_{54}=1.36; P=.18) and hippocampus (control group, 784±89 attomoles; suicide group, 827±71; t_{42}=1.7; P=.09). The results for mRNA levels of Rap-1, including results expressed as a ratio with cyclophilin, are given in **Figure 3**A and B, respectively. Comparison of the total control group with the total suicide group showed that the mRNA level of Rap-1 was decreased in PFC (t_{54}=6.13, P<.001) and hippocampus (t_{42}=8.69, P<.001) of the suicide group. Similar results were obtained when the mRNA level of Rap-1 was calculated as a ratio to cyclophilin (PFC: t_{54}=5.22, P<.001; hippocampus: t_{42}=8.29, P<.001). When the mRNA data from the 2 cohorts were analyzed separately, the absolute amount of Rap-1 mRNA was significantly decreased in PFC and hippocampus of the suicide group as compared with the control group in each cohort with a similar magnitude (PFC: Maryland cohort, t_{20}=4.07, P=.001; Budapest cohort, t_{32}=3.75, P=.001; hippocampus: Maryland cohort, t_{20}=6.77, P=.001; Budapest cohort, t_{20}=5.51, P=.001) (**Table 3**).

mRNA LEVELS OF Epac

The mRNA levels of Epac were determined in PFC and hippocampus. The amplification products for Epac arose from the mRNA template at 366 bp, and the digestion

Table 1. Characteristics of Depressed Suicide Victims and Normal Control Subjects*

Patient No./ Sex/Age, y	PMI, h	Brain pH	Cause of Death	Toxicology Results (at Time of Death)
			Suicide Group†	
1/F/72	4	5.9	Poisoning	Barbiturate, ethanol
2/M/45	5	5.7	Poisoning	Benzodiazepines + barbiturate
3/M/48	4	5.9	Poisoning	Barbiturate + benzodiazepine + ethanol
4/M/47	6	5.8	Hanging	NA
5/M/66	6	5.9	Hanging	Ethanol
6/M/42	3	6.0	Hanging	NA
7/F/28	6	6.0	Poisoning	Meprobamate, benzodiazepine, propranolol hydrochloride, ethanol
8/M/60	3.5	6.5	Hanging	Ethanol
9/M/44	4	6.4	Hanging	Ethanol
10/M/77	4	5.9	Hanging	NA
11/M/36	6	6.6	Hanging	NA
12/M/52	3	6.8	Hanging	Ethanol
13/M/49	6	6.7	Hanging	Ethanol
14/F/45	6	7.0	Poisoning	Clonazepam, metoprolol tartrate, meprobamate
15/M/49	5	6.2	Hanging	NA
16/M/43	4	6.6	Hanging	Ethanol
17/M/42	4	6.7	Hanging	NA
18/F/22	16	5.3	Drug overdose	Propranolol
19/M/24	7	5.6	GSW	None
20/M/21	17	6.1	GSW	None
21/M/27	24	6.4	GSW	None
22/M/38	24	6.3	Drug overdose	Ethanol, diphenhydramine hydrochloride
23/F/36	10	6.5	GSW	Butalbital, diphenhydramine, acetaminophen
24/F/41	27	5.9	Drug overdose	Amitriptyline hydrochloride, desipramine hydrochloride, diphenhydramine, nortriptyline hydrochloride, pseudoephedrine hydrochloride, salicylate meglumine, ethanol
25/F/44	11	5.6	Drug overdose	Nortriptyline
26/F/46	16	6.1	Drug overdose	Nortriptyline
27/F/46	21	5.3	Drug overdose	Amitriptyline, desipramine, ethanol
28/M/53	23	6.1	Jumped	None
			Control Group‡	
1/F/64	4	6.0	Myocardial infarction	None
2/M/52	4	6.2	Myocardial infarction	None
3/F/33	3	6.2	Myocardial infarction	None
4/F/60	3	6.0	Circulatory failure	None
5/F/76	3	6.0	Accident	None
6/M/50	NA	6.3	Acute heart failure	None
7/M/64	1.5	6.4	Myocardial infarction	None
8/M/78	1.5	6.5	Myocardial infarction	None
9/M/84	1.5	6.3	Myocardial infarction	None
10/F/78	1.5	6.2	Acute heart failure	None
11/M/65	1	6.5	Acute heart failure	None
12/F/58	1	5.8	Acute heart failure	None
13/M/51	1	6.4	Acute heart failure	None
14/M/74	3	6.6	Acute heart failure	None
15/M/71	2	6.6	Acute heart failure	None
16/M/52	1.5	5.8	Acute heart failure	None
17/F/80	1	6.5	Acute heart failure	None
18/M/22	19	6.2	GSW	None
19/F/63	30	5.7	Ovarian cancer	None
20/M/31	8	5.6	GSW	None
21/M/33	15	6.0	GSW	Acetaminophen
22/M/37	5	6.6	ASCVD	None
23/F/65	23	5.6	ASCVD	None
24/M/38	16	5.8	Lung sarcoidosis	None
25/F/40	7	6.5	ASCVD	None
26/M/23	15	6.7	GSW	None
27/M/37	9.5	6.1	ASCVD	None
28/F/42	23	6.2	Pneumonia	None

Abbreviations: ASCVD, atherosclerotic cardiovascular disease; GSW, gunshot wound; NA, not available; PMI, postmortem interval.

*The Budapest cohort included subjects 1 through 17, 26, and 27 of the suicide group and subjects 1 through 17 of the control group. The Maryland cohort included subjects 18 through 25 and 28 of the suicide group and subjects 18 through 28 of the control group.

†Mean ± SD age, 44 ± 13 years; 9 women and 19 men; PMI, 9.7 ± 7.7 hours; brain pH, 6.14 ± 0.44.

‡Mean ± SD age, 54 ± 18 years; 11 women and 17 men; PMI, 7.5 ± 8.3 hours; brain pH, 6.19 ± 0.32.

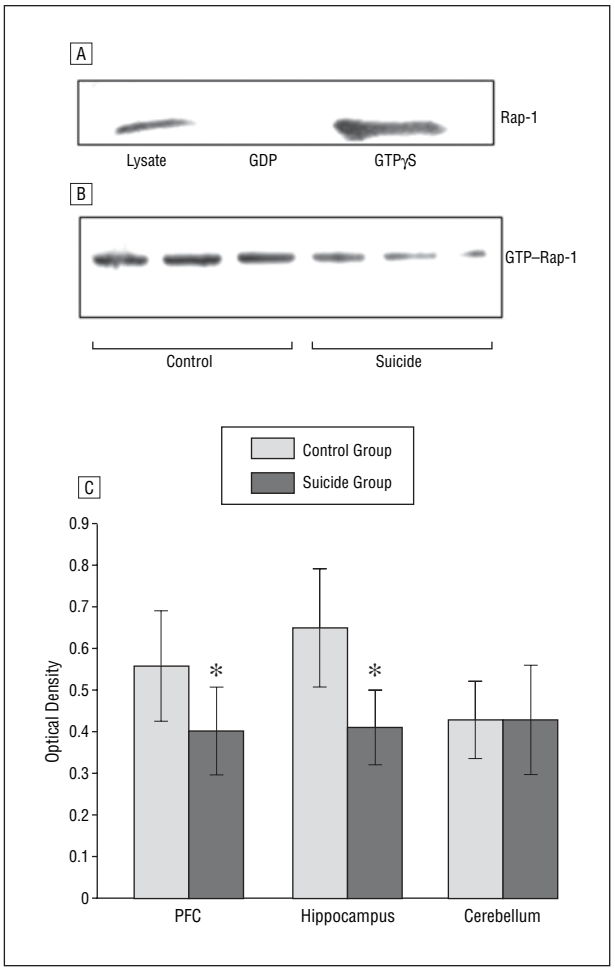

Figure 1. Rap-1 activation in postmortem brain of depressed suicide victims. A, Representative Western blot showing the activation of Rap-1. Glutathione-*S*-transferase (GST) fusion protein containing the Rap-1 binding domain (RBD) of human guanine nucleotide exchange factor specific for the Ras protein Ra1 (Ra1GDS) was used to affinity precipitate active Rap-1 (GTP–Rap-1). The GST-RalGDS-RBD was incubated with lysate and an immobilized glutathione disk. The pulled-down active Rap-1 was detected by Western blot using specific Rap-1 antibody. For control experiments, lysates were treated with guanosine-*S*′-O-(3-thio)-triphosphate (GTPγS) or guanine nucleotide diphosphate (GDP) to activate or inactivate Rap-1. B, Representative Western blots showing active Rap-1 in 3 control subjects and 3 depressed suicide victims. GTP indicates guanine nucleotide triphosphate. C, Mean ± SD values of active Rap-1 (measured as optical density of GTP–Rap-1 on Western blots) in prefrontal cortex (PFC), hippocampus, and cerebellum of depressed suicide victims and normal controls. The PFC and cerebellum from 28 depressed suicide victims and 28 normal controls and hippocampus from 22 depressed suicide victims and 22 normal controls were used. Asterisk indicates $P<.001$.

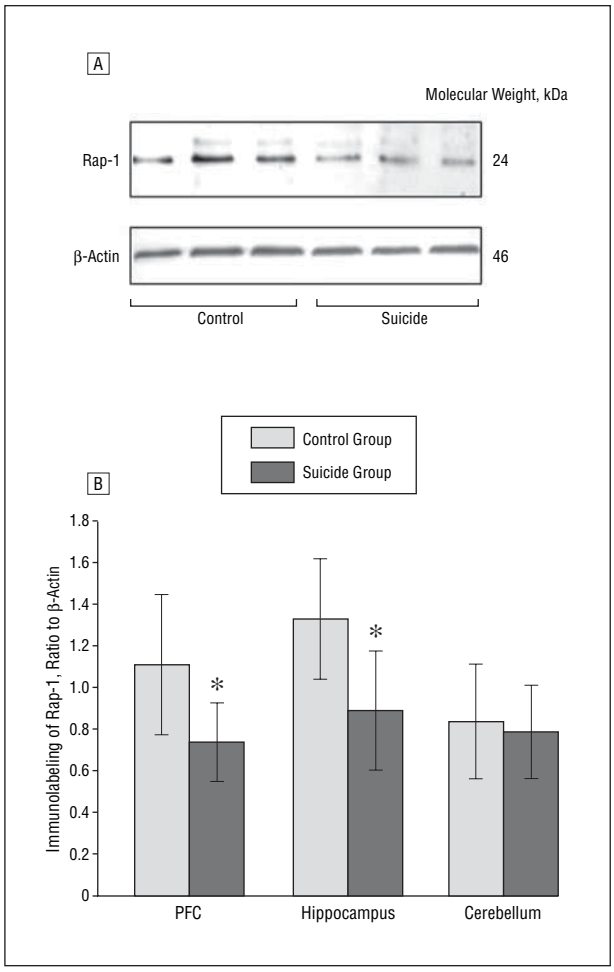

Figure 2. Protein levels of Rap-1 in postmortem brain of depressed suicide victims. A, Representative Western blots showing the immunolabeling of Rap-1 in prefrontal cortex (PFC) of 3 depressed suicide victims and 3 normal controls. Protein samples were subjected to 10% polyacrylamide gel electrophoresis and transferred to enhanced chemiluminescence nitrocellulose membranes, which were then incubated with primary antibody specific for Rap-1 and secondary anti–rabbit antibody. The membranes were stripped and probed with β-actin primary and anti–mouse secondary antibodies. The bands were quantified as described in the "Methods" section. Ratios of the optical density of Rap-1 to that of β-actin were calculated. B, Mean ± SD values of Rap-1 immunolabeling in PFC, hippocampus, and cerebellum of normal control subjects and depressed suicide victims. The PFC and cerebellum from 28 depressed suicide victims and 28 normal controls and hippocampus from 22 depressed suicide victims and 22 normal controls were used. The suicide group was compared with the control group. Asterisk indicates $P<.001$.

products arose from complementary RNA at 193 + 173 bp (data not shown). Comparison of mRNA levels of Epac between the total control group and the total suicide group did not show any significant differences in either PFC or hippocampus, whether analyzed independently (PFC: $t_{54}=0.77$, $P=.44$; hippocampus: $t_{42}=0.12$, $P=.90$) or as a ratio to cyclophilin (PFC: $t_{54}=1.09$, $P=.28$; hippocampus: $t_{42}=0.52$, $P=.60$). Similarly, mRNA levels of Epac were not significantly different between the control and suicide groups when the Maryland (PFC: $t_{20}=0.19$, $P=.85$; hippocampus: $t_{20}=0.52$, $P=.61$) or the Budapest (PFC: $t_{32}=1.17$, $P=.25$; hippocampus: $t_{20}=0.66$, $P=.51$) cohort was analyzed separately (Table 3).

PROTEIN LEVELS OF EPAC-1 AND EPAC-2

Since the mRNA analysis of Epac did not distinguish between Epac-1 and Epac-2, we determined the immunolabeling of Epac-1 and Epac-2 individually by Western blot in all 3 brain areas, ie, PFC, hippocampus, and cerebellum. Representative Western blots of Epac-1, Epac-2, and β-actin are shown in **Figure 4**A. Comparison of their expression levels showed that the level of Epac-1 was lower than that of Epac-2 in all brain areas. Comparison between the control and suicide groups showed that the protein level of Epac-1 expressed as a ratio to β-actin was not altered in PFC ($t_{54}=0.13$, $P=.89$), hippocampus ($t_{42}=0.25$, $P=.80$), or cerebellum ($t_{54}=1.72$, $P=.91$) (Figure 4B). On the other hand, the protein level of Epac-2

Table 2. Protein Levels of Rap-1 and Epac in PFC, Hippocampus, and Cerebellum of Depressed Suicide Victims and Normal Control Subjects Obtained From 2 Cohorts of Brain Samples

Measures	Protein Levels,* Mean ± SD	
	Control Group	Suicide Group
PFC		
Budapest cohort (n = 17)		
Rap-1	1.07 ± 0.29	0.71 ± 0.19†
Epac-1	0.64 ± 0.20	0.63 ± 0.19
Epac-2	1.03 ± 0.22	1.37 ± 0.43‡
Maryland cohort (n = 11)		
Rap-1	1.20 ± 0.40	0.79 ± 0.21§
Epac-1	0.59 ± 0.22	0.57 ± 0.12
Epac-2	1.07 ± 0.22	1.43 ± 0.23‖
Hippocampus		
Budapest cohort (n = 11)		
Rap-1	1.34 ± 0.29	0.83 ± 0.30‖
Epac-1	0.64 ± 0.17	0.68 ± 0.25
Epac-2	0.93 ± 0.22	1.72 ± 0.62‖
Maryland cohort		
Rap-1	1.32 ± 0.31	0.96 ± 0.27§
Epac-1	0.73 ± 0.19	0.72 ± 0.27
Epac-2	1.05 ± 0.28	1.81 ± 0.52‖
Cerebellum		
Budapest cohort (n = 17)		
Rap-1	0.88 ± 0.28	0.81 ± 0.26
Epac-1	0.76 ± 0.32	0.69 ± 0.17
Epac-2	0.83 ± 0.39	0.95 ± 0.33
Maryland cohort (n = 11)		
Rap-1	0.80 ± 0.29	0.75 ± 0.22
Epac-1	0.76 ± 0.21	0.60 ± 0.15
Epac-2	1.03 ± 0.48	1.21 ± 0.35

Abbreviation: PFC, prefrontal cortex.
*Ratio to β-actin.
†$P<.001$.
‡$P=.008$.
§$P=.007$.
‖$P=.001$.

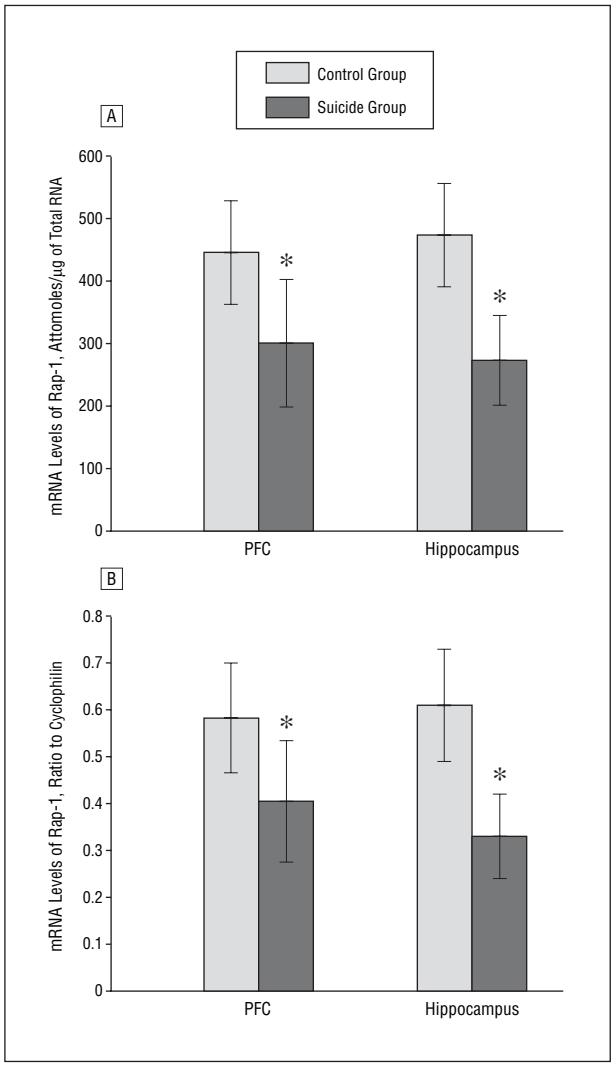

Figure 3. Messenger RNA (mRNA) level of Rap-1 in postmortem brain of depressed suicide victims. Mean ± SD value of Rap-1 mRNA (A) or expressed as a ratio to cyclophilin (B) in prefrontal cortex (PFC) and hippocampus of normal control subjects and depressed suicide victims. The PFC and cerebellum from 28 depressed suicide victims and 28 normal controls and hippocampus from 22 depressed suicide victims and 22 normal controls were used. The suicide group was compared with the control group. Asterisk indicates $P<.001$.

was significantly increased in PFC ($t_{54}=4.32$, $P<.001$) and hippocampus ($t_{42}=5.85$, $P<.001$) in the suicide group without any change in cerebellum ($t_{54}=1.30$, $P=.19$) (Figure 4C). A similar magnitude of increase was observed when the protein level of Epac-2 was calculated separately in the Maryland (PFC: $t_{20}=3.77$, $P=.001$; hippocampus: $t_{20}=4.21$, $P=.001$; cerebellum: $t_{20}=0.96$, $P=.35$) or the Budapest (PFC: $t_{32}=2.8$, $P=.008$; hippocampus: $t_{20}=3.9$, $P=.001$; cerebellum: $t_{32}=0.92$, $P=.36$) cohort (Table 2). The OD ratios of Epac-1 and Epac-2 with respect to pooled samples run on the same gel showed similar changes (data not shown) to those observed when the ratio to β-actin values was used.

EFFECTS OF CONFOUNDING VARIABLES

The effects of potential confounding variables, namely, age, sex, PMI, pH of the brain, antidepressant treatment, and method of suicide, were evaluated with respect to Rap-1 activation and expression levels of Rap-1 and Epac-2, in which we had found differences between the control and suicide groups.

We found no significant effects of age or PMI on Rap-1 activation, mRNA or protein levels of Rap-1, or protein levels of Epac-2 either in PFC or hippocampus (data not shown). Measurement of tissue pH after death serves as an important marker of premortem hypoxia and acidosis and has been used extensively as a measure of agonal state.[41] No significant correlation between pH of the brain and Rap-1 activation, mRNA or protein levels, or protein levels of Epac-2 was observed in either PFC or hippocampus (data not shown), which suggests that the changes in Rap-1 and Epac-2 were not related to the agonal state.

There were 12 men and 10 women in the Maryland cohort, and 24 men and 10 women in the Budapest cohort. Comparison studies showed no significant differences in Rap-1 or Epac measures between men and women in PFC or hippocampus (data not shown) in the Maryland cohort. However, a slight but significantly higher level of Rap-1 protein was observed in women as compared with men in

Table 3. mRNA Levels of Rap-1 and Epac in Total RNA Isolated From PFC and Hippocampus of Depressed Suicide Victims and Normal Control Subjects Obtained From 2 Cohorts of Brain Samples

Measures	mRNA Levels,* Mean ± SD	
	Control Group	Suicide Group
PFC		
Budapest cohort (n = 17)		
Rap-1	0.61 ± 0.13	0.42 ± 0.16†
Epac	0.33 ± 0.12	0.37 ± 0.09
Maryland cohort (n = 11)		
Rap-1	0.55 ± 0.11	0.37 ± 0.09†
Epac	0.31 ± 0.10	0.32 ± 0.07
Hippocampus		
Budapest cohort (n = 11)		
Rap-1	0.59 ± 0.12	0.34 ± 0.10†
Epac	0.37 ± 0.08	0.39 ± 0.12
Maryland cohort (n = 11)		
Rap-1	0.62 ± 0.13	0.32 ± 0.08†
Epac	0.38 ± 0.06	0.34 ± 0.09

Abbreviations: mRNA, messenger RNA; PFC, prefrontal cortex.
*Ratio to cyclophilin.
†P = .001.

PFC (t_{20}=2.3, P=.03) but not in hippocampus (t_{20}=0.73, P=.47) in the Budapest cohort, without any difference in the level of Epac-2 in either PFC or hippocampus.

To examine whether the method of suicide had any effect on the measures of Rap-1 in which we found changes, we compared the depressed suicide victims who died by violent means (n=16) with those who died by drug overdose or poisoning (n=11). No significant differences in Rap-1 activation, mRNA or protein levels of Rap-1, or protein levels of Epac-2 were observed between these 2 groups (data not shown).

For the Maryland cohort, we had information about the antidepressant medication taken 6, 3, and 1 month before death. Of the 11 patients in the suicide group, 4 showed positive results of antidepressant toxicology studies at the time of death (cases 24-27). Patients 25 and 27 were treated with antidepressants (tricyclics) 6, 3, and 1 month before death, whereas patients 24 and 26 were given perphenazine or tricyclics only at 6 and 3 months but not 1 month before death. Comparison of the subjects in the suicide group who showed positive antidepressant toxicology results at the time of death with those who did not showed no significant differences in Rap-1 and Epac measures. As indicated in Table 1, in the Budapest cohort, antidepressant toxicology results were not available in 6 cases (4, 6, 10, 11, 15, and 17), but toxicology results of other subjects in the suicide group showed no indication of antidepressant use at the time of death.

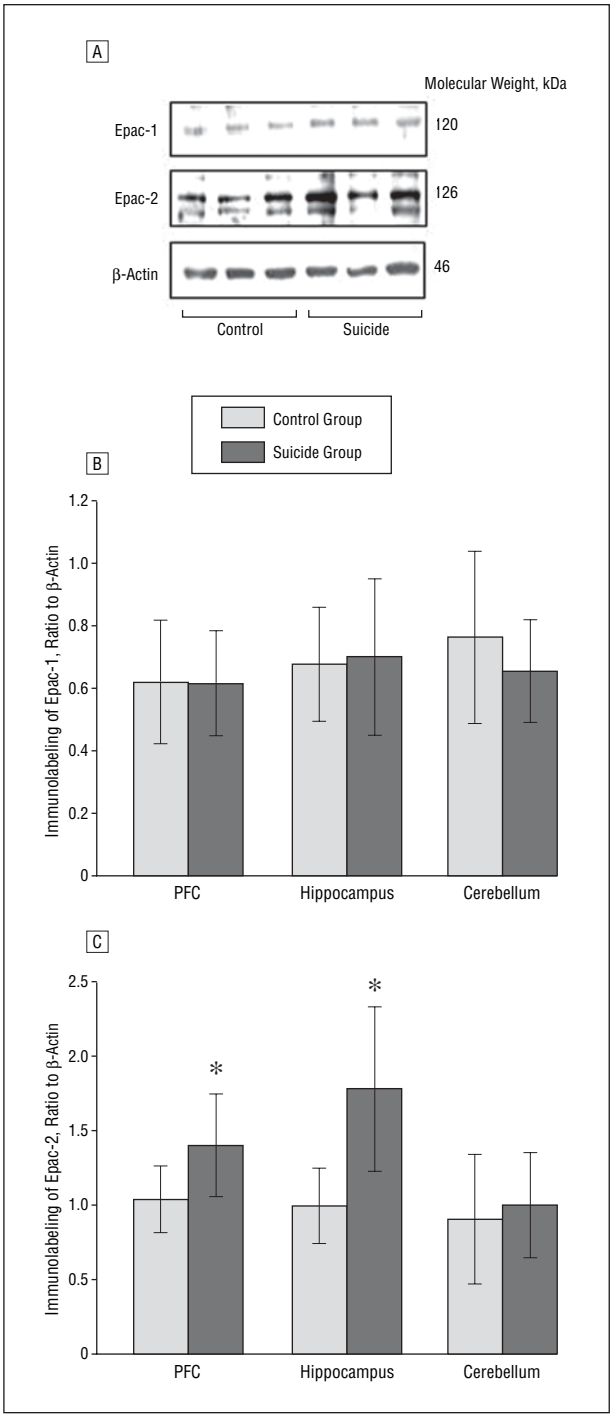

Figure 4. Protein levels of Epac-1 and Epac-2 in postmortem brain of depressed suicide victims. A, Representative Western blots showing immunolabeling of Epac-1, Epac-2, and β-actin. B and C, The mean ± SD protein levels of Epac-1 (B) and Epac-2 (C) in prefrontal cortex (PFC), hippocampus and cerebellum of normal control subjects and depressed suicide victims are shown. The PFC and cerebellum from 28 depressed suicide victims and 28 normal controls and hippocampus from 22 depressed suicide victims and 22 normal controls were used. The depressed group was compared with the control group. Asterisk indicates P<.001.

COMMENT

The results obtained in the current investigation indicate intriguing differential regulation of Rap-1 and Epac in PFC and hippocampus of depressed suicide victims. Activation of Rap-1 was significantly decreased in PFC and hippocampus in the suicide group. We also observed that the mRNA level of Rap-1 was decreased, along with a decrease in its protein levels in these 2 brain areas. These results suggest that the decreased protein level of

Rap-1 could be associated with reduced transcription and that the reduced activation of Rap-1 could be due to its reduced expression. These changes were not apparent in cerebellum, which suggests brain region–specific changes in Rap-1 in depressed suicide victims. These changes were not related to age, PMI, pH of the brain, or method of suicide, which indicates that the observed changes are not secondary to these confounding variables. A decrease in Rap-1 was present in all depressed suicide victims whether they were diagnosed during their lifetime or had a firm diagnosis of major depression at the time of death. Comparison of depressed subjects who showed positive antidepressant toxicology findings at the time of death with those who did not disclosed that none of the measures was affected by the positive antidepressant toxicology finding. Interestingly, of the 4 subjects, 2 had been taking antidepressant medication for at least 6 months before death, and even those 2 subjects showed differences in Rap-1 similar to those observed in subjects who showed positive toxicology results at the time of death. Moreover, the Rap-1 measures were determined in 2 different cohorts of brain samples, one obtained from Maryland and the other from Budapest. The magnitude of the decreases in Rap-1 was similar in these 2 cohorts, which supports and confirms our findings that depression is associated with decreased Rap-1 activation and expression.

Epac is a GEF directly activated by cAMP. This protein contains a cAMP-binding site and a domain that is homologous to known GEFs for Rap-1 or Ras. The cAMP strongly induces the GEF activity of Epac toward Rap-1 via conformational change.[22-24] Since the cAMP analogue selectively inhibits the cAMP-mediated activation of Epac without affecting PKA activity,[42] it has been shown that Epac mediates some of the cAMP-associated functions previously thought to be induced by PKA.[43] Given that Epac activates Rap-1, we determined Epac at both transcriptional and translational levels. The mRNA level of total Epac was not significantly different between the suicide and control groups.

The Epac family consists of Epac-1 and Epac-2. Both of these proteins contain a C-terminal catalytic region responsible for nucleotide exchange and an N-terminal inhibitory regulatory region,[25,44,45] and both can activate Rap-1. The difference between Epac-1 and Epac-2 lies in their regulatory regions. Whereas Epac-1 consists of a disheveled domain and one cyclic nucleotide monophosphate–binding domain, Epac-2 has a disheveled domain sandwiched between 2 cyclic nucleotide monophosphate–binding domains.[25] Also, Epac-1 is ubiquitously expressed, whereas Epac-2 is primarily expressed in brain and adrenal glands.[22] To further examine whether expression of either Epac was different in depressed suicide victims, we determined the immunolabeling of Epac-1 and Epac-2 with the use of human-specific antibodies. Comparison of protein levels of Epac-1 and Epac-2 between the control and suicide groups showed differential regulation such that, although the level of Epac-1 was not changed, the level of Epac-2 was significantly increased in PFC and hippocampus of depressed suicide victims. No changes were apparent in cerebellum, pointing out that change was brain region specific in both Rap-1 and Epac.

The fact that we did not observe significant changes in Epac mRNA level suggests that the increase in Epac-2 is probably not associated with altered gene transcription. The functional significance of the differential increase in only Epac-2 is not clear; however, given that both Epac-1 and Epac-2 activate Rap-1 and that Epac-2 is highly expressed in adult brain,[22] this selective increase in Epac-2 is quite interesting. Because activation and expression of Rap-1 is decreased while the level of Epac-2 is increased in PFC and hippocampus of depressed suicide victims, there is a possibility that the level of Epac-2 may be increased as a result of a compensatory response to decreased Rap-1 activation or expression.

A number of studies show decreased PKA activity in fibroblasts of depressed patients.[30-33] We have also reported reduced tritiated cAMP binding and catalytic activity of PKA,[28] along with selectively decreased expression of certain catalytic and regulatory subunits of PKA,[29] in postmortem brains of depressed suicide victims. In these same subjects, we have now shown increased Epac-2 and decreased Rap-1 activation and expression. Since PKA phosphorylates Rap-1 at its C-terminal Ser-80 residue[15] and makes it sensitive to Smg GDS (a small-molecular-weight GTP-binding protein guanine-nucleotide dissociation stimulator) to stimulate its GDP/GTP exchange reaction,[46] although the idea is speculative, it is possible that the decrease in Rap-1 activation could be due to decreased PKA. Further studies are required to confirm this possibility. Another likely possibility is that the reduced expression of Rap-1 may account for its reduced activation.

In recent years, several studies have suggested altered levels of Rap-1 in platelets of bipolar and depressed patients. For example, Perez et al[47,48] reported significantly increased immunolabeling of Rap-1 in platelets of patients with bipolar disorder but no change in patients with unipolar or bipolar disorder with psychotic features.[49] On the other hand, they found significantly lower protein level of Rap-1 in untreated depressed patients compared with untreated euthymic and healthy subjects.[37] So far there have been no studies of Rap-1 in brains of patients with mental disorders. Ours is, to our knowledge, the first central nervous system study not only showing decreased transcription of Rap-1 but also suggesting that functions of Rap-1 may be diminished in postmortem brains of depressed suicide victims. In this study we observed decreased Rap-1 values in depressed patients, which is contrary to the observation in bipolar disorder.[47,48] This raises the interesting possibility that depression may be associated with decreased Rap-1 values but that bipolar disorder may have the opposite effect.

The functional significance of Rap-1 in depression is yet to be explored; however, recent studies[11,36] have implicated Rap-1 in various physiologic functions, including cell survival, proliferation, differentiation, cell adhesion, and synaptic plasticity. These functions of Rap-1 are mediated through the activation of signaling mechanisms, the most important one being the extracellular signal-regulated kinase (ERK) pathway.[50] It has been shown that PKA stimulates the ERK pathway in neuronal cells expressing B-Raf via Src-dependent activation of Rap-1.[13] In cultured striatal neurons, Rap-1 has been proposed to be a key component of cAMP signaling, culminating in the phos-

phorylation of ERK and cAMP response element binding protein.[21] In addition, Grewal et al[51] showed calcium ion (Ca^{2+})–mediated PKA-dependent activation of Rap-1/B-Raf/ERK signaling in hippocampal neurons and suggested that this pathway may be an important regulator of activity-dependent neuronal functions. Therefore, Rap-1 links the PKA and ERK signaling systems through B-Raf, and at this level, these 2 signaling systems cross talk to each other. As is well documented,[52-54] in addition to synaptic plasticity, ERK signaling is critical in many physiologic functions, including neuroprotection, proliferation, and cell survival. Morozov et al[55] showed that mice with a dominant interfering mutant of Rap-1 had a reduced basal level of phospho-ERK in hippocampus, which was associated with deficient learning and memory storage. It is possible that this decreased activation could be associated with decreased activation of Rap-1. In fact, our group reported significantly decreased activation of ERK-1 and ERK-2 in PFC and hippocampus but not in cerebellum of depressed suicide victims.[56] In addition, our group recently reported significantly decreased activation of B-Raf in postmortem brain of the same suicide group[57] in which we herein report decreased Rap-1 activation and expression. It is possible that the decrease in Rap-1 could be associated with the decreased B-Raf activation, for Rap-1 may act as a downstream regulator of the substrate B-Raf, which could indicate decreased functional response of Rap-1 in depressed suicide victims. In addition to activation by ERK, Rap-1 is activated by neurotrophic factors, their mediated tyrosine kinases, and neuron-specific Ca^{2+}-calmodulin kinase Gr.[11,58-60] Rong et al[61] recently showed that nerve growth factor receptor TrkA induces multiple signals to activate Rap-1 through phospholipase Cγ1 in PC12 cells. York et al[62] showed that Rap-1 is involved in regulating cAMP-induced neurite outgrowth. Rap-1 also has been implicated in cAMP-induced activation of protein kinase B/Akt.[63] We and other investigators have shown involvement of phospholipase Cγ,[64] neurotrophic factors,[35] cAMP response element binding protein,[34] and Akt[65,66] in the pathophysiology of depression or suicide. In view of these findings, our observed decrease in Rap-1 activation may have significant implications in the pathophysiology of depression.

In conclusion, our study shows that brain in depressed patients is associated with differential regulation of Rap-1 and Epac, such that activation and expression of Rap-1 are decreased whereas expression of Epac-2, but not Epac-1, is increased. These results raise the interesting possibility of the involvement of Rap-1 and Epac in the pathophysiology of depression.

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